Cardiac Troponin I in Standardbred Racehorses

by

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ABSTRACT

CARDIAC TROTONIN I IN STANDARDBRED RACEHORSES

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We investigated the use of cardiac troponin I (cTnI) assays in horses and their application in detection of exercise-associated myocardial damage. Available assays were designed for use in humans; therefore, study phase I involved analytical validation of current assays in the horse. Linearity, limit of quantification, and short and long-term precision were evaluated using the American Society for Veterinary Clinical Pathology (ASVCP) guidelines, with equine myocardium and serum as test substrates. Two assays, including a high-sensitivity assay, underwent full analytical validation and performed with clinically acceptable accuracy and precision. Linearity was evaluated in 2 additional assays and was found to be within acceptable limits. Equine cTnI was not detected by 1 assay, and recovery was poor in a second. Appropriate choice of validated assays is essential for clinically valid decision making. Validated contemporary and high-sensitivity assays were used in phase II to establish the normal post-exercise cTnI release curve. Moderate increase in cTnI concentration of limited duration was detected within 1 hour post-exercise and peaked at 2-6 hours. Concentrations returned to near baseline within 24 hours. In phase III horses were sampled pre and post-race in conjunction with ECG monitoring to investigate the cTnI exercise response. Associations between resting and post-race cTnI concentration, demographic variables, and race variables were investigated using multivariable linear
regression models. Moderate increase in cTnI of limited duration was detected in most horses monitored, but some developed elevations suggesting myocardial damage. Concentrations increased significantly with age and were higher in trotters than pacers. Post-race cTnI concentrations were significantly increased with presence of exercise-associated complex ventricular arrhythmia and finishing distanced relative to either condition alone or the absence of both. Results were consistent with exercise-induced myocardial damage, though causation could not be confirmed and the clinical significance of these findings is unknown. Further longitudinal studies are needed to investigate associations and establish causation. Equine clinicians should be aware of the presence and timing of exercise-associated cTnI release when evaluating horses post-exercise. Some cTnI elevation may be a usual feature of the response to maximal effort in the horse.
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Statement of Work Done

The study concept and design for chapters 2-4 were a combined effort by Dr. Tanya Rossi and the advisory committee: Dr. Glen Pyle, Dr. Grant Maxie, Dr. Peter Kavsak, Dr. David Pearl, and Dr. Peter Physick-Sheard. The animal use protocol and funding proposals for these projects were written by Dr. Tanya Rossi, in collaboration with Dr. Peter Physick-Sheard. Industry support from manufacturers was procured by Dr. Tanya Rossi with advice from Dr. Peter Physick-Sheard and Dr. Peter Kavsak. Consent and cooperation from training facilities and the racetrack were obtained by Dr. Peter Physick-Sheard. The animal use protocol was written by Dr. Tanya Rossi and Dr. Peter Physick-Sheard.

Review of current literature on cardiac troponins, exercise-associated sudden death, and cardiac injury was performed by Dr. Tanya Rossi with suggestions from the advisory committee. Data collection and sample preparation for chapter 2 was performed by Dr. Tanya Rossi and Dr. Glen Pyle. Data collection for chapter 3 was performed by Dr. Tanya Rossi and the following research assistants: Emma Chappell, Megan Ballantine, and Nicole Gee. Data for chapter 4 were collected by Dr. Tanya Rossi and the following research assistants: Catherine Nicoll, Melitza Soley, Megan Ballantine, Catherine Belanger, Kathleen Taylor, Ashley Lamond, Kim Lambert, Melanie Scott, Kristen Frederick, and Vivek Basu. The electrocardiogram (ECG) girths were designed by Dr. Peter Physick-Sheard and Emma Chappell, and later constructed, tested and refined by Dr. Tanya Rossi, Catherine Nicoll, and Melitza Soley. Race line data for chapters 3 and 4 were obtained and cleaned by Dr. Peter Physick-Sheard, and coded by Dr. Tanya Rossi.

Interpretation of ECGs was initially performed by Dr. Physick-Sheard as data were collected and later by Dr. Tanya Rossi. All data from chapters 2-4 were cleaned and coded by Dr. Tanya Rossi. Determination of serum cardiac troponin I (cTnI) concentrations was performed
by Lorna Clark and Gary Hickey using commercial automated immunoassay analyzers. Statistical analysis of data for all chapters was performed by Dr. Tanya Rossi with advice from Dr. David Pearl, Dr. Peter Kavsak, and Dr. Peter Physick-Sheard. All figures for the thesis were created by Dr. Tanya Rossi. All thesis chapters were written by Dr. Tanya Rossi with input and editing provided by the advisory committee.

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Chapter 1: Troponin assays and assessment of the equine myocardium

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Introduction:

Detection of myocardial injury is an essential clinical activity, and is facilitated by assays for cardiac biomarkers. In 2000, cardiac troponin assays were adopted as the test of choice for detection of acute myocardial injury in humans. Numerous troponin assays have been developed for use in human medicine, each requiring extensive testing and validation before being made available on the open market. The adoption of troponin testing in veterinary medicine followed shortly after its development for use in humans, providing a much-needed
means of detecting and following myocardial damage in horses. However, application of these tests in veterinary medicine has exclusively involved use of assays designed for, and clinically validated in, human patients. There is no mandated requirement for test validation in veterinary medicine, and, while many tests have been shown to be capable of detecting equine troponin, the wide diversity of available tests, lack of validation, absence of protocols for their use, and lack of standardisation make their application potentially problematic. Analytical validation of troponin assays, establishment of reference intervals in healthy populations, and determination of responses to exercise and disease are necessary to confirm assays are accurate and reliable for use in horses.

Detection of Myocardial Damage

Although clinically apparent disease of the myocardium arises infrequently in the horse, episodes of subclinical damage are suspected to occur based on postmortem findings of ventricular myocardial fibrosis[1-3]. When significant arrhythmias are identified during systemic disorders, such as disturbances of homeostasis or toxicity, possible primary myocardial involvement may be overlooked [4-7]. Viral infections of horses, such as equine influenza, African horse sickness, and equine viral arteritis, as well as intoxications associated with consumption of ionophores, oleander, or blister beetle contaminated hay, can have direct effects on various tissues, including the equine myocardium, causing inflammation, degeneration, and/or necrosis [6; 8-13]. Clinical manifestations are primarily those of rhythm disturbance, except in severe cases where overt heart failure may develop. Of importance, but often overlooked, is involvement of the myocardium in a range of systemic conditions. When an animal has to deal with systemic disease, the heart sustains an increased workload in the face of homeostatic disturbances and toxicities, and may also be subject to
direct insults such as exposure to cardiotoxic agents. This more subtle or occult (background or secondary), yet potentially cumulative, myocardial damage may be of particular significance in a performance animal, such as the horse. The clinician must be constantly alert to the possibility of myocardial impacts. Presence of actual or incipient myocardial damage is not difficult to anticipate, but confirmation and assessment of degree and course of damage are necessary to assess prognosis and monitor clinical progress, and this requires a suitable test.

Cardiovascular disease, a significant source of morbidity and mortality in humans, encompasses a broad range of lesions and clinical circumstances. Among these is the phenomenon of sudden cardiac death, most often attributed to coronary artery disease and myocardial infarction in humans [14]. In cases where no definitive cause can be assigned at postmortem, differential diagnoses may include ion channel abnormalities and cardiac rhythm disturbances, as in young human athletes [15-18]. However, these are presumptive diagnoses and have not been confirmed in the horse [14]. In contrast to humans, coronary artery disease and myocardial infarction are rare in horses, although functional variations in heart sounds and rhythm are common [19]. Cardiovascular disease is thus infrequently diagnosed as a primary cause of death in horses, even in cases of sudden, unexpected death, where cardiovascular involvement may be overlooked in favor of more apparent findings. Sudden death in horses is also a rare event, usually occurring in the context of intense exercise, when numerous other competing factors are in play [20; 21].

In a study of racing fatalities in Victoria, Australia, Boden et al. found that sudden death accounted for 26% of trackside deaths [22]. Unpublished analysis of the Ontario Racing Commission Death Registry reveals a sudden death risk of 0.07 deaths/1,000 starts for the Ontario Standardbred, and 0.36 deaths/1,000 starts for the Ontario Thoroughbred, accounting for 12% of total trackside mortality. Although cases are rare, these deaths frequently occur
during or immediately following races and in view of the general public. Such events can affect public perception of animal welfare in the racing industry, as well as raising concerns about rider/driver safety.

Studies investigating sudden death in apparently healthy racehorses have found signs at postmortem consistent with cardiac involvement, including pulmonary congestion and hemorrhage, pulmonary edema, and rupture of pulmonary or thoracic vessels [18; 20; 22; 23]. In almost half of the cases described in these studies, however, cause of death was not apparent. Gross postmortem findings are similarly ambiguous in many cases of sudden cardiac death in humans in the absence of concomitant coronary artery disease [24]. It can take several days for even microscopic changes to become apparent in myocardium [25]. Histopathology can thus be unrewarding. Given the peracute nature of sudden death, fatal rhythm disturbance must be considered in cases where there is no clear, definitive cause [24]. This hypothesis may be supported by results of a recent study that recorded electrocardiogram (ECG) tracings in Standardbred racehorses during scheduled races [26]. The study found clinically significant, complex ventricular rhythm disturbances during the cool-down period in 18% of race events. These disturbances involved 16% of the horses monitored during the study. Although none of the affected horses died and the presence of the arrhythmias was clinically inapparent, the arrhythmias occurred at a point during recovery from intense exercise that has been associated with a peak in sudden deaths [22; 27]. This suggests the possibility that such disturbances in rhythm may have the potential to become malignant, and raises the question of whether they are a physiologic response to intense exercise or pathologic and associated with myocardial injury. If the exercise-associated arrhythmias are pathologic, then myocardial damage may have precipitated rhythm disturbance. Alternatively, damage may follow rhythm disturbance. Detection of underlying myocardial injury would clearly require a suitable test.
Numerous imaging, biochemical, and other diagnostic tests are available to clinicians for detection of myocardial damage and dysfunction in humans, but for logistical or biological reasons these are not currently used in equine medicine. Body surface ECG has been used in horses for many decades and is a reliable tool for detection of rate and rhythm abnormalities. However, body surface ECG recordings represent only a small fraction of ventricular myocardium in the horse, as the structure of the conduction system leads to significant mirror image cancellation of electrical activity [28; 29]. As a result, the body surface ECG is insensitive to changes in chamber size and to myocardial injury without concomitant rhythm disturbance. Echocardiography is widely used for detection of structural and functional cardiac abnormalities and is sensitive and specific for changes in chamber size, wall thickness and left ventricular function [30; 31]. Extensive shoulder musculature in the horse coupled with cranial orientation of the right ventricle and its anatomy constrain imaging of the right ventricle, limiting detection of changes in that chamber. In addition, though useful for gross changes in structure and function, echocardiography is relatively insensitive to myocardial compromise at the cellular level. Imaging modalities such as cardiac MRI could, in theory, detect occult myocardial damage in horses. However, cost, limited availability of equipment, logistical difficulties, and necessity for general anesthesia make such tests unfeasible as screening tools at this time.

Biochemical markers have long been used to diagnose myocardial damage in human and veterinary medicine [32-35]. Biomarker assays are often inexpensive and are easily accessible to ambulatory practitioners. Serum enzymes, such as lactate dehydrogenase (LDH) isoenzymes, and aspartate transaminase (AST), have been used to diagnose myocardial infarctions in humans since the early 1950s [32; 33]. Serum elevations of these enzymes are sensitive indicators of cell necrosis and enzyme leakage. As they are present in many tissues, however, elevation in serum activity is not a specific indicator of cardiac damage. Elevations
are seen in cases of skeletal muscle damage, hepatic disease, endocrine disorders, and pulmonary conditions [32; 33]. In addition, it often takes hours to days for levels to exceed reference limits, making these enzymes of limited value in emergency room settings where rapid intervention is necessary. These enzymes were subsequently replaced by creatine kinase (CK), followed by the more cardiac specific creatine kinase myocardial band (CK-MB) [36]. CK-MB is highly sensitive for myocardial damage and can be detected in the circulation rapidly (4-6 hrs) after injury in humans [37; 38]. In otherwise healthy individuals, CK-MB is also more specific than CK for cardiac damage. However, CK-MB is also expressed in skeletal muscle and in cases of concurrent disease, such as neuromuscular disease or renal failure, elevations may result from skeletal muscle damage alone, leading to false positives [39; 40]. These findings make CK-MB a less than ideal choice for detection of myocardial injury, especially in the face of intense exercise [41]. The current standard in human medicine is the cardiac troponin assay, which, as of 2000, was declared the preferred biomarker for diagnosis of acute myocardial infarction by joint consensus of the European Society of Cardiology and the American College of Cardiology [25].

**What are Cardiac Troponins?**

Troponins are globular proteins found in striated muscle that regulate the calcium-dependant myosin-actin interaction necessary for muscle contraction. Along with tropomyosin and actin, they are the major components of the thin filament within the myofibril. Three polypeptides make up the troponin complex, troponins I, T, and C. These subunits form complexes in a 1:1:1 ratio in the contractile apparatus of myocytes [42]. Troponin T binds the troponin complex to tropomyosin and actin, whereas troponin I inhibits activity of the actinomyosin ATPase, reducing myofilament interaction. When the troponin C subunit binds calcium,
changes in the troponin conformation allow myosin crossbridge formation and muscle contraction. The troponin polypeptides are highly conserved across mammalian species. For example, nucleotide and amino acid sequencing has shown a > 90% homology between equine, feline, and canine cardiac troponin I (cTnI) [43]. This homology suggests the potential for human assays to detect cTnI in other species, but does not guarantee their suitability as diagnostic tests.

Troponin subunits exist in two populations within the myocyte, a small pool of free cytosolic subunits, and a larger pool existing in complex within the myofibrils. The cytosolic pool represents only 2-4% of troponin I within the cell, and the purpose of this pool is unknown [38]. However, it may have a significant effect on the kinetics of troponin I release into the circulation. Myocardial damage in which there is a loss of cell membrane integrity results in two phases of cTnI release. A mild-moderate rise in circulating concentration occurs early, within 1-2 hrs post-injury, and is thought to represent the release of cTnI from the cytosolic pool [44-46]. Some data exist which suggest this initial release of cTnI may be the result of reversible anoxic injury to myocardial cells, resulting in formation of membrane blebs. When ischemia is reversed, the blebs are either reabsorbed or shed, and the cell does not undergo necrosis [46; 47]. However, further work using myocardial tissue is necessary to fully investigate this theory. A more substantial rise is seen at 4-6 hrs post-injury, and represents cTnI released from the degradation of myofibrils. This second rise is more prolonged because of ongoing cellular degeneration, and in some cases from ongoing myocardial insult.

cTnI was found to have a circulating half-life of 0.47 hrs in horses, based on a study using intravenous infusions of exogenous cTnI [48]. However, these results may not reflect in situ release and clearance, as pure cTnI subunits were administered as a bolus. In vivo, cTnI is primarily released in complex with other troponin subunits, protecting it from protease
degradation and potentially delaying clearance [49]. These results differ from a study on rats and dogs, using similar methodology, which found cTnI half-lives of 0.8, and 1.85 hrs, respectively [50]. In the kidneys, cTnI is cleared through glomerular filtration and conforms to a first-order elimination model, where clearance rate is dependent on plasma concentration, in all species studied [48; 50; 51]. Renal dysfunction has the potential to interfere with elimination of cTnI, however, whether increased levels in patients with kidney failure reflect decreased clearance or myocardial damage has not been confirmed [52; 53].

**Troponin I as a Cardiac Biomarker**

The potential for troponin I to serve as a marker of myocardial damage was first recognized in the late 1980s, when researchers were looking for an ideal cardiac biomarker [54; 55]. The impetus behind this search was the need for a sensitive and specific diagnostic indicator of myocardial damage that could be utilized in an emergency room setting. As stated earlier, methods of detecting myocardial damage at the time included serum concentration or activity of creatine kinase-myocardial band (CK-MB), CK, and lactate dehydrogenase (LDH) isoenzymes 1 and 2. These isoenzymes are fairly sensitive to myocardial necrosis but are not unique to cardiomyocytes, and therefore serum elevations are not specific for cardiac damage [39; 40; 56]. This lack of specificity becomes problematic when trying to detect cardiac damage in the face of neuromuscular disorders, myopathies, intense exercise, chronic renal failure, or endocrine disorders. A more analytically specific marker of myocardial damage was needed for accurate diagnosis of cardiac injury.

An ideal biomarker for myocardial damage needs to fulfill certain criteria in order to be used clinically with confidence [57]. It must be specific for cardiac tissue, which is achievable only if it has a distinct cardiac isoform not present in other tissues. Troponin I and T have
cardiac-specific isoforms distinguishable from those of skeletal muscle, allowing
differentiation between myocardial and skeletal muscle damage. The cardiac isoform of
troponin I is not expressed in either healthy or diseased skeletal muscle, unlike cardiac
troponin T which is expressed in skeletal muscle in cases of chronic muscular disease, such
as polymyositis, muscular dystrophy, and other neuromuscular diseases[58; 59]. The
presence of cTnT in skeletal muscle occurs due to re-expression of the fetal troponin T
isoform (cTnT) as a consequence of repeated cellular injury [58]. Unlike cTnT, cTnI is not
expressed in skeletal muscle during fetal development and therefore is not re-expressed in
chronic muscle disease [58; 60]. Troponin C is found in the same isoform in both cardiac and
slow-twitch skeletal muscle, making changes in its activity less specific for myocardial injury
[61].

An ideal biomarker must be present at only low concentrations in the plasma of healthy
individuals and should be rapidly released into the bloodstream after injury. Low resting
levels allow for clearer cut-points between normal and abnormal to be established, thereby
increasing diagnostic confidence. However, low concentrations can be a drawback if most of
the reference population is below the detection limit of available assays. This makes it
impossible to detect significant changes in troponin levels below this limit. Studies in
clinically healthy humans have shown that cTnI is present at or below the detection limit of most commercial assays. In a healthy reference population of Standardbred racehorses at rest, basal cTnI concentrations were below the detection limit of the test (present at concentrations
less than 0.03 ug/L), using the Stratus CS cTnI immunoassay [62].

Newer generation “high-sensitivity” assays are now in routine clinical use and have been
used in research studies, including a study by Nostell et al. involving endotoxin in ponies
[63]. Some ambiguity exists concerning the use of the term “high-sensitivity” and it has been
used, inappropriately, to denote newer generation tests with lower detection limits. As of
2012, an assay must meet two criteria to be designated “high-sensitivity”: total imprecision at the 99th percentile must be \( \leq 10\% \), and a measurable concentration of cTnI should be obtainable by the assay, in \( \geq 50\% \) of healthy individuals [64; 65]. The development of progressively higher sensitivity assays will potentially lead to a greater understanding of the natural history of cTnI. The ability of assays to detect resting levels in clinically “healthy” individuals will necessitate a new definition of what is considered normal; a definition complicated by the presence of subclinical disease in the population.

In humans, both CK-MB and cTnI are rapidly released into the bloodstream following cardiac injury, allowing early diagnosis and therapeutic intervention [38]. No studies assessing the troponin I release curve post-injury have been performed in horses. However, release and clearance curves have been established for humans and laboratory animals, and suggest detectable increases in serum cTnI occur within 1-4 hours after myocardial insult, with peak levels occurring within 6-24 hours, depending on the species [38; 66; 67]. The insult in these studies ranged from acute infarction to experimentally induced toxic damage. The observed release time may have been impacted by the type and severity of myocardial injury and should be taken into account when interpreting these studies.

In some cases of myocardial injury, particularly those in which damage is minor or subclinical, evidence of cardiac disease may not persist after the initial insult. If the biomarker of choice does not last long in circulation, clinicians may not be able to confirm their diagnosis, or potentially may misdiagnose the condition. Adequate persistence in circulation is therefore important when selecting an ideal biomarker. Unlike CK-MB, which returns to baseline within 48-72 hours, measurable amounts of cTnI persist in the blood for 7-10 days post-injury, allowing delayed diagnosis [54; 68]. As with release times, these numbers may vary based on the type and severity of myocardial injury and whether it is a single event or an ongoing pathologic process.
In addition to diagnosis, an ideal cardiac biomarker can provide prognostic information if serum concentration strongly correlates with lesion size or severity of disease. A 2006 study by O’Brien et al. found that cTnI values correlated closely with the size and severity of myocardial lesions in groups of laboratory mice, rats, and dogs with experimentally induced cardiac toxicity [66]. Similarly, a positive correlation between peak cTnI levels and infarct size on cardiac MRI was observed in a study using a canine model of acute myocardial infarction [69]. Although few studies investigating the correlation between cTnI levels and severity of cardiac disease have been published in non-laboratory animals, there are numerous examples in the human literature. In human patients presenting with acute chest pain, peak circulating cTnI concentration was positively correlated with severity of outcome, including mortality at 30 days and 6 months, revascularization, and progression to chronic cardiac disease [70]. Even low troponin concentrations suggested a worse prognosis over undetectable concentrations [71]. A similar association was found in patients with acute heart failure, with mortality increasing with increasing cTnI values [72]. A study in dogs presented for cardiovascular examination found that cTnI concentration at initial consult was negatively correlated with survival time [73]. Dogs that responded well to treatment had significantly lower cTnI values on recheck appointment; the same was not true for dogs that responded poorly. Mean survival time between the two groups differed significantly, with dogs that had a reduction in cTnI concentration on follow-up living longer on average than dogs that did not show this improvement. Two observational studies of colic in horses found that higher cTnI concentrations, either at admission or post-operatively, were associated with ventricular arrhythmias and non-survival to discharge [74; 75]. These findings suggest cTnI can be used as a prognostic indicator in non-human species with cardiac disease.
cTnI fulfills all criteria for an ideal marker of myocardial injury. For this reason, it has become the preferred biomarker in human medicine and is an established tool for diagnosis and surveillance of cardiac disease.

**Use of Troponin in Horses:**

Although all currently available commercial troponin I assays were designed for human patients, there are numerous reports of their use in horses. Published cases have found elevations of cTnI above the presumed reference interval in horses with piroplasmosis, atypical myopathy, ruptured aortic jet lesions, structural cardiac disease, neoplasia, and after transvenous electrical cardioversion [4; 10; 76-80]. Serum concentrations of cTnI showed a statistically significant increase after intravenous infusion of endotoxin in ponies in one study [63]. The elevations were followed by ventricular arrhythmias in all subjects. These findings are indicative of myocardial dysfunction resulting in rhythm disturbance. In a study by Divers et al., horses were given various doses of sodium monensin via nasogastric tube in conjunction with either corn oil or water [10]. cTnI concentration was measured throughout the study, and was found to be increased in horses exhibiting clinical signs of cardiac disease or failure. The highest cTnI concentrations were observed in the animals that progressed to fulminant heart failure. No increase was observed in horses given low doses of monensin that did not exhibit clinical signs of myocardial damage.

A recent study compared cTnI results between 2 assays in healthy horses and in those with suspected myocardial damage [80]. Animals in the latter group had significantly higher cTnI concentrations than animals in the former group. The study showed moderate correlation in cTnI results between assays, however, individual troponin values differed substantially between tests. Interpretation of these findings is difficult, as inclusion criteria for the
myocardial damage group were not clearly stated, and in most cases, myocardial damage was not confirmed. Inclusion of clinically healthy animals with structural cardiac anomalies in the diseased group may have diminished the difference between groups. A similar study was performed, comparing results between a cTnI assay and a high-sensitivity cTnT assay in healthy horses, horses with structural cardiac anomalies, and those with suspected myocardial disease [81]. A moderate correlation in results was found between the 2 assays, and horses in the diseased group had significantly higher levels of both analytes than those in the first 2 groups. The cTnI assay used in these studies has not undergone analytical validation for use in the horse; therefore, interpretation of findings must be performed with caution. A study of assay precision and linearity has been performed in horses on the high-sensitivity cTnT assay and found acceptable repeatability and linearity [82]. However, estimates of precision were made using a small number of repetitions (n=5) contrary to the American Society for Veterinary Clinical Pathology (ASVCP) guidelines which recommend at least 20 [83]. Between-run precision was determined over 3 days, rather than the minimum of 5-20 recommended. This study used serum from patients with atypical myopathy in which it is expected that skeletal troponin T would be released into circulation, creating the potential for cross-reactivity between troponin T isoforms.

Several studies have been published exploring the effect of exercise on cTnI levels in horses [62; 84; 85]. The results have been variable, with 2 studies finding mild to moderate increases after racing or endurance events, and one finding no significant difference. The lack of agreement could be due to differences in methodology, exercise intensity or duration, population studied, sample times, or assays used. Standard sensitivity cTnI assays may have minimum detection limits that lie above the resting level of healthy subjects, which means mild-moderate elevations in cTnI levels could be missed [62; 86; 87]. Further studies using a standardized approach, based on knowledge of the troponin I release curve and assay
sensitivity and precision are necessary to determine the significance of exercise-associated cTnI release.

These case reports and studies suggest that at least some commercial cTnI assays could be useful diagnostic tools in equine medicine. However, caution must be employed before basing clinical decisions on assays designed for another species, and with one exception, not validated for use in the horse [62]. Several issues associated with use of cTnI assays should be taken into account when interpreting results.

**Issues Associated with Measuring Troponin I:**

**Analytical Validation:** Analytical validation, also referred to as “method validation”, is defined by Westgard as “the process of testing a measurement procedure to assess its performance and to validate that performance is acceptable” [88]. This process seeks to quantify the analytical error inherent in laboratory tests, to determine whether a test is suitable for its intended use. Without validation, the user is ignorant of the reliability of a test, and therefore, the predictive value of test results. Although there are no formal requirements for test validation in veterinary medicine, the American Society for Veterinary Clinical Pathology (ASVCP) has published suggested guidelines for introducing new assays into veterinary diagnostic laboratories [83]. These guidelines should be followed when adapting human assays for use in animals, as well as when introducing new species-specific laboratory tests.

The guidelines recommend that studies of long- and short-term precision, limit of quantification, limit of detection, linearity, analyte recovery, and interference be performed.
before considering inclusion of a test in the diagnostic laboratory [83]. Precision or replication experiments determine the variance or imprecision of a diagnostic method [89]. This imprecision represents variation in laboratory protocol, test performance, and environmental conditions. These studies are performed over several time periods (short- or long-term). Short-term testing is usually carried out within a single day or control period, and imprecision should be minimal because conditions and protocol will not differ significantly among replicates. Long-term precision is carried out over a period of several days or weeks, and is considered a better estimate of test precision as it encompasses the variability of a test in different conditions [89].

Linearity experiments assess whether results are accurate within the reportable range of a test. The results of a series of sample dilutions plotted against the expected results should produce a straight line if the test is linear [90].

Limit of quantification, also called functional sensitivity, limit of detection, and limit of blank, describes the lowest level of analyte a test can measure. It is important to recognize that while all these terms are used in the literature to describe test performance, they have different definitions and refer to different aspects of validation. The Limit of Quantification refers to “the lowest amount of analyte that can be quantitatively determined with stated acceptable precision and trueness” [91]. If clinically significant changes occur below the level of functional sensitivity, valuable diagnostic information will be lost.

Limit of detection is the “lowest amount of analyte in a sample that can be detected with (stated) probability (usually 95%), although not quantified as an exact value” [91]. This definition differs from that of limit of quantification in the accuracy of results. While the test can detect analyte at the limit of detection, it is with greater imprecision and lower accuracy than at the higher, limit of quantification.
Limit of blank refers to the “highest measurement result that is likely to be observed (with a stated probability) for a blank sample” [91]. The limit of blank reflects the random error or “background noise” inherent in the test. When using a validated human assay, the limit of blank will already have been established by the manufacturer, however laboratories are advised to verify the limit of blank wherever possible [92].

The percentage of analyte in a sample that is detected by a test is referred to as the test recovery. Factors such as test matrix or interference from other substances can affect recovery and the accuracy of results [93]. Therefore, interference experiments should be run with common biological substances, such as bilirubin, hemoglobin, and lipids, to determine their effect on results. These validation experiments are best achieved by using a standard solution where approximate analyte quantity is known, and in the case of veterinary medicine, the standard should come from the species of interest. Without the information provided by test validation, it is impossible to determine the predictive value of assay results for confirming myocardial injury.

**Epitopes:** Tests used to detect and measure proteins and polypeptides most often employ immunologic strategies (for example, sandwich immunoassays), and use carefully selected antibodies. The similarity in cTnI amino acid sequence between humans and horses means protein/polypeptide sites (epitopes) targeted by test (assay) antibodies for human use are likely to be effective in horses [43]. This has been used to justify the use of human cTnI assays in horses without appropriate laboratory validation. However, good homology does not guarantee epitopes will be accessible to antibodies, as small changes in amino acid sequence can affect polypeptide folding and potentially obstruct binding sites. Even single amino acid substitutions or deletions can alter antibody affinity for an epitope, affecting analytic sensitivity and test precision [94].
Standardisation: Many of the available cTnI assays are chemiluminescent immunoassays. Automated analyzers apply patient samples to plates or introduce beads coated with capture antibodies against cTnI. The capture antibodies bind to the cTnI and the plates or sample cups are then washed to remove unbound antigen. Acridium or enzyme-linked detection antibodies are next introduced which bind to the cTnI. The antibody-cTnI complexes are then incubated with reagents to catalyze a chemiluminescent reaction. The light generated by this reaction is measured (relative light units; RLU) and used to determine the concentration of cTnI in the sample.

There are many commercial cTnI assays on the market made by several different manufacturers, each assay being designed to run on dedicated, proprietary analyzers. These tests also use unique, proprietary antibodies. A major theme, not only in veterinary medicine but also in human medicine, is the lack of standardisation across assays. The consequence is the inability to compare cTnI values from different analysers, creating the potential for confusion and misinterpretation. A recent study compared the results of 6 different cTnI assays run on patient samples, and found a 5-7 fold difference in results from the same sample [95]. This lack of agreement is the result of a number of factors, starting with the tendency for assays to each use different antibodies for capture and detection of cTnI. These antibodies target various epitopes on the N or C terminus of the protein, and are either monoclonal, polyclonal, or a mixture thereof, and vary in the indicator molecule used. This diversity of antibodies and epitopes naturally leads to a lack of concordance, and variation in their ability to detect different forms of cTnI released into the circulation.

Standard Reference Material: The lack of a standard reference material for calibration is a problem that further complicates the issue. Ideally, this standard would mimic the composition of cTnI forms released into the circulation after myocardial damage [49].
Unfortunately, attempts to produce such a reference material have so far failed, and there has been no standardization or harmonization of cTnI assays [96].

**Free and Complex Troponin I:** cTnI is released into the circulation in several forms after myocardial injury [49; 97; 98]. In addition to free troponin I subunits (which make up only a small percentage of troponin I released), cTnI is often released in tertiary or binary complex with troponin T and C, the most common form being a complex of C and I subunits [49; 98]. The existence of complexes may have a profound effect on assay results, as presence of troponin C or T could restrict access to target epitopes for antibody binding. Conversely, some assays use antibodies that preferentially bind to cTnI in complex, leading to more variation in assay results [98].

**Target Degradation:** The N and C termini of cTnI are susceptible to degradation by proteases, both at the site of myocardial damage and in serum [97; 99]. During myocardial necrosis, proteolytic enzymes are released from lysosomes and act on contractile proteins within hours after the initial insult. Additionally, proteases existing in serum in healthy animals further act on cTnI in circulation. A study by Katrukha *et al.*, found that fragmentation of cTnI occurred within 2 hours of tissue necrosis and was most profound in free cTnI rather than in complex [97]. This fragmentation was most apparent in regions at the extreme end of the C and N-termini, whereas amino acid residues in the more central regions were largely preserved, likely because of protection from proteases. The effect of degradation on assay antibody immunoreactivity was dependant on the region targeted by the test. Increasing discordance in test results between assays with time was observed [97]. These findings indicate that observable peak release time and elimination curves will vary with the assay used. Therefore, interpretation of results and thus clinical diagnosis and case management are highly dependent on assay.
Chemical Modifications: Chemical modification of proteins, such as phosphorylation of serine groups, oxidation and reduction, and N-terminus acetylation, can occur both intracellularly and in the circulation [99; 100]. A study from Labugger et al. found that some assay antibodies changed their immunoreactivity to cTnI after dephosphorylation of serum elements, while other assays were not affected [99]. This suggests that only epitope sequences that include phosphorylation sites would be susceptible to variability and imprecision by this means. A study comparing diseased and healthy myocardium in humans found that healthy tissue contained higher concentrations of phosphorylated cTnI [101]. These results suggest that assay performance may differ in cases of chronic cardiac disease. Little is known about the effects of oxidation and reduction on assay results, although the potential clearly exists for such modification to interfere with findings.

Standard Sample Protocols: A further consideration is lack of a standard laboratory protocol for collection of patient samples. The diversity of cTnI assays on the market means that preferred sample type (serum versus plasma, and anticoagulant type) varies by assay. Some assays are able to process either plasma or serum whereas others can also use whole blood. However, sample additives such as anticoagulants, as well as by-products of clot formation, can have an effect on results. Several studies have documented lower recoveries for heparinized samples compared with serum in both healthy subjects and those with cardiac disease [102-104], although results were not consistent across the literature, and some studies found no difference or higher values in heparinized samples [105; 106]. It is likely that these discrepancies are in part the result of antibody differences in the assays used, as heparin is thought to interfere in some cases by binding and restricting access to target epitopes [103].

Anticoagulant: EDTA can affect cTnI results by chelation of circulating calcium ions, as
maintenance of troponin complexes is calcium dependant [107]. Free troponin I released into the circulation because of low calcium concentrations is vulnerable to degradation by proteases. This could have profound effects on measurable troponin levels as free troponin I may have a shorter half-life than that in complex. Test antibodies may vary in their affinity for free cTnI [97].

As anticoagulants have been shown to affect assay results, the logical choice would be to use serum. However, coagulation products such as fibrin have the potential to influence results as they are prone to non-specific binding by assay antibodies [108; 109]. This can cause falsely elevated levels and could give rise to clinical misdiagnoses, though these false positives are considered uncommon and seem to be the consequence of incomplete clot formation and centrifugation [108; 109]. Since blood samples taken under field conditions are often exposed to extreme temperatures, inappropriate handling, and incomplete centrifugation, care should be taken to establish consistent sampling protocols to ensure accurate assay results. Further studies examining the extent of fibrin and anticoagulant interference in humans and in the horse are required in order to determine the most appropriate protocol and sample to be used in clinical settings.

**Conclusion and Future Work:**

cTnI is a sensitive and specific marker of myocardial injury and its measurement is the test of choice in human medicine. Several case reports and observational studies have documented its potential use as a diagnostic tool in equine medicine. However, offering guidance on use of human assays in clinical settings is inadvisable at this time as work still needs to be done to validate commercial assays to ensure they are reliable and accurate when used in the horse. Sample collection protocols also need to be established. As a number of different assays exist
on the market, none of them standardised, care must be taken to establish laboratory protocols and reference intervals that are specific to each assay. Future studies should include establishing resting reference intervals in “healthy” populations using high-sensitivity assays. Additionally, the pattern of equine cTnI release after exercise, and the elimination curve in normal, exercising animals should be investigated to determine the most appropriate times to sample animals. This pattern of release and elimination should be compared with that of animals with cardiac disturbance or injury. These data would facilitate accurate diagnosis of myocardial damage and minimise false-positive and false-negative results. Further studies using high-sensitivity assays are needed to fully investigate the relationship between exercise and cTnI concentrations, and to assess any relationship between cTnI release, rhythm disturbances, and myocardial damage. As such the objectives of this thesis are:

1. To analytically validate commercial troponin I assays for use in the horse.
2. To plot the magnitude and time course of cTnI release and clearance after maximal effort, using a validated cTnI assay.
3. To examine cTnI levels pre- and post-maximal effort in a population of racehorses competing in scheduled competition.
4. To compare the magnitude and time course of cTnI elevation in a group of horses experiencing arrhythmia with that of randomly selected horses not exhibiting arrhythmia from the same maximal intensity event.

Manufacturer’s Details:

a. Stratus CS, Siemens Diagnostic Healthcare, Newark, Delaware, United States of America.

References:


Chapter 2: Analytical validation of cardiac troponin I assays for use in the horse

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Abstract:

Human cardiac troponin I (cTnI) assays have been used in equine medicine, often without prior analytical validation for equine use. In the absence of appropriate validation, the clinical significance of assay results is uncertain and can lead to misdiagnosis. This study followed the American Society for Veterinary Clinical Pathology guidelines and investigated linearity, precision, limit of quantification (LoQ), and comparative recovery for several commercial cTnI assays. Clinically acceptable linearity was observed in assays A-D, whereas assay E did not detect equine cTnI in any sample. Comparative recovery revealed 1-3 fold differences between assay results, and low analyte recoveries (2.2-3.4%) were observed in assay F. Precision was investigated in assays A and B, and found to be within acceptable limits. LoQ was 1.53 ng/L for assay A, and 0.031 µg/L for assay B. Assays A and B performed within
clinically acceptable limits and were deemed suitable for use in equine medicine. Assays C and D did not undergo full validation but had acceptable linearity, which indicates potential as equine diagnostic tests. Assays E and F are unsuitable for use in horses due to issues with detection of equine cTnI. The variability in results between assays indicates that reference intervals and cut-offs for diagnostic decision making are assay specific and should be established prior to adoption by diagnostic laboratories.

**Keywords:** Analytical Validation , Cardiac Biomarkers, Equine Myocardium Disease, Troponin,

**Introduction:**

Cardiac troponins are globular proteins, which along with tropomyosin and actin form part of the thin filament of the contractile apparatus in striated muscle. The troponin complex is made up of three polypeptide subunits: troponin I, T, and C, that regulate the calcium dependant actin-myosin interaction necessary for muscle contraction (1). Troponin I and T have cardiac specific isoforms that are distinguishable from those of skeletal muscle, allowing differentiation between myocardial and skeletal muscle damage. The analytical specificity of cardiac troponins and their release during cardiac injury has led to the development of commercial immunoassays and their adoption as markers of myocardial injury.

Cardiac troponin I (cTnI) assays are important for the detection of myocardial damage and have been in widespread use in human medicine since the 1990s (2-4). In 2000, cardiac troponins were adopted as the biomarkers of choice for detection of myocardial infarction in human patients by joint consensus of the European Society of Cardiology, and American College of Cardiology (4). Use of cTnI assays in veterinary medicine followed shortly after their introduction, and studies have documented their use in several non-human species,
including the horse (5-8). However, the incorporation of cTnI for use in animals has relied exclusively on the adoption of assays designed for and validated in humans, often without performing analytical validation in the species of interest. This is potentially problematic, as although the amino acid sequence is well conserved between humans and other domestic species, amino acid substitutions and deletions do exist at target epitopes (9). In addition, species-specific physiology, disease states, and composition of the test matrix (plasma or serum), may have unforeseen effects on assay performance. In order to identify and quantify potential sources of error, the American Society for Veterinary Clinical Pathology recommends that analytical validation be performed before adoption of a new diagnostic test or method for routine laboratory use (10). In the absence of analytical validation and subsequent epidemiologic (clinical) validation, the clinician or researcher is unaware of the reliability of the test and therefore of the predictive value of a given result.

There are currently several published studies describing the clinical use of cTnI assays in the horse, but only two detailing full or partial analytical validation (11-15). The results of these studies were consistent with the hypothesis that commercial cTnI assays are capable of detecting myocardial damage in horses. However, since the diagnostic accuracy of these assays depends on their analytical performance, validation should be performed prior to assessing their suitability as diagnostic tests. The objective of this study was to analytically validate commercial cardiac troponin I assays for use in the horse.

**Methods:**

Animal use and study protocol were approved by the University of Guelph’s Animal Care Committee, Animal Utilization Protocol #1339.

*Preparation of test substrates and matrix*
Serum, myocardium, and skeletal muscle were collected from a single donor horse. Inclusion criteria included: apparently healthy, less than 2 years old with no history of race training. Physical examination was performed prior to tissue collection to exclude clinically apparent systemic illness or cardiac dysfunction. Prior to anesthesia a 14 gauge intravenous catheter was placed in a jugular vein using aseptic technique, and a 4.1 mL extension set attached. The filly was pre-medicated with xylazine\(^a\), and general anesthesia induced with intravenous ketamine hydrochloride\(^b\) and diazepam\(^c\), followed by maintenance with isoflurane inhalant. Following induction, a second I.V. catheter was placed in the contralateral jugular vein and whole blood was collected into sterile 1L blood collection bags. The process was stopped once 10 L of blood was obtained as blood viscosity compromised further collection. The horse was then euthanized with pentobarbital sodium\(^d\) IV and immediately transferred to a postmortem suite.

A gross postmortem examination was performed and the heart extracted within 20 minutes of euthanasia to reduce degradation of cardiac troponin complex. The heart was immediately frozen at -80°C. A portion of the triceps brachii was also extracted and frozen.

The collected whole blood was allowed to clot for 1 hour at room temperature, and then for 12 hours at 4°C to allow full maturation of the clot and maximum serum yield. All steps were carried out at room temperature unless otherwise stated. The serum was first manually separated from the mature clot, which was then centrifuged at 2,981g for 10 minutes and the serum decanted. Serum was passed through a fibrin filter to remove any remaining fibrin, and divided into 100 mL aliquots in separate vials. Serum was stored at -80°C until use.

*Isolation of Cardiac Troponin Complex*

Isolation of crude cardiac troponin complex from harvested myocardium was achieved using the protocol described by J.D Potter *et al.*, (16). Modifications to the protocol were made to
accommodate the volume of tissue used, and are listed in the summary below (16). All steps were carried out at room temperature unless otherwise stated. Briefly, myocardium from the left ventricular free wall was homogenized, using a micro homogenizer, in 4°C buffer solution (1% Triton X-100, 50 mM KCl, and 5 mM Tris, pH 8) and centrifuged at 11,000g for 15 mins at 4°C. The pellet was re-suspended in buffer solution and the process was repeated 8 times. The pellet was transferred to a Buchner funnel and washed with chilled 95% ethanol under suction 3 times. The process was repeated using diethyl ether in place of ethanol and the resultant etherized sample was allowed to dry overnight at 4°C. The etherized substrate was re-suspended in 200:1 volume/weight high-salt solution (1M KCl, 20 mM Tes, pH 7, and 15 mM BME) rather than 20:1. The rationale for this change was a smaller mass of myocardium was used in the current study, and the sample volume would have been too low in a 20:1 solution to accurately test and adjust pH. The substrate was allowed to extract in the high-salt solution overnight at 4°C. The sample was centrifuged at 11,000g for 20 mins at 4°C and the pellet discarded. Ammonium sulfate (1.62 M) was added to the supernatant and the pH adjusted to 7.02. The sample was centrifuged at 11,000g for 20 mins at 4°C and the pellet discarded. Ammonium sulfate (0.55 M) was added to the supernatant and the sample was centrifuged at 11,000g for 15 mins at 4°C. The resultant pellet was stored in polypropylene microcentrifuge tubes at -20°C until use. As the substrate of interest was the crude cardiac troponin complex, further purification through dialysis and chromatography was not attempted.

**Immunoblotting**

Western blot analysis was performed using a modified protocol described by Pyle et al. (17). The gels were loaded with homogenized murine myocardium, and the equine troponin isolate. The samples were run on 12% SDS-polyacrylamide gels and the proteins transferred to a nitrocellulose membrane. The membrane was incubated in a 1:1000 solution of anti-TnI
antibodies overnight at 4°C. The membrane was then incubated in a 1:5000 solution of secondary antibodies conjugated with horseradish-peroxidase. Protein bands were detected using Western Lighting. Immunoblot images were captured using the FluorChem 9900 gel documentation system. Images were viewed on ImageJ.

Preparation of cTnI Spiked Serum:

Low cTnI serum was used as a matrix for the spiked samples. In the absence of a gold standard, the cTnI concentration in this serum was measured by the high-sensitivity assay A. Affinity of assay A for equine cTnI was later confirmed through analytical validation. The crude troponin complex was suspended in this matrix and mixed using a vortex spinner. The concentration of cTnI in the spiked serum was ascertained using assay D due to availability of assay at the time. Serial dilutions were produced from this spiked serum to make up various concentrations of cTnI for use in the validation process. The samples were then frozen at -80°C until analysis.

Measurement of cTnI:

Hospital laboratories and manufacturers were recruited for participation in the analytical validation of commercial cTnI assays. The number of validation experiments performed depended on both the performance of the assays and the continued participation of these industry partners. Six (6) assays currently being used in veterinary and human hospital laboratories were included in this study (assays A-F) (Table 2.1). Samples were prepared by the researchers and shipped to the laboratories on dry ice. Analysing laboratories were blinded as to the concentration of the samples.

Linearity Experiment:
Linearity was assessed in assays A-E. The methodology described in guidelines published by the American Society for Veterinary Clinical Pathology (ASVCP) was employed in the assessment of assay linearity (10). Five concentrations of cTnI spiked serum, spanning the full range of the tests, as reported by the manufacturer, were prepared. Three replicates of each concentration were run on assays A-E. The values for each concentration were plotted against the expected value and the plot visibly inspected for outliers, and linearity. Curve fitting was performed with linear regression using Origin 8.6 software.

Recovery:

Recovery was compared between assays by running aliquots of five concentrations of cardiac troponin spiked serum on assays A-F. For each concentration, three replicates were run and the mean calculated. Results for each assay were compared to a high-sensitivity referent (assay A) and recovery expressed as a percentage of the referent.

Short- and Long-Term Precision:

Short-term precision was determined for assays A and B. As recommended in the ASVCP guidelines, 2 concentrations of cTnI were used: a low concentration close to the limit of quantification, and a second concentration in the presumed pathologic range based on the human literature (18). Twenty replicates of each sample were tested within a single 8 hour control period. Long-term precision was determined for assay B using the same samples. Twenty replicates were run over a 20-day period for both concentrations. The mean concentration, standard deviation, and coefficient of variation (CV) were calculated using Microsoft Excel 2010.

Limit of Quantification (LoQ):
A spiked serum sample of low cTnI concentration was used to determine the limit of quantification for assays A and B. Serial dilutions of the spiked serum were prepared using isotonic saline, and 20 replicates of each dilution were tested within a single control period. The mean concentration, standard deviation, and CV were calculated for each dilution using Microsoft Excel 2010. The greatest dilution (lowest concentration) with a CV of < 20% was accepted as the limit of quantification.

Pre-analytical Storage:

Post-exercise serum samples from 9 Standardbred racehorses were used to assess the effect of pre-analytical storage temperature on assay results. Samples were collected from horses competing in a regularly scheduled 1 mile race at Mohawk Racetrack in southern Ontario. In agreement with industry partners (track management, horsemen, and the Ontario Racing Commission) participation in the study was made a condition of race entry. For each horse, whole blood samples were collected via jugular venipuncture into 2 red top vacutainer tubes. The first tube was allowed to clot for 45 mins at room temperature, and the serum was then separated and divided into 2 aliquots. One aliquot was immediately stored at -80°C and used as the control group. The second aliquot was placed in a -20°C freezer for 30 days and then stored at -80°C until analysis. The second tube was divided into 2 aliquots before clot formation. The aliquots were stored either at room temperature (24°C) or 4°C for 24 hours, and then centrifuged at 3000g for 15 mins. Serum was then separated and stored at -80°C until immediately before analysis. All samples were analysed using assay A as it had the highest analytical sensitivity of assays tested. The difference between each group and the control group stored at -80°C was assessed using a mixed linear regression model with horse modelled as a random intercept. Statistical analysis was performed using STATA 13®.

Results:
Preparation of test substrate and matrix

Physical examination of the donor filly revealed bilateral angular limb deformities of the forelimbs. All other findings were within normal limits. Postmortem examination documented moderate-severe, bilateral carpal valgus, and vargus of the metacarpal-phalangeal joints. No other clinically significant abnormalities were found.

The serum harvested from this filly was tested using assay A and found to have a cTnI concentration above the limit of blank of 0.88 ± 0.32 ng/L (mean ± standard deviation [SD]).

Western blot analysis:

Protein bands of ~23kDa consistent with presence of cTnI were detected in the lanes loaded with the crude equine troponin isolate (Figure 2.1). A protein bands of ~24kDa was detected in the lane loaded with murine myocardium.

Linearity

Results for assay E were under the detection limit of the test for all sample concentrations. Linearity results for assays A-D are shown in Figure 2.2. Least squared linear regression revealed R² values between 0.965-0.996 (mean = 0.977 ± 0.006 SD) for these assays.

Comparative Recovery

Comparative recoveries for assays A-D and F compared to assay A was between 0.9%-323% (Mean = 145.75 ± 28.6) (Table 2.2). Results were below the detection limit for 2 samples using assay F and were not included in the analysis. A result for the highest concentration sample was not obtained for assay D.

Short- and Long-Term Precision
The short-term or “within run” precision for assay A was $1.6 \pm 0.2$ ng/L (CV = 13.6%) and $203.4 \pm 56.3$ ng/L (CV = 2.8%). Short-term precision for assay B was $26.2 \pm 0.2$ µg/L (CV = 0.8%) and $0.058 \pm 0.003$ µg/L (CV = 5.7%). Long-term or “between run” precision for assay B was $25.6 \pm 0.32$ µg/L (CV = 1.2%) and $0.044 \pm 0.009$ µg/L (CV = 20.9%).

Limit of Quantification

The limit of quantification (LoQ) for assay A, calculated during a single 8-hour control period, was determined to be $1.53 \pm 0.002$ ng/L (CV = 17.6%). Using the same procedure, the LoQ for assay B was $0.031 \pm 0.002$ µg/L (CV = 7.5%).

Pre-analytical Storage

None of the horses used for this experiment showed signs of myocardial disease at the time of sampling. Samples were thawed prior to analysis, and run in a single 8-hour control period using assay A. Mean cTnI concentrations, in ng/L, for the -80°C, -20°, 4°C, and 24°C groups were $5.11 \pm 1.64$, $4.62 \pm 1.41$, $4.75 \pm 1.59$, and $4.22 \pm 0.49$ respectively. No statistically significant difference was found between storage groups ($p \geq 0.05$).

Discussion:

This study relied on cTnI spiked serum for the majority of the analytical validation procedures. Efforts were made to select a healthy donor animal of racehorse breeding that had not participated in race training, as this could potentially result in elevated cTnI concentrations. Use of myocardium and serum from a single animal is a potential limitation of this study as these tissues may not account for normal variation in troponin I amino acid sequence in the equine population. However, to the authors’ knowledge, there are no reports of sequence variation in the absence of myocardial disease in either the human or equine literature. The decision to use a single donor was based on ethical concerns, such as limiting
animal usage, as well as, economic constraints. Western blot analysis of the troponin complex isolate from this filly revealed protein band of approximately 23kDa, which was consistent with cTnI (molecular weight = 24kDa). Cross-reactivity with other proteins is possible; especially with skeletal troponin I. However, the exclusive use of myocardium for cTnI isolation, combined with the molecular weight of the resulting protein band, make this scenario unlikely. These results indicate that the protocol was successful in isolating cTnI from the myocardium.

Examination of linearity data for assays A-D revealed a linear relationship between the observed and expected cTnI values ($R^2 = 0.965-0.996$). These results suggest that these assays are capable of detecting equine cTnI, and there is a strong linear relationship between the “real” and observed concentration of cTnI throughout the reportable range of each assay. Although visual examination of the line graphs (Figures 2.2) shows linearity was not perfect, the assays performance is clinically acceptable.

Assay recovery results, expressed in Table 2.2 as comparative recovery, varied widely among the assays tested, with assay F having the lowest recovery and assay B the highest. This discrepancy in results between assays has been observed in other studies using human and canine patient samples, where differences of 2-5 fold between assay results were found (19, 20). Since there is no gold standard, it is difficult to determine which assay most accurately measures cTnI concentration. This lack of standardization between assays is likely due to differences in antibody configuration and epitope targets, and the absence of a standard reference material (21, 22). Some assays may be better at detecting cTnI fragments in serum, or cTnI that is in complex with other troponin subunits. Therefore, cTnI reference intervals should be developed for each laboratory population, using a single assay, and comparisons of results between assays for clinical purposes should be avoided.
Despite using many of the same epitopes as assays with acceptable performance, assay E did not detect equine cTnI in any of the samples. These results may reflect the way in which these epitope targets are used. Both assay E and C employ antibodies against amino acid residues 87-91 in the cTnI polypeptide (23). Examination of the amino acid sequence of equine cTnI, compared to that of the human protein, reveals a single substitution of glutamic acid for alanine at residue 91 (9). However, assay C utilizes this epitope along with amino acids 24-40 as targets for its 2 capture antibodies, while it is the only epitope target of assay E’s detection antibody. Similarly, assay F uses the residue 87-91 epitope as the target for its capture antibody (23). The comparative recovery of assay F in this study was much lower (2.2–3.4%) than other assays tested, indicating a poor analytical sensitivity for equine cTnI. These examples suggest that the amino acid substitution decreases or negates the affinity of assay antibodies for this epitope and impacts the accuracy of assays that rely solely on this affinity for antigen capture or detection. This lack of detection precluded further validation, and assays E and F were deemed unsuitable for use in equine medicine.

ASVCP guidelines state that a test must have a CV of \( \leq 20\% \) within the reportable range to be considered clinically acceptable (10). Assessment of short-term precision in assays A and B found a CV of \(<15\%\) at both a high and low cTnI concentration, indicating acceptable performance. Results for the long-term precision experiment in Assay B showed a low CV (1.2\%) for the high concentration sample, but a CV slightly above the acceptable cut-off for the low concentration (20.9\%). These results emphasize the need for a well-regulated quality control program and for a strict procedural protocol in order to minimize sources of analytical variation and maximize precision.

A LoQ of 0.031\( \mu \)g/L was found for assay B, which is in agreement with the LoQ in human samples (23). This suggests the analytical sensitivity of this assay is similar in humans and horses. Assay A had a LoQ of 1.53 ng/L, which is an order of magnitude lower than Assay B.
This higher sensitivity allows the clinician to detect changes in cTnI concentration that are below the detection limit of most commercial assays, potentially leading to fewer false-negatives, both in diagnosis and clinical progression. In addition, higher sensitivity tests will provide information on biological variability, and a better understanding of basal cTnI concentrations in sub-populations such as performance animals.

Analysis of the impact of pre-analytical storage conditions on cTnI results revealed no difference between groups. These findings are consistent with those of human studies that found minimal changes in cTnI concentration on the basis of pre-analytical storage (24, 25). Storage groups in our study were designed to mirror common conditions in equine practice where serum cannot be immediately separated or frozen. Results suggest that short-term storage under these conditions does not significantly impact results. However, the sample size was small (n = 9) and these samples were taken from clinically healthy horses with low cTnI concentrations. Studies should be performed using larger study populations, and include horses with myocardial disease to further assess the impact of pre-analytical storage.

The objective of this study was to perform analytical validation on commercial cTnI assays. The extent of analytical validation was dependant on the continued participation of third party laboratories, which was not always feasible, and therefore some of the assays did not go through full analytical validation. However, assays A and B were fully validated and determined to have clinically acceptable performance when used on equine samples. Although not fully validated in this study, results of the linearity experiment suggest that assays C and D may be suitable for use in equine medicine. Full analytical validation should be performed before including these assays into the diagnostic laboratory. Population specific reference intervals should be established for each assay and diagnostic cut-offs determined. In human medicine, the 99th percentile of the reference population is typically accepted as the cut-off for myocardial injury (4, 20). These guidelines were validated by clinical studies.
which investigated the diagnostic sensitivity and specificity at different cut-offs, in patients presenting with possible myocardial infarctions (26, 27). Further studies into appropriate cut-offs in equine patients should be performed to assess case characteristics and optimise diagnostic accuracy.

**Footnotes:**

a. Rompun™ (Xylazine) 100mg/ml Injectable, Bayer, Shawnee Mission, KS, USA.
b. Ketalar™ (Ketamine Hydrochloride) 10mg/ml Injection, Pfizer Inc., New York, NY, USA.
c. Diazepam injection, Sandoz Canada Inc, Boucherville, QC, Canada
d. Pentobarbital Sodium Injection, Merck Animal Health, Kirkland, QC, Canada
e. Micro Homogenizer, Omni International, Kennesaw, GA, USA
f. Fisherbrand™ Snapcap™ 1.5ml microcentrifuge tubes, Fisher Scientific, Pittsburgh, PA, USA
g. Troponin I (C-4): sc-133117, Santa Cruz Biotechnology Inc., Dallas, TX, USA.
h. Anti-Mouse IgG (Fab specific)–Peroxidase antibody produced in goat, Sigma-Aldrich Co., St.Louis, MO, USA.
i. FluorChem 9900 gel documentation system, Protein Simple, San Jose, CA, USA.
k. Assay A: Abbott ARCHITECT STAT High Sensitivity Troponin-I assay, Abbott Park, IL, USA.
l. Assay B: Siemens Dimension Vista® Troponin-I assay, Siemens Healthcare Diagnostics Inc, Newark, De, USA.
m. Assay C: Abbott ARCHITECT STAT Troponin-I assay, Abbott Park, IL, USA.
n. Assay D: Beckman Access AccuTnI, Beckman Coulter Inc., Brea, CA, USA.
o. Assay E: Ortho Vitros ECi ES Troponin-I assay, Ortho Clinical Diagnostics, Raritan, NJ, USA.

p. Assay F: Siemens IMMULITE® 1000 Troponin I assay, Siemens Healthcare Diagnostics Inc, Newark, De, USA.

q. Origin 8.6 software, OriginLab Corp., Northampton, MA, USA.

r. STATA 13 Data Analysis and Statistical Software, StataCorp, College Station, TX, USA.
Figures and Tables:

Figure 2.1: Western blot analysis of the equine cardiac troponin isolate.

Gel wells were loaded with murine cTnI (left) in well 1, and equine cardiac troponin isolate in wells 2 – 5.
Figure 2.2: Linearity results for Assays A-D.

Results are stated in µg/L and represented by solid dots. Dashed line represents fitted line using linear regression.
Table 2.1: cTnI assays evaluated in this study.

<table>
<thead>
<tr>
<th>Assay</th>
<th>Assay Name</th>
<th>Manufacturer</th>
<th>LoD*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Assay A</td>
<td>ARCHITECT STAT High Sensitivity Troponin I</td>
<td>Abbott</td>
<td>0.0012</td>
</tr>
<tr>
<td>Assay B</td>
<td>Dimensions Vista® Troponin I</td>
<td>Siemens</td>
<td>0.015</td>
</tr>
<tr>
<td>Assay C</td>
<td>ARCHITECT STAT Troponin I</td>
<td>Abbott</td>
<td>0.009</td>
</tr>
<tr>
<td>Assay D</td>
<td>Access AccuTnI</td>
<td>Beckman Coulter</td>
<td>0.01</td>
</tr>
<tr>
<td>Assay E</td>
<td>Vitros ECi ES Troponin-I</td>
<td>Ortho Clinical Diagnostics</td>
<td>0.012</td>
</tr>
<tr>
<td>Assay F</td>
<td>IMMULITE® 1000 Troponin I</td>
<td>Siemens</td>
<td>0.15</td>
</tr>
</tbody>
</table>

* Manufacturer’s stated Limit of Detection (LoD) in µg/L from human studies.
Table 2.2: Comparison of cTnI results between commercial assays.*

<table>
<thead>
<tr>
<th>Assay A</th>
<th>Assay B</th>
<th>Assay C</th>
<th>Assay D</th>
<th>Assay F</th>
</tr>
</thead>
<tbody>
<tr>
<td>100% (24.6*)</td>
<td>290% (71.45)</td>
<td>91.3% (22.47)</td>
<td>-</td>
<td>2.7% (0.67)</td>
</tr>
<tr>
<td>100% (17.98)</td>
<td>323% (58.05)</td>
<td>86.4% (15.54)</td>
<td>238% (42.73)</td>
<td>2.2% (0.55)</td>
</tr>
<tr>
<td>100% (11.6)</td>
<td>276% (32.04)</td>
<td>90.1% (10.45)</td>
<td>268% (31.12)</td>
<td>3.4% (0.39)</td>
</tr>
<tr>
<td>100% (4.49)</td>
<td>283% (12.69)</td>
<td>60.6% (2.72)</td>
<td>212% (9.53)</td>
<td>- (&lt;0.2)</td>
</tr>
<tr>
<td>100% (0.022)</td>
<td>68.2% (0.015)</td>
<td>0.9% (0.0002)</td>
<td>182% (0.04)</td>
<td>- (&lt;0.2)</td>
</tr>
</tbody>
</table>

* cTnI results are presented in brackets as µg/L. Assay A acts as the referent.
References:


Chapter 3: Post-exercise cardiac troponin I release and clearance in normal Standardbred racehorses

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Formatted for submission to the Equine veterinary journal.

Abstract:

Reasons for performing study: There are currently no studies detailing cardiac troponin I (cTnI) release and clearance in normal horses post-exercise using an analytically validated assay. These data are essential for selecting appropriate sampling times in equine athletes with suspected myocardial injury.

Objective: To plot the magnitude and time course of cTnI release and clearance after maximal effort, using a validated cTnI assay.

Study Design: Descriptive longitudinal study.

Methods: Five clinically normal Standardbred racehorses in full race training were included in the study. Physical examinations were performed and blood samples were taken via jugular venipuncture pre-exercise. Horses were exercised in harness at near-race intensity in groups
on a training track. A second blood sample was taken immediately post-exercise and an intravenous catheter was then placed in a jugular vein. Blood samples were taken hourly for 24 hours and serum samples stored at -80°C until analysis. All samples were analyzed using the validated high-sensitivity cTnI assay (assay A) and a contemporary sensitivity cTnI assay (assay B).

**Results:** Mean resting cTnI was 1.33 ± 0.6 standard deviation (SD) ng/L (range, 0.82 - 2.33 ng/L) using assay A. All horses were below the detection limit at rest using assay B. Peak elevation occurred 2 - 6 h post-exercise with both assays (mean, 4.6 ± 1.7 h SD and 4.0 ± 2 h SD, respectively). Mean peak increase in cTnI was 11.96 ± 9.41 SD ng/L (range, 1.72 - 23.76 ng/L) using assay A. Peak levels were detectable in 3 of the horses using assay B, and were between 0.039 - 0.051 µg/L (mean 0.043 ± 0.006 µg/L). All horses returned to baseline within 24 h.

**Conclusions:** All horses experienced an increase in cTnI post-exercise, with the peak occurring 2 - 6 h post-exercise. Cardiac troponin I elevation was detected earlier using the high-sensitivity assay, which may convey a diagnostic advantage. Targeted studies are needed to determine the significance of these increases.

**Ethical Animal Research:** The study protocol was submitted to the University's Animal Care Committee, which adheres to the ethical guidelines set forth by the Canadian Council on Animal Care, reviewed by the committee, and approved. Explicit informed consent was obtained in writing for all client-owned animals.

**Declaration of Competing Interests:** Dr. Kavsak has received grants/honoraria/consultant/advisor fees from Abbott Laboratories, Abbott Point of Care, Beckman Coulter, Ortho Clinical Diagnostics, Randox Laboratories, Roche Diagnostics, Siemens Healthcare Diagnostics, and the Canadian Agency for Drugs and Technologies in Health. He is listed as an inventor on patents filed by McMaster University related to laboratory testing.
in acute cardiac care. No funding was received from the manufacturers of the assays used in this study.

**Source of Funding:** Funding for this study was provided by Equine Guelph. Support in kind was provided by Siemens Diagnostics Inc. through performing assays or provision of assay kits.

**Keywords:** Horse, cardiac troponin I, biomarkers, release curve, exercise physiology.
Introduction

In 2000, cardiac troponin I (cTnI) was chosen as a biomarker of choice for detection of myocardial injury in humans [1]. Since that time, human cTnI assays have been adopted for use in veterinary medicine, often without established protocols or analytical validation [2-5]. An important step in establishing assay protocols is determining when to sample post-injury, for which knowledge of the cTnI release curve is necessary. In human and laboratory animals, detectable increases in cTnI occur within 1 - 4 h after myocardial insult, with peak levels occurring at 4 - 24 h, depending on species [3; 6; 7]. Recommended sampling times for human patients with symptoms suggestive of acute coronary syndrome (3-6 h), anticipate this estimated peak release time, decreasing the incidence of false-negatives and improving diagnostic accuracy of cTnI assays[8-10]. To date, no studies have been performed to determine the equine cTnI release curve.

Since the introduction of cTnI testing, newer and more analytically sensitive cTnI assays have been developed and used in human medicine. Most recently, assays labelled as “high-sensitivity” have been in development, with some already in use in equine research [11; 12]. For an assay to be characterized as “high-sensitivity,” it must meet the following criteria: “the total imprecision (coefficient of variability, CV) at the 99th percentile must be ≤ 10%, and a measurable concentration of cTnI should be obtainable by the assay in ≥50% of healthy individuals.”[11; 13]. The ability of high-sensitivity assays to detect cTnI in normal individuals at rest suggests changes in cTnI could be more rapidly detected post-insult, leading to earlier therapeutic intervention.

In addition to assay validation and development of sampling protocols, knowledge of cTnI levels in subsets of the population, such as athletes, is important for diagnostic accuracy. Exercise-associated increases in cTnI have been reported in the human and veterinary
literature; however, these increases were not consistent across various reports [14-21]. Differences in study design, assay sensitivity, and exercise intensity may explain the variation. Whether this increase in cTnI is a physiologic response to exercise, or represents myocardial injury, is unknown [18; 22; 23]. Exercise-associated cTnI release presents a challenge for practitioners diagnosing myocardial disease in equine athletes. Knowledge of release and clearance after intense exercise would aid practitioners in applying and evaluating assay results in horses that have undergone recent exercise.

The objective of this study was to plot the magnitude and time course of cTnI release and clearance after maximal effort using validated cTnI assays. Samples were analysed using a high-sensitivity cTnI assay and a contemporary cTnI assay.

Methods

Animals

Standardbred racehorses, ≥2 years, in race condition were eligible for inclusion in this study. Exclusion criteria included signs of systemic illness or lameness, a heart murmur greater than 3/5, or presence of an irregularly irregular heart rhythm that did not resolve with exercise. Horses with a history of systemic illness or poor performance in the current race season (per owner/trainer history, and raceline data) were also excluded. Additionally, any horse that did not complete the race mile was excluded from analysis. A convenience sample of 5 horses, from a single training facility§ was included in this study. Data on the horses’ racing performance for the current race year were drawn from racelines provided by Standardbred Canada§. Animal use and study protocols were approved by the University of Guelph’s Animal Care Committee, Animal Utilization Protocol #1339.

Study protocol
A physical examination was performed on all study horses on the day of data collection, with particular focus on the cardiovascular system. A blood sample was taken immediately following examination via jugular venipuncture. Subjects were then equipped with an electrocardiogram (ECG) girth and monitor and exercised by their regular drivers on a half-mile dirt training track. Each horse completed a warm-up jog of at least 15 minutes, and a mile at sub-maximal intensity followed by a cool-down of light jogging/walking. A second blood sample was taken immediately following horses’ leaving the training track. The ECG girth was removed 15 minutes after completion of exercise. A 14 gauge intravenous catheter was placed in a jugular vein using aseptic technique, and a 4.1 mL extension set attached for ease of sampling. Hourly blood samples were taken via catheter for 24 hours, starting 1 hour post-exercise, using the following technique. The catheter and extension set were flushed with 10 mL of sterile saline, and then 10 mL of waste blood was drawn from the catheter and discarded. Ten mL of blood were then withdrawn and injected into a red top vacutainer tube, after which the catheter was flushed with sterile saline. The catheter was removed at the end of the sampling period and the site monitored for heat, pain, or swelling for 7 days.

All blood samples were allowed to clot for 30 - 60 minutes at room temperature, and then centrifuged for 15 minutes at 3,000 g. Serum was separated, snap frozen in liquid nitrogen, and stored at -80°C until analysis.

Electrocardiogram

Prior to harnessing, each horse was fitted with a purpose-designed saddle cloth and girth containing integrated electrodes constructed of conductive silicone rubber. Two channels of ECG data could be collected using this equipment, each with a reverse base-apex configuration. Positive lead electrodes were located to either side of the dorsal midline and were integrated into the upper half of the girth. Each lead shared a common negative
electrode situated ventrally, beneath the girth, and forming the upper surface of a standard foam girth channel. Electrodes were connected to a two channel data logger\(^e\), mounted in the racing cloth, which digitised the signal at 2,000 samples per second, per channel, with 24 bit resolution. Data were stored on a micro-SD card for later analysis.

**Cardiac troponin I assay**

Serum samples were analysed using 2 cTnI assays: high-sensitivity assay A\(^f\) (Juravinski Hospital, Hamilton, Ontario), and assay B\(^g\) (Siemens Healthcare Diagnostics Inc, Newark, Delaware). Analytical validation using equine cTnI was performed by the researchers on both assays, prior to sample analysis and demonstrated acceptable analytical performance [Chapter 2]. The limits of detection for assays A and B are 1.2 ng/L (0.0012 µg/L), and 0.015 µg/L respectively.

**Data analysis**

Results were plotted against sample time for each horse to create a visual representation of the cTnI release curve. Mean value among horses for each sample time was calculated along with standard deviation of the mean; a composite cTnI release curve was then plotted. Peak cTnI release time was defined as time at the sample point with the highest cTnI result after exercise, and was determined separately for each horse. Delta troponin level, defined as change in cTnI over baseline, was calculated for the peak. For each horse, levels of cTnI for each time point were also divided by the area under the curve, and these normalized values were used to create plots of individual release curves, together with a composite plot of the full set of 5 normalized values. Curve fitting was performed on the downward slope of each of the individual release curves using least- squared linear regression and a model for first-order exponential decay. Residual plots were used to determine goodness of fit for the composite curve. Curve fitting and analysis was performed using Origin 8.6 software\(^h\).
The cTnI half-life was determined by first calculating the elimination rate constant ($k_E$) for the downward slope of the composite cTnI release curve. The equation $T_{1/2} = \ln2/k_E$, where $T_{1/2}$ is the cTnI half-life, was used to calculate half-life [24].

ECG data were downloaded using dedicated proprietary software (MIE Medical Research, Leeds, UK), for on-screen visualization, then exported into 2-column ASCII format. A high-pass digital filter was applied to eliminate baseline (low frequency) drift in each channel. A QRS recognition algorithm written for the purpose applied a 250 Hz low-pass digital filter, then achieved QRS recognition by detecting a gradient threshold. Accurate QRS recognition was confirmed by ensuring that waveform height at the gradient threshold met a minimum threshold deviation from baseline and by confirming the presence of a point of zero gradient in the ECG signal adjacent to the gradient threshold. During analysis, thresholds were adjusted dynamically according to the mean amplitude of the block of signal being examined. Identified beats were superimposed on the original ECG signal to confirm accurate QRS recognition. Errors were corrected using the same algorithm, reapplied to the immediately adjacent ECG. If recognition could not be achieved reliably, related segments of data were discarded and the signal loss interval annotated accordingly.

**Results**

*Descriptive statistics*

Five horses (three geldings, one mare, and one stallion) were included in the study. Descriptive statistics for age, sex, gait, career earnings, raceline information, and physical examination findings, are summarized in Table 3.1. None of the horses in the study had a history of poor performance in the current race year and no medications were being administered. There were no significant findings on physical examination. Horse 2 exhibited second-degree AV block at rest, which disappeared when the horse was trotted. Horse 3 had a
grade 1/5 murmur over the right AV valve that did not worsen with exercise. All horses finished the mile working at close to their maximal intensity. It is accepted in the industry that horses do not achieve their best mile time on training tracks given inferior track conditions, and despite working at similar intensities. Thus, individual times were slower than each horse's best race time for the season (Table 3.1). Horses warmed up for a mean of 28.8 ± 8.4 standard deviation (SD) mins (range 15 - 36 mins), and finished the mile in a mean of 2:09 mins (range 2:02 - 2:23 mins). Individual mile times in this study differed by a mean of 14.32 ± 3.24 (SD) seconds (range 4.6 - 24) from the horses’ best race times that season and were classified as near-maximal intensity.

ECG results

Equipment failure occurred in 2 horses, preventing analysis of cardiac rhythm during exercise and recovery. No significant rhythm abnormalities were found in horses 1 & 5. Horse 4 exhibited brief runs of polymorphic ventricular tachycardia (2-4 beats) in the 5-minute period after cessation of exercise. None of the horses showed signs of circulatory limitation (weakness, syncope) during cool-down.

Cardiac troponin results

Serum samples were stored at -80°C for 8 - 14 months before being thawed and assayed in batches. Results were above the limit of detection (1.2 ng/L) in most horses and sampling times using assay A (Table 3.2). Resting cTnI levels were 0.82 - 2.33 ng/L (mean 1.38 ± 0.6 (SD) ng/L). All horses exhibited an increase in cTnI after exercise with peak elevation occurring 2 - 6 h post-exercise (mean 4.6 ± 1.7 (SD) h, Figures 3.1-3.7). Mean peak delta cTnI was 11.96 ± 9.41 (SD) ng/L (range, 1.72 - 23.76 ng/L). The downward slope of the cTnI release curves best fit an exponential model, with the coefficient of determination (R^2 ranging
from 0.54 - 0.99, \( R^2 \) for composite curve = 0.99). The cTnI half-life for the composite curve for assay A was calculated to be 6.4 h.

Using assay B, cTnI was below the limit of detection (0.015 ug/L) in all horses at rest (Table 3.3). Horses 1 and 5 were below the limit of detection at all time-points (Table 3.3) and so were excluded from further analysis. Horses 2 - 4 exhibited an increase in cTnI levels after exercise with the peak occurring 2 - 6 hours post-exercise (mean 4 ± 2 (SD) h). The cTnI level decreased to below the assay's limit of detection at a mean of 9 ± 1.15 h post-exercise. As the true baseline level of cTnI could not be ascertained in these horses, peak levels are presented as the absolute peak cTnI level rather as delta cTnI levels. The mean peak cTnI level was 0.043 ± 0.006µg/L (range 0.039 - 0.051µg/L). Curve fitting was not attempted on assay B as many of the results were below the detection limit and the shape of the curve could not be determined.

Discussion

Cardiac troponin I was released post-exercise in all horses examined. The magnitude of release varied widely between horses, with a range at peak increase of 1.72 - 23.76 ng/L over baseline using assay A. This increase may represent physiologic release, as a result of working at the edge of aerobic capacity, or be the result of myocardial tissue disruption. The significance of exercise-associated troponin release has been debated in the human literature for several years [18; 19; 22; 23]. The lack of other signs of cardiac disease and the short period of cTnI elevation compared to that seen in myocardial disease may indicate that these elevations are a physiologic response to exercise[22; 25]. Hickman et al. proposed that short periods of cTnI elevation could be the result of membrane bleb formation and shedding during periods of transient cellular ischemia, such as in exercise [25; 26]. Conversely, some
studies have found evidence of cardiac dysfunction in addition to biomarker release, suggesting that this release may not be benign [16, 17].

Peak cTnI levels were reached 2 - 6 h post-exercise and remained above baseline for close to 24 h. This is in agreement with a study by Durando et al. which investigated cTnI levels after near-maximal intensity exercise in Standardbred racehorses [15]. Additionally, in laboratory rats, cTnI levels peaked at 4-6 h after a single episode of myocardial injury, which would suggest that the kinetics of release are similar between species [3].

Regardless of the significance, exercise-associated cTnI release should be taken into account when evaluating horses for cardiac damage, and serial samples taken beyond the 24 hour period after exercise. Our small study population precluded multivariable analysis, and effects of age, sex, arrhythmia, and race variables were not formally assessed. Anecdotally, the older horses in this study (4 - 8 years) had higher delta cTnI than the 3 year olds (7.89 - 23.76 ng/L vs. 1.72 - 4.02 ng/L). This difference could represent cumulative myocardial damage over the horses’ race careers, associated with repeated bouts of maximal intensity exercise, or may be incidental. One horse in our study experienced brief runs of polymorphic ventricular tachycardia during exercise recovery, but did not show clinical signs of cardiovascular impairment. This arrhythmia was similar to that found in other apparently healthy Standardbred racehorses during race recovery, and which also did not result in clinical signs [27]. The delta cTnI in this horse was 7.89 ng/L, which did not exceed that of the other horses, and its release curve did not differ visually. Further studies are under way to assess any association between cardiac arrhythmias and cTnI release.

Cardiac troponin I levels were below the detection limit at most time-points using assay B, which limited data interpretation. Similar to assay A, peak cTnI levels occurred at 2 - 6 hours post-exercise. Assay A detected a rise in cTnI earlier than assay B, and levels were above the
limit of detection between 2-9 hours post-exercise in only 3 horses using assay B. Both assays could be suitable tests for clinical use, in performance animals with actual myocardial injury if serial samples were used, as serial samples would differentiate between sustained, ongoing myocardial injury and a single bout of release. The resting range of cTnI levels in healthy individuals will need to be determined before the suitability of these tests can be confirmed. However, the ability of high-sensitivity assays to detect cTnI in normal, resting animals make them essential for use in studies on the kinetics of cTnI.

Curve fitting was performed on the downward slope of both the individual cTnI release curves and the composite curve. An exponential model was found to be the best fit in all instances. This is consistent with first-order elimination and indicates that a constant percentage of plasma cTnI is eliminated from the circulation per unit time. These findings are in agreement with a study in ponies by Kaus et al. and with studies in laboratory animals [28; 29]. If cTnI is eliminated by first-order kinetics, our findings are suggestive of a single brief period of cTnI release, rather than sustained release.

This study found a cTnI plasma half-life of 6.4 hours, based on the elimination phase of the composite curve. These findings are in contrast to a previous study in ponies, in which recombinant cTnI was administered as an intravenous bolus and that determined a half-life of 0.47 hours [28]. In a rat study using exogenous cTnI, a half-life of 0.8 hours was found; a second rat study using a single episode of induced damage found a half-life of 6 hours[3; 29]. The reason for these differences may be that clearance of recombinant cTnI subunits is not representative of in vivo clearance, as the majority of naturally occurring circulating cTnI is found in complex with other troponin subunits [30]. Additionally, free cTnI subunits are more vulnerable to degradation by serum proteases, which may have shortened apparent half-life in some studies [31]. The half-life in our study may have also been affected by several further factors. As the study design was observational, exercise was used to stimulate cTnI
release, limiting control over timing and duration of release. Release of cTnI may have been more prolonged than in studies using exogenous cTnI. Calculation of pharmacokinetic half-life assumes the volume of distribution remains constant throughout the sampling period. Fluid shifts that take place during exercise and recovery between the extra- and intracellular spaces may have resulted in a variable volume of distribution and affected half-life [32]. In addition to these fluid shifts, renal blood flow and glomerular filtration rate decrease during high intensity exercise, and fluctuate between increased and decreased during recovery [33]. Changes in renal function may affect clearance of cTnI, and alter half-life, however, this is not supported by the literature [34; 35]. These factors could impact calculation of half-life in all studies, making it difficult to determine true half-life of cTnI after a single injection or a single, brief episode of myocardial compromise. Further studies are required to resolve these issues.

A negative deflection or “notch” was observed around the time of peak in the individual cTnI release curves, typically 2 - 4 hours post-exercise. This notch may reflect inherent imprecision of the assay and could be to the result of random error. However, this notch could also reflect fluid shifts occurring during the post-exertional recovery phase, resulting in an increase in plasma volume and decrease in plasma cTnI concentration. Standardbred racehorses undergo rapid shifts in fluid volume in both the extra- and intracellular space during exercise and recovery [32]. Fluid shifts include a mean decrease in plasma volume of 26.9% immediately post-race that is partially restored within 2 hours. Fluid shifts could also have blunted the peak of the release curve, resulting in underestimation of peak release. It is interesting that this notch was not detected in the composite graph, which may reflect variation in its timing between individuals. Investigation of the cTnI release curve in larger study populations could determine the biological significance of this negative deflection.
Although this study included horses considered typical of the Ontario Standardbred racehorse population, we used a convenience sample and findings may not be fully representative of the broader Ontario Standardbred racehorse population. This was at inception a Standardbred study, aimed at determining the cTnI release curve, if any, in that population after intense physical effort. Therefore, exercise was limited to Standardbred harness racing, and no additional disciplines or exercise intensities were studied. The cTnI release curve may vary with the horses’ discipline and level of training, and no attempt should be made to apply our findings directly to horses performing light or submaximal effort. Further studies are needed to explore the effect of exercise intensity and duration on cTnI release in the horse.

Samples were stored at -80°C for several months before analysis, which may have affected assay results. However, previous reports have determined that long-term storage of serum samples at this temperature had little or no impact of cTnI results [3; 36] [Chapter 2]. Another limitation is our small sample size. Larger sample sizes would allow a more in-depth examination of cTnI release and clearance, provide greater insight into the significance of this release, and clarify the effect of demographic and performance variables.

**Conclusions**

This study reveals that horses may experience a rise in cardiac troponin I post-exercise with peak levels occurring 2 - 6 hours after cessation of intense activity. This elevation was detectable within 1 hour after exercise using the high-sensitivity troponin-I assay, and in 3 of 5 horses at 2 - 6 hours post-exercise using assay B. Characterization of release is highly dependent on the analytical sensitivity of the assay, and sampling protocol, therefore researchers interested in the kinetics of cTnI should consider using high-sensitivity assays. Exercise-associated myocardial damage, where present, can be detected earlier using a high-
sensitivity assay. Knowledge of the post-exercise cTnI release curve should aid practitioners in accurately assessing myocardial status in performance animals.

**Acknowledgements:** This study was made possible by the participation of Robert Fellows Stables and the hard work of our summer students. A special thanks to Lorna Clark at Juravinski Hospital for running our serum samples.

**Footnotes**

a. Robert Fellows Stables, Rockwood, ON, Canada

b. Standardbred Canada, Mississauga, ON, Canada

c. 14 ga x13 cm (5.25 in) polyurethane catheter-over-needle, Mila International Inc., Erlanger, KY, USA

d. Elastosil LR 3162, Wacker Chemical Corporation, Adrian, MO

e. MIE Data Logger, MIE Medical Research, Leeds, UK

f. Abbott ARCHITECT STAT High Sensitivity Troponin-I assay, Abbott Park, IL, USA

g. Siemens Dimension Vista® Troponin-I assay, Siemens Healthcare Diagnostics Inc, Newark, DE, USA

h. Microcal OriginPro 8.6, OriginLab Corporation, Northampton, MA

**References**


Figures and Tables:

**Table 3.1**: Description of signalment, raceline information, and training mile time of study horses.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Horse 1</th>
<th>Horse 2</th>
<th>Horse 3</th>
<th>Horse 4</th>
<th>Horse 5</th>
<th>Mean</th>
<th>SD^1</th>
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<td>4</td>
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<td>3</td>
<td>4.8</td>
<td>2.2</td>
</tr>
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<td>Sex^2</td>
<td>G</td>
<td>G</td>
<td>G</td>
<td>H</td>
<td>M</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gait^3</td>
<td>P</td>
<td>T</td>
<td>P</td>
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<td>P</td>
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<td>Race starts</td>
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<td>9</td>
<td>38.6</td>
<td>34.4</td>
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<td>161.1</td>
<td>3.6</td>
<td>168.1</td>
<td>40.8</td>
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<td>Resting heart rate^5</td>
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<td>Respiratory rate^6</td>
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<td>114.7</td>
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</tr>
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</table>

1. Standard deviation
2. G= gelding, H= horse, M= mare
3. P= pacer, T= trotter
4. Earnings in thousands, recorded in Canadian dollars (CAD)
5. Beats/minute
6. Breaths/minute
7. Measured in seconds
Table 3.2: Assay A cardiac troponin I results* by time post-exercise. Mean and standard deviation presented (all concentrations are above limit of blank >0.6 ng/L).

<table>
<thead>
<tr>
<th>Time</th>
<th>Horse 1</th>
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<th>Horse 3</th>
<th>Horse 4</th>
<th>Horse 5</th>
<th>Mean</th>
<th>SD2</th>
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</tr>
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<td>1.07</td>
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*ctTnI results in ng/L

1. Time post-exercise in hours
2. Standard deviation of the mean
Table 3.3: Assay B cardiac troponin I results** by time post-exercise. Mean and standard deviation presented.

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**cTnI results in ug/L

1. Time in hours
2. Mean calculated based on horses with measurable cTnI levels
3. Standard deviation of the mean
Figure 3.1: Cardiac troponin I release curve for horse 1, presented as cTnI level by time after exercise (Assay A).
Figure 3.2: Cardiac troponin I release curve for horse 2, presented as cTnI level by time after exercise (Assay A).
Figure 3.3: Cardiac troponin I release curve for horse 3, presented as cTnI level by time after exercise (Assay A).
Figure 3.4: Cardiac troponin I release curve for horse 4, presented as cTnI level by time after exercise (Assay A).
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Chapter 4: Effect of racing on cardiac troponin I concentration and associations with cardiac rhythm in Standardbred racehorses

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Abstract

Reason for performing study: There are currently no studies investigating the effect of race-intensity exercise on cardiac troponin I (cTnI) concentrations in healthy Standardbred racehorses using a validated, high-sensitivity cTnI assay. Use of these assays allows determination of a resting reference interval and 99th percentile. Information on the relationship between demographic variables and cTnI concentrations would aid practitioners and researchers in interpreting cTnI results.

Accumulating evidence indicates maximal athletic effort can be associated with myocardial dysfunction and damage in humans; a recent equine study documented complex ventricular
arrhythmias during race recovery, a period associated with a peak in sudden deaths in both humans and horses. Investigating the impact of maximal effort on myocardium and possible association of myocardial injury with arrhythmia requires a high-sensitivity cardiac troponin assay.

**Objectives:** i) to determine resting cTnI and effect of racing on cTnI in Standardbred horses using a validated high-sensitivity assay, ii) to explore associations between exercise-associated arrhythmia and cTnI concentration, and iii) to compare the magnitude and time course of cTnI release in horses with exercise-associated ventricular arrhythmia with that of control horses.

**Study design:** Prospective cohort study.

**Methods:** Pre- and post-race cTnI concentrations were measured in 158 routine race starts. Continuous ECG monitoring was applied to sampled horses during rest, warm-up, race, and race recovery, and screened for presence of complex ventricular arrhythmia. A subset of the study population, selected based on presence or absence of arrhythmia, underwent extended cTnI sampling at 2-6, 12, 24, and 36 hrs. Demographic data and performance data collected from racelines were used in multivariable linear regression modeling to explore associations with post-race cTnI concentration and arrhythmia.

**Results:** Mean±SD cTnI concentration was 2.21±2.44 ng/L (range = 0.22-37.61) at rest; mean post-race concentration was 19.54±176.37 ng/L (range = 0.48-2168). There was a statistically significant increase in mean cTnI concentration post-race of 18.34±181.07 ng/L compared to pre-race (p<0.0001). Trotting gait and age were positively associated with resting cTnI concentration in multivariable analysis. Post-race cTnI concentration was positively associated with pre-race concentration, and there was a significant interaction between arrhythmia status and finishing distanced. No difference was observed in the time
course of cTnI release between arrhythmia cases and controls. The magnitude of cTnI release in extended sampling was greater for control horses vs. cases (41.17±48.23 SD vs. 9.81±11.87 SD in ng/L).

**Conclusions:** Use of a high-sensitivity cTnI assay allowed for detection of cTnI concentration in all study horses and determination of a reference interval. Variance in the resting population was high and population distribution was right-sided with a long right tail. Maximal intensity exercise resulted in elevations in cTnI. A synergistic effect was observed in horses that finished distanced and also experienced arrhythmia with the presence of both associated with higher cTnI release than the presence of either alone or the absence of both. Arrhythmia was not observed to affect the time course or magnitude of cTnI release in the first 36 hours post-race.

**Ethical Animal Research:** The study protocol was submitted to the University's Animal Care Committee, which adheres to the ethical guidelines set forth by the Canadian Council on Animal Care, reviewed by the committee, and approved. Explicit informed consent was obtained through race entry for all client-owned animals and written consent obtained for all animals participating in extended sampling.

**Declaration of Competing Interests:** Dr. Kavsak has received grants/honoraria/consultant/advisor fees from Abbott Laboratories, Abbott Point of Care, Beckman Coulter, Ortho Clinical Diagnostics, Randox Laboratories, Roche Diagnostics, Siemens Healthcare Diagnostics and the Canadian Agency for Drugs and Technologies in Health. He is listed as an inventor on patents filed by McMaster University related to laboratory testing in acute cardiac care. No funding was received from the manufacturers of the assays used in this study.
**Source of Funding:** Funding for this study was provided by Equine Guelph. Support in kind was provided by Siemens Diagnostics Inc. through performing assays or provision of assay kits.

**Keywords:** Biomarker, Cardiac troponin, Exercise-associated arrhythmia, Exercise physiology, Horse, Sudden death.

**Introduction:**

Cardiac troponin I (cTnI) is currently considered the gold standard for detection of myocardial injury as defined in the Third Universal Definition of Myocardial Infarction [1]. The recent introduction of high-sensitivity cTnI assays has allowed resting concentrations to be determined in healthy populations [2; 3]. Aside from aiding in the determination of reference intervals and the 99th percentile cut-off, higher sensitivity has provided insight into factors that may have an effect on resting concentrations, such as age, sex, and race [2-4]. Better understanding of the impact of demographic factors improves diagnostic accuracy and reduces false negative results. As interest in cTnI assays for detection of myocardial injury in horses has risen in recent years, such factors need to be explored and reference intervals and cut-offs established. Additionally, the effect of maximal intensity exercise needs to be investigated, and clinicians and researchers made aware of impacts on cTnI concentrations. Accumulating evidence indicates that near-maximal intensity exercise may be associated with myocardial dysfunction and injury [5-10]. Potential clinical consequences in horses may include poor performance, exercise-associated pulmonary hemorrhage, or sudden death [11-15]. Although considered a rare event, a study by Boden et al revealed that ~26% of track-side mortality was classified as sudden death [13]. These sudden deaths often occur during live racing and in view of the public, which impacts lay perceptions of the racing industry, as well as horse and handler welfare.
Several postmortem studies of trackside mortality in horses, including those described as sudden death, have found signs consistent with cardiac involvement such as rupture of pulmonary and thoracic vessels, pulmonary congestion and edema, and pulmonary hemorrhage [13; 16; 17]. However, a definitive cause of death was not found in almost half of cases. The peracute nature of exercise-associated sudden death, coupled with the scarcity of clinically significant postmortem findings, is suggestive of fatal cardiac rhythm disturbance. However, histopathology of the myocardium in cardiac cases is often unrewarding as myocardial tissue changes can take hours to days to become apparent [18].

Several studies investigating cardiac rhythm during and after exercise in performance horses have revealed single ventricular ectopic beats, punctuated deceleration, and complex ventricular arrhythmias [5; 19-21]. These studies included an investigation of presumptively healthy Standardbred racehorses during live racing, which revealed complex ventricular rhythm disturbances in the immediate post-race recovery period in 18% of horses and 16% of race events [5]. Although none of the horses in this study suffered sudden death, these arrhythmias occurred in the period during recovery associated with a peak incidence of sudden death in humans and horses [13; 22]. These findings suggest that in some circumstances rhythm disturbances may be pathologic and reflect, or lead to, myocardial injury. A sensitive and specific marker of myocardial damage is necessary to further explore this interpretation.

Studies investigating the effect of intense exercise on cTnI concentrations have been performed in horses and humans with variable results [6; 8; 23-26]. The lack of agreement between studies may be the result of differences in study populations, sample timing, lack of analytical validation of assays, assay sensitivity, or type of exercise examined. Recent studies have identified the peak timing of cTnI release post-exercise in horses as being 2-6 hours, which should increase the accuracy of future study results [23][Chapter 3]. Additionally, the
introduction of high-sensitivity cTnI assays able to detect cTnI at a far lower concentration than previous assays should allow for comparisons of resting and post-exercise levels of cTnI.

The objectives of this study were to measure resting cTnI and determine the effect of racing on cTnI in Standardbred horses using a validated high-sensitivity assay and to examine the association between exercise-associated arrhythmia and cTnI concentration in these horses. Additionally a small subpopulation of horses was sampled to compare the time course and magnitude of cTnI release in horses experiencing rhythm disturbance with control animals.

Methods:

Animals and race selection

Standardbred horses ≥ 3 years, racing at Mohawk racetrack* in southern Ontario between August-October 2014, were eligible for inclusion in this prospective cohort study. In collaboration with track management, the horsemen, and the Ontario Racing Commission, study participation was made a condition of race entry in posted sampled races, and thus owner/trainer consent was obtained at time of race entry. A convenience sample of 2 races per study night was identified by track management, selected to allow sufficient time between races to equip and sample horses. Stakes races and races for 2-year-old horses were excluded. A small subpopulation of horses was selected for extended sampling based on presence of post-race arrhythmia and owner co-operation. Informed written consent was obtained from owners/trainers for extended sampling at the time of enrolment. Animal use and study protocols were approved by the University of Guelph’s Animal Care Committee, Animal Utilization Protocol #1339.

Study protocol
Horse identity was confirmed on arrival at the racetrack by freeze brand and comparison with the race schedule. Study protocol was explained to grooms and trainers by members of the research team before proceeding. A pre-race blood sample was obtained via jugular venipuncture and collected into a red top vacutainer tube. In cases in which horses were selected by track management for routine TCO₂ screening, the track technician collected a second tube for the study to minimise venipuncture.

An adjustable electrocardiogram (ECG) girth was applied to the horse prior to harnessing and an attached Holter monitor set to record. Horses were then harnessed by their grooms and warmed up as usual. The race then proceeded as scheduled. The Holter monitors recorded continuously throughout warm-up, the race event, and for 15 mins after returning to the paddock. ECG recordings were immediately reviewed by a member of the team (PPS) and those with arrhythmia identified to recruit horses for extended sampling.

A second blood sample was collected via jugular venipuncture as close to 2 hours post-race as possible and precise time of sampling from the end of the race recorded. Blood samples were allowed to clot for 45-60 mins at room temperature and then centrifuged for 15 mins at 3,000 x g. Serum was separated and aliquoted into 1.5 mL microcentrifuge tubes, then snap frozen in liquid nitrogen and stored at -80°C until analysis.

**Extended sampling**

Horses identified with arrhythmia and control horses running in the same race were selected for further measurement of cTnI concentration. Blood samples were collected via jugular venipuncture at 2-6, 12, 24, and 36 hours post-race and processed as above. Wherever possible, samples were taken by the research team. In cases where logistics precluded this approach, the trainer was given written instructions for sample collection and timing, and samples were stored at 4°C until retrieved and processed by researchers.
**ECG equipment**

The ECG equipment consisted of an adjustable elastic girth embedded with four electrically conductive silicone rubber electrodes. Electrodes were positioned to provide two inverse base-apex leads, with positive electrodes lying dorsally and a single negative electrode positioned ventrally. An additional ground electrode was positioned on the right thorax between the withers and ventrum. A two channel solid state Holter monitor was connected to the leads and attached to the race harness at the girth.

**cTnI analysis**

Serum samples were analysed using two cTnI assays: a high-sensitivity assay A and a contemporary sensitivity assay B. Analytical validation using equine cTnI was previously performed by the researchers on both assays, and demonstrated acceptable analytical performance [Chapter 2]. Concentrations below 0.6 ng/L (0.0006 µg/L) for Assay A, and 0.015 µg/L for Assay B are reported as <0.6 ng/L and <0.015 ug/L, respectively.

**99th Percentile**

The 95th and 99th Percentile of the resting cTnI concentrations was calculated using StataIC 14.1 statistical software. Outliers with probable or confirmed myocardial disease, based on ECG assessment and later race performance, were excluded from the data set. Data distribution was graphed, and data were transformed if necessary to create an approximately normal distribution before calculating the 99th percentile.

**ECG analysis**

ECG recordings were downloaded from Holter monitors to a laptop computer in proprietary format for on-screen display. An initial visual analysis of ECGs from the end of pre-race warm-up to as far into post-race recovery as possible before harness removal was performed.
at the track to identify subjects for extended sampling. A second beat-by-beat analysis of the entire tracing was performed at a later time to confirm presence/absence of a rhythm disturbance. An arrhythmia case was defined as torsades-like polymorphic ventricular tachycardia, clusters of multiform ventricular premature beats, re-entrant phenomenon, or non-sustained monomorphic ventricular tachycardia [5].

Race data

Demographic and performance data for horses were drawn from on-line records provided by Standardbred Canada. Independent variables used in statistical analysis were sex, age, gait, Lasix use, post position, finish position, finish time, parked at the 2nd quarter, interruption (breaking) of gait in the final stretch, purse, and finished distanced.

Statistical analysis

The difference between pre and post-race cTnI serum concentrations was assessed using a paired t-test. Normality of the difference between pre and post-race cTnI was tested using a Shapiro-Wilks test of normality. Data were transformed if the distribution was non-parametric. Two linear regression models: one with pre-race cTnI, and one with post-race cTnI as dependant variables were performed as follows. Univariable linear regression was performed on each explanatory variable. For the pre-race cTnI analysis explanatory variables tested are listed in Table 4.3. Explanatory variables tested in the post-race cTnI analysis are listed in Table 4.5. Linearity was assessed for each continuous variable by introduction of a quadratic term and visual inspection of a Lowess curve. If the variable was not linear, then the variable was categorized based on quartiles. Statistical significance for initial modeling was set at $\alpha = 0.2$. Multivariable linear regression was performed including all explanatory variables significant in the univariable analysis. Variables of interest were added into the multivariable model to check for statistical significance. Subsequently, variables that were
not significant at \( p<0.05 \) were removed from the model in a stepwise manner, checking for confounding. Confounding was defined a change in other variable coefficients of \( \geq 20\% \) upon removal of the confounder from the model. Identified confounders were kept in the final model. Two-way interaction terms were generated for all retained variables and tested for significance using a partial F test. Contrast statements were generated for any significant interactions between categorical variables, and predicted curves were graphed for continuous variables. Model fitness was tested by visual inspection of standardized residuals, leverage, Cook’s D, Dfits, and Dfbeta. Outliers were identified and scrutinized for errors in data coding. The significance of outliers in the model was assessed by running the model with outliers as a fixed effect. Outliers were removed from the data set if coding errors were identified. Normal distribution of standardized residuals was assessed by histogram and a Shapiro-Wilk’s test. A Cook-Weisberg test was performed to test for homoscedasticity. Clustering by horse or race ID was determined via a multi-level model with random intercepts for horse and race ID, and Akaike information criteria were used to determine the best model. Fit of multilevel model was determined by visual inspection of best linear unbiased predictions (BLUPs). All statistical modeling was performed using StataIC 14.1 statistical software. Post-race cTnI release curves were generated for horses participating in extended sampling. Results were plotted against sample time for each horse to create a visual representation of the cTnI release curve. Mean values among case and control horses for each sample time were calculated along with standard deviation of the mean; a composite cTnI release curve was then plotted. Graphs were produced using Origin 8.6 software.

**Results:**

*Descriptive statistics*
The unit of interest in this study was race start. Data were collected from 145 horses in 158 race starts and 18 race events. Thirteen horses were sampled twice. Demographic and performance data are summarized in Table 4.1. Descriptive data for Assay A cTnI results are presented in Table 4.2.

**Cardiac rhythm**

ECG recordings were of non-diagnostic quality during race and recovery in 20/158 race starts (12.6%). 16/138 (11.6%) of race starts met study criteria for an arrhythmia case (Table 4.1). One horse developed atrial fibrillation in addition to polymorphic ventricular tachycardia in the post-race recovery period. Diagnostic recordings were obtained in the pre-race rest period in 154/158 race starts (97.5%). Analysis of resting tracings revealed the following arrhythmias in the 154 race starts: second-degree atrioventricular block in 12 horses (7.8%) cases, sinoatrial block in 2 horses (1.3%), multiple isolated supraventricular premature beats (SVPB) in 10 horses (6.5%), and single ventricular ectopic beats in 5 horses (3.2%). Four horses experienced multiple resting arrhythmias including: single SVPBs as well as a ventricular ectopic (n = 1), second-degree heart block in addition to multiple lone SVPBs (n = 2), and second-degree heart block and multiple lone ventricular ectopics (n= 1).

**Pre-race cTnI analysis**

Resting cTnI concentrations were obtained in 147 race starts. Eleven resting samples were lost or excluded due to accidental destruction (n = 8), failure to yield adequate serum (n= 2), or evident systemic disease (n = 1). Data for assay B were transformed using a log 10 transformation to create a normal distribution before calculating the 95th and 99th percentile. The 95th and 99th percentile of the upper limit of the resting cTnI concentration range for assays A were 5.5 and 10.5 ng/L respectively. The 99th percentile for assay B was 0.025 µg/L. Most resting cTnI concentrations were below the limit of detection (0.015 µg/L) using
assay B, which precluded further analysis. Associations between resting cTnI results and demographic variables (assay A), were investigated using linear regression. As few stallions (n=9) were sampled in this study, geldings and stallions were grouped together as “male”.

Resting cTnI concentrations were not normally distributed and were transformed using a log 10 transformation to meet model assumptions. Results of univariable analysis are summarized in Table 4.3. Variables for gait and age were included in the multivariable model (Table 4.4) and significant positive associations between trotters and resting cTnI concentration, as well as increasing age and resting cTnI concentration were found. One horse was dropped from the data set before analysis because of a high cTnI concentration (53 ng/L), which was suggestive of active myocardial disease or injury. The subsequent model fit was assessed as described and no significant (> 3 standard deviations from mean) outliers were identified.

Post-race cTnI results

Many post-race cTnI results were under the limit of detection using assay B, which precluded analysis.

Post-race cTnI concentrations were obtained using assay A in 151 race starts. A histogram of the change in cTnI concentration (delta) between pre and post-race is shown in Figure 4.1. Results were not normally distributed and were transformed using a log 10 transformation. A paired t-test comparing the log 10 of resting and post-race cTnI concentrations showed an increase in cTnI within 2 hours after racing to be significant (Mean = 2.15 ng/L, 95% CI =1.87-2.49 ng/L, p<0.0001).

Results of univariable linear regression are summarized in Table 4.5. Gait, log 10 pre-race cTnI concentration, finish time, and finished distanced were significant and were included in
multivariable analysis (Table 4.6). Arrhythmia and sample time were introduced as variables of interest. A positive association between log10 pre-race and post-race cTnI concentrations was found to be statistically significant in the final model (p<0.0001). The interaction term for arrhythmia*finished distanced was also found to be significant (p<0.0001) (Table 4.7). A contrast statement table was set up and all combinations of arrhythmia and finished distanced were tested for significant differences. Sample time was found to be a confounder of arrhythmia and finished distanced and was retained in the final model.

Model diagnostics identified one data point as having a high leverage in the model. This horse had a high cTnI concentration post-race (2,168.85 ng/L), and had developed polymorphic ventricular tachycardia and re-entrant phenomenon during race recovery. However, removal of this point from the model did not change the significance of any variables and therefore it was retained in the final analysis. Tests for model fitness, with the exception of the Shapiro-Wilkes test of normality (p=0.01), revealed that the model satisfied the assumptions of linear regression. Results from the multilevel random effect model showed no clustering by race ID or horse.

Extended sampling

Data were collected from 6 case horses and 5 controls. Samples were collected for all time points for 5/6 cases and 4/5 controls. The 2-6 hour sample was missing in 1 case and the 36 hour sample in 1 control. Samples taken by trainers were picked up and processed within 24 hours of collection. All horses reached peak cTnI concentrations at 2-6 hours post-race. Mean peak cTnI concentration in ng/L for cases and controls was 9.81±11.87 SD and 41.17±48.23 SD respectively. The composite release curves for cases and control are shown in Figure 4.3. The small sample size precluded further analysis.
**Discussion:**

A positive association between age and resting cTnI concentration was found to be statistically significant in our multivariable model. A similar observation has been found in human populations and is usually attributed to the increased prevalence of occult coronary artery disease in aged populations [2; 4]. In horses, the association may be the result of changes in myocardial turnover or the cumulative effect of longer racing careers and exercise-associated cardiac remodeling [27; 28]. An association was also found between gait and resting cTnI concentration, with trotters having higher concentrations than pacers. Although both groups are classed as ‘Standardbreds’, they are largely distinct genetic populations.

Despite determining a resting range of cTnI concentrations in our study population, a diagnostic cutoff could not be established in absence of clinical validation. In human medicine, the 99th percentile of the cTnI reference interval is used as a cut-off for diagnosis of myocardial infarction, and indicates a range above which results should be interpreted as diagnostic of disease, or positive [1; 2]. However, this cutoff should not be implemented in equine medicine without performing clinical validation. A disease of interest should first be identified in horses and studies performed to determine the diagnostic sensitivity and specificity of the assay for that disease. Receiver operator curves would then identify the ideal cutoff value with reference to the consequences of a false-positive or false-negative diagnosis. These criteria are challenging in equine medicine as diseases of myocardium are uncommon and often occur secondary to toxicity or systemic disease, rather than a disease with a single known etiology. Gold standards with which to confirm myocardial disease are also lacking given that diagnostic imaging for myocardium is not available for horses and
postmortem investigation is often unrewarding. Consequently, clinical use of the 99th percentile in this study is not recommended.

However, the 99th percentile is useful for comparing populations and proposing variables that may impact resting cTnI concentrations. Analysis of resting cTnI concentrations in this study found a 99th percentile of 10.4 ng/L for assay A and 0.025 µg/L for assay B. Studies investigating the 99th percentile in humans for assay A have derived concentrations of 19-23 ng/L, higher than the results of this study [29; 30]. This difference may reflect species effects or demographic differences in sample populations. For example, the human 99th percentile concentration for males is usually higher than that for females (27 vs. 11 ng/L & 36 vs. 15 ng/L in the cited studies), possibly given the higher incidence of ischemic heart disease in men, larger heart size or differences in circulating catecholamines [2-4; 29-31]. Our study population was 94% mares and geldings, which may have decreased the 99th percentile concentration. Alternatively, coronary artery disease has not been described in horses, and its prevalence in the human population may account for the difference [3; 31]. Additionally, an association between age and cTnI concentration has been observed, with older subjects typically having higher concentrations [2; 4]. Our population consisted of young racehorses (3-12 years). Our 99th percentile for assay B was similar to that published for human populations (21 ng/L & 22 ng/L) [29; 32].

A statistically significant increase in cTnI concentration was observed within 2 hours post-race in this study. The median increase (delta) in concentration was 1.36 ng/L with wide individual variation (0-2165 ng/L)(Figure 4.1). The distribution of delta cTnI was right-sided with a long right tail and was approximately normal in a log 10 transformation. Post-race 9.2% of subject had cTnI concentrations exceeding the 99th percentile. The observations in this right tail represent horses with large increases in cTnI post-race and may be the result of a separate mechanism of cTnI release from that in other horses, or an extreme expression of a
single mechanism. Mild increases in cTnI concentration may occur because of increased cell membrane permeability as a result of changes in oxygen tensions or local pH during exercise [33; 34]. However this theory is controversial and high cTnI release may represent irreversible myocardial injury. Horses with moderate-high delta cTnI concentrations may be experiencing myocardial necrosis because of pre-existing cardiac disease, psychologic stress, or the lack of aerobic capacity necessary for racing.

The delta cTnI concentrations seen in this study agree with previous studies on the impact of exercise on cTnI release in horses and humans, and suggest a possible relationship between intense exercise and myocardial injury [8; 9; 23-25]. Findings in a previous study investigating the cTnI release curve after near-maximal intensity exercise suggest that these elevations in concentration do not coincide with the timing of hemoconcentration after exercise cessation [Chapter 3] [35]. Increase in cTnI concentration is thus likely not the result of fluid shifts out of the vascular space, but may represent actual damage to myocytes. In most cases the increase in cTnI concentration was mild-moderate; the clinical significance of repeated episodes of cTnI release is yet to be determined. However, clinicians should be aware that exercise-associated cTnI elevations exist and be aware of their timing when making clinical decisions in performance horses. Further, longitudinal studies comparing horses with high delta cTnI concentrations to those without should be conducted to further investigate the clinical significance of these differences.

The incidence of complex ventricular arrhythmia in this study was 12%, lower than the 18% previously described by Physick-Sheard and McGurrin [5]. This lower incidence may be reflective of sample selection or may be the result of differing track conditions, or random error. Non-diagnostic ECG recordings in both studies may have introduced sampling bias in the form of response bias, affecting the internal validity of results [24].
All horses exhibiting complex ventricular arrhythmia spontaneously reverted to sinus rhythm without clinical intervention. One horse developed atrial fibrillation during race recovery and did not revert to sinus rhythm during the period of ECG monitoring. This horse was unfortunately lost to follow-up. Moderate-severe pulmonary edema developed in another horse, coinciding with the timing of torsade-like polymorphic tachycardia. This horse reverted to sinus rhythm and was treated by its regular veterinarian for pulmonary edema.

Multivariable analysis found post-race cTnI concentrations were greater in horses with complex ventricular arrhythmia that finished distanced, than horses with either variable alone or the absence of both. This relationship indicates that in some cases, complex ventricular arrhythmia may be associated with sufficient myocardial damage to result in poor race performance, although the direction of this relationship remains unclear. Also, although arrhythmia may follow damage, it is possible this association is not a simple sequential relationship given that arrhythmia may precipitate greater myocardial injury, resulting in more episodes of arrhythmia. Physick-Sheard & McGurrin have suggested that these arrhythmias may be triggered by autonomic imbalance and increased vagal tone with contributions from psychologic stress [5]. Psychological stress has been shown to trigger ventricular arrhythmias, and to result in myocardial dysfunction possibly due to acute increases in sympathetic drive and catecholamine release [36-38]. The psychological stress and related myocardial injury resulting from finishing distanced may have led to an increased incidence and severity of arrhythmia, which in turn increased myocardial damage. The consequence of this effect may have been a more severe and sustained release of cTnI from cardiac myocytes, given that the magnitude of cTnI release correlates with severity of myocardial injury [39; 40].

The interaction between arrhythmia and finishing distanced was based on a small number (n = 3) of horses who were positive for both. However, the magnitude of cTnI release in these
horses was such that the biological significance of this association should not be ignored. Case-control studies, as well as, longitudinal studies with larger sample sizes should be performed to further investigate the biological and clinical significance of these findings.

There was no difference in the time course of cTnI release during the first 36 hours post-race between arrhythmia cases and controls in this study. All horses reached peak concentrations 2-6 hours post-race, which is in agreement with previous studies [23][Chapter 3]. Controls had a higher magnitude of cTnI release versus cases (41.17±48.23 SD vs. 9.81±11.87 SD in ng/L), which may reflect differences in subject selection between groups, or the small sample size. Case horses were selected based on the presence of arrhythmia and owner willingness to participate. These horses were often stabled long distances from the racetrack, therefore researchers relied on trainers to take and store blood samples for up to 24 hours. Controls were selected on the absence of arrhythmias, owner participation, and proximity to the racetrack, which allowed researchers to collect and process samples. Differences in sample handling and storage may have affected cTnI concentrations. However, previous studies have shown that pre-analytical storage for time periods and at temperatures similar to those in this study did not affect cTnI concentrations [41][Chapter 2]. Further investigations with a larger sample size should be performed to determine the effect of arrhythmia on the cTnI release curve.

The results of this study are based on a convenience sample of racehorses competing at a single Ontario racetrack. This represents a possible study limitation, as the population may not be representative of all Standardbred racehorse populations. Efforts were taken to ensure that the study population was similar to the general racetrack population in terms of age, gait, and sex. However, lack of 2-year-old racehorses and stakes races in this study may have decreased the number of intact males and impacted other aspects of the sample population. Use of a single racetrack reflected logistical constraints, but impacts the external validity of
our results. Further multicentre studies are needed in order to investigate the generalizability of our findings in multiple populations.

The difference between pre- and post-race cTnI concentration may have been underestimated in this study as many horses were sampled before the known peak of cTnI release post-exercise (2-6 hrs) [23][Chapter 3]. At the time of sampling, Mohawk Racetrack did not offer on-track stabling; therefore all horses were driven in for their race and returned to their home stable the same night. Often horses stayed in the race paddock for only ~1 hour post-race before leaving. This presented a challenge for researchers that resulted in horses being sampled <2-4 hours post-race. Sample time was included in the multivariable analysis to control for this potential limitation.

This study demonstrates that race intensity exercise in Standardbred horses may be associated with elevation in serum concentration of cTnI, as determined using a validated assay, and provides guidance on the range of elevations that might be encountered. The magnitude of this elevation of some individual horses whose performance has caused no current concern may nonetheless be experiencing active myocardial disease. Routine Standardbred racing is occasionally associated with the development of complex ventricular arrhythmias during race recovery. In some of these cases, arrhythmias are associated with elevations in serum cTnI concentration suggestive of myocardial damage. Practitioners evaluating horses post-race should be aware of the possibility of exercise-associated arrhythmia and exercise-associated cTnI release when making diagnostic and therapeutic plans. The causal relationship between cTnI release and arrhythmia remains unclear, and further studies are needed before drawing firm conclusions.

Footnotes

a. Mohawk Racetrack, Woodbine Entertainment Group, Campbellville, ON, Canada
b. Elastosil LR 3162, Wacker Chemical Corporation, Adrian, MO, USA

c. Trillium 5000, Forest Medical, East Syracuse, NY, USA

d. Abbott ARCHITECT STAT High Sensitivity Troponin-I assay, Abbott Park, IL, USA

e. Siemens Dimension Vista® Troponin-I assay, Siemens Healthcare Diagnostics Inc, Newark, DE, USA

f. Standardbred Canada, Mississauga, ON, Canada

g. StataIC 14.1, StataCorp LP, College Station, TX, USA

h. Microcal OriginPro 8.6, OriginLab Corporation, Northampton, MA, USA

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racehorse fatality in flat starts in Victoria, Australia (1989-2004). *Equine Veterinary

racehorse fatality in jump starts in Victoria, Australia (1989-2004). *Equine Veterinary


Preanalytical storage does not affect 99th percentile cardiac troponin T concentrations measured with a high-sensitivity assay. Clinical Chemistry 59, 442-443.
Tables and figures:

Table 4.1: Descriptive statistics for signalment, race information, and arrhythmia.

<table>
<thead>
<tr>
<th>Variable</th>
<th>n (%)</th>
<th>Mean±SD (range)¹</th>
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</thead>
<tbody>
<tr>
<td><strong>Gait:</strong> Trotter</td>
<td>46 (29.1%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>112 (70.9%)</td>
<td></td>
</tr>
<tr>
<td><strong>Sex:</strong> Mare</td>
<td>48 (30.4%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>9 (5.7%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>101 (63.9%)</td>
<td></td>
</tr>
<tr>
<td>Lasix</td>
<td>34 (21.5%)</td>
<td></td>
</tr>
<tr>
<td><strong>Age</strong>²</td>
<td></td>
<td>4.88±2.01 (3-12)</td>
</tr>
<tr>
<td><strong>Purse</strong>³</td>
<td></td>
<td>11,759±3,770 (8,000-18,000)</td>
</tr>
<tr>
<td><strong>Finished Distanced</strong>⁴</td>
<td>7 (4.5%)</td>
<td></td>
</tr>
<tr>
<td><strong>Parked at Half-Mile</strong>⁵</td>
<td>58 (36.8%)</td>
<td></td>
</tr>
<tr>
<td><strong>Finish Time</strong>⁶</td>
<td></td>
<td>114.99±2.48 (109.6-120.8)</td>
</tr>
<tr>
<td><strong>Arrhythmia</strong></td>
<td>16 (11.6%)</td>
<td></td>
</tr>
<tr>
<td><strong>Sample Time:</strong> &lt;1 hour</td>
<td>96 (61.9%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>≥1 hour</td>
<td>59 (38.1%)</td>
</tr>
</tbody>
</table>

¹ Population mean with standard deviation and range, ² Age in years, ³ Purse in Canadian dollars, ⁴ Finished Distanced = When a horse finishes more than 35 lengths behind the winner., ⁵ Parked = When a horse cannot find a position along the rail in a race and is forced to race outside those on the inside. ⁶ Finish time in seconds.
Table 4.2: Descriptive statistics for the assay A cardiac troponin I results.

<table>
<thead>
<tr>
<th>Variable</th>
<th>n</th>
<th>Median (IQR)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-Race cTnI*</td>
<td>147</td>
<td>1.58 (1.11-2.31)</td>
</tr>
<tr>
<td>Post-Race cTnI*</td>
<td>151</td>
<td>3.27 (1.92-9.64)</td>
</tr>
<tr>
<td>Absolute Delta cTnI*</td>
<td>143</td>
<td>1.36 (0.49-2.81)</td>
</tr>
<tr>
<td>Relative Delta cTnI**</td>
<td>143</td>
<td>79.8 (34.4-180.1)</td>
</tr>
</tbody>
</table>

1 Population median with interquartile range (IQR), * cTnI results stated in ng/L, ** Results stated in %
Table 4.3: Results of univariable linear regression analysis with log 10 pre-race cTnI as the dependant variable.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Coefficient (95%CI)$^1$</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>0.04 (0.01-0.06)</td>
<td>0.002$^*$</td>
</tr>
<tr>
<td>Gait: Trotter</td>
<td>0.09 (-0.01-0.19)</td>
<td>0.09$^*$</td>
</tr>
<tr>
<td>Pacer</td>
<td>Referent</td>
<td></td>
</tr>
<tr>
<td>Sex: Female</td>
<td>0.03 (-0.07-0.13)</td>
<td>0.524</td>
</tr>
<tr>
<td>Male</td>
<td>Referent</td>
<td></td>
</tr>
</tbody>
</table>

Outcome variable is the log transformation of pre-race cTnI concentration. $^1$ Model coefficient with the 95% confidence interval, $^*$ p-value of $\leq$0.2 was considered significant for inclusion in multivariable linear regression.
Table 4.4: Results of the final multivariable linear regression analysis with log 10 pre-race cTnI as the dependant variable.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Coefficient (95%CI)</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>0.02 (0.01-0.06)</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td>Gait: Trotter</td>
<td>0.13 (0.03-0.23)</td>
<td>0.013*</td>
</tr>
<tr>
<td>Pacer</td>
<td>Referent</td>
<td></td>
</tr>
<tr>
<td>Intercept</td>
<td>-0.26 (-0.15-0.1)</td>
<td>0.69</td>
</tr>
</tbody>
</table>

Outcome variable is the log transformation of pre-race cTnI. * Model coefficient with the 95% confidence interval. * p-Value ≤0.05 was considered significant.
Table 4.5: Results of univariable linear regression analysis with log 10 post-race cTnI as the dependant variable.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Coefficient (95% CI)(^\dagger)</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Race Date</td>
<td>0.996(^\dagger)</td>
<td></td>
</tr>
<tr>
<td>Post Number</td>
<td>0.473(^\dagger)</td>
<td></td>
</tr>
<tr>
<td>Gait: Trotter Pacer</td>
<td>0.14 (-0.004-0.29)</td>
<td>0.058*</td>
</tr>
<tr>
<td>Finish Position</td>
<td></td>
<td>0.51(^\ddagger)</td>
</tr>
<tr>
<td>Lasix</td>
<td>-0.009 (-0.17-0.15)</td>
<td>0.936</td>
</tr>
<tr>
<td>Log 10 Pre-cTnI</td>
<td>0.67 (0.47-0.88)</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td>Purse(^3): 0-8,000</td>
<td>Referent</td>
<td>0.62(^\dagger)</td>
</tr>
<tr>
<td>8,001-10,000</td>
<td>-0.1</td>
<td></td>
</tr>
<tr>
<td>10,001-15,000</td>
<td>-0.02</td>
<td></td>
</tr>
<tr>
<td>15,001-18,000</td>
<td>0.05</td>
<td></td>
</tr>
<tr>
<td>Age(^4): 3-4</td>
<td>Referent</td>
<td>0.7(^\ddagger)</td>
</tr>
<tr>
<td>5-6</td>
<td>-0.02 (-0.19-0.16)</td>
<td></td>
</tr>
<tr>
<td>7-12</td>
<td>-0.03 (-0.19-0.13)</td>
<td></td>
</tr>
<tr>
<td>Sex: Male Female</td>
<td>Referent</td>
<td>0.267</td>
</tr>
<tr>
<td>Arrhythmia</td>
<td>0.12 (-0.09-0.34)</td>
<td>0.27</td>
</tr>
<tr>
<td>Finish Time(^5)</td>
<td>0.01 (-0.004-0.04)</td>
<td>0.17*</td>
</tr>
<tr>
<td>Parked at Half-Mile</td>
<td>-0.06 (-0.17-0.04)</td>
<td>0.25</td>
</tr>
<tr>
<td>Finished Distanced</td>
<td>0.40 (0.10-0.69)</td>
<td>0.0009*</td>
</tr>
<tr>
<td>Sample Time: &lt;1 Hour</td>
<td>Referent</td>
<td>0.29</td>
</tr>
<tr>
<td>≥1 Hour</td>
<td>0.14 (0.01-0.28)</td>
<td></td>
</tr>
</tbody>
</table>

Outcome variable is the log transformation of post-race cTnI concentration. \(^\dagger\) Model coefficient with the 95% confidence interval, \(^\dagger\) p-Value results from partial F test for categorical variables, \(^3\) Purse in Canadian dollars, \(^4\) Age in years, \(^5\) Finish time in seconds, \(^*\) P value of \(\leq 0.2\) was considered significant for inclusion in multivariable linear regression.
Table 4.6: Results of the final multivariable linear regression analysis with log 10 post-cTnI as the dependent variable.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Coefficient (95% CI)</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Log 10 Pre-race cTnI</td>
<td>0.62 (0.47-0.78)</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td>Arrhythmia</td>
<td>0.03 (-0.13-0.19)</td>
<td>0.722</td>
</tr>
<tr>
<td>Finished Distanced</td>
<td>0.04 (-0.22-0.31)</td>
<td>0.763</td>
</tr>
<tr>
<td>Arrhythmia x Distanced</td>
<td>2.46 (1.84-3.07)</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td>Sample Time: &lt; 1Hour Referent</td>
<td></td>
<td>0.065</td>
</tr>
<tr>
<td>≥1 Hour</td>
<td>0.09 (-0.01-0.19)</td>
<td></td>
</tr>
<tr>
<td>Intercept</td>
<td>0.36 (0.27-0.44)</td>
<td>&lt;0.0001*</td>
</tr>
</tbody>
</table>

Outcome variable is the log 10 transformation of post-race cTnI. 

1 Model coefficient with the 95% confidence interval, * p-Value ≤0.05 was considered significant.
Table 4.7: Contrast statement showing the interaction between the explanatory variables: finished distanced and arrhythmia in the post-race cTnI model.

<table>
<thead>
<tr>
<th>Contrast tested</th>
<th>Coefficient*</th>
<th>P-Value**</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>(A+/D+) vs. (A-/D-)</td>
<td>2.52</td>
<td>&lt;0.0001</td>
<td>1.99-3.05</td>
</tr>
<tr>
<td>(A+/D+) vs. (A+/D-)</td>
<td>2.49</td>
<td>&lt;0.0001</td>
<td>1.95-3.04</td>
</tr>
<tr>
<td>(A+/D+) vs. (A-/D+)</td>
<td>2.48</td>
<td>&lt;0.0001</td>
<td>1.90-3.07</td>
</tr>
<tr>
<td>(A+/D-) vs. (A-/D-)</td>
<td>0.03</td>
<td>0.72</td>
<td>-0.13-0.19</td>
</tr>
<tr>
<td>(A-/D+) vs. (A-/D-)</td>
<td>0.04</td>
<td>0.76</td>
<td>-0.22-0.31</td>
</tr>
<tr>
<td>(A+/D-) vs. (A-/D+)</td>
<td>-0.01</td>
<td>0.94</td>
<td>-0.31-0.30</td>
</tr>
</tbody>
</table>

*Outcome variable is the log 10 transformation of post-race cTnI. 1 Model coefficient with the 95% confidence interval, ** p-Value ≤0.05 was considered significant.
Figure 4.1: Histogram showing the distribution of delta cTnI concentrations for pre and post-race.*

* Two Observations with high delta cTnI concentrations (116 and 2,165 ng/L) were omitted from the histogram as their inclusion precluded observation of the distribution.
Figure 4.2: Mean cTnI release curves for arrhythmia and control horses. Error bars represent standard deviation for each data point.
Chapter 5: Conclusions

Key Findings:

Analytical Validation of Assays:

cTnI assays are often employed in the equine literature without first performing analytical validation [Chapter 1]. The validity of these studies hinges on the reliability and performance of these assays. In Chapter 2, analytical validation of 2 cTnI assays confirmed their ability to reliably detect equine cTnI with acceptable precision and analytic sensitivity. Linearity was tested and confirmed in 2 additional assays, which suggests these assays may be suitable for use in horses with additional validation. A fifth assay failed to detect equine cTnI in any of the concentrations tested and is therefore not recommended for use in horses. The variability of performance between assays demonstrates the need for analytical validation before use of human cTnI assays in non-human species.

Differences in Assay Results

Recovery of cTnI varied widely between assays (1-3 fold differences). Similar findings have been reported in the human literature and are usually attributed to differences in epitope targets, reference materials, and analytical techniques. Reference intervals should be established for each assay and direct comparison of results between assays avoided [1,2]. One assay had very low recovery and was deemed unsuitable for use in horses.

Effect of Amino Acid Substitution in Different Assays
Two of the assays examined in Chapter 2 either did not detect equine cTnI or had low recoveries. Both of these assays used the same cTnI epitope, whether for their capture or detection antibody. Both assays also used a single antibody target for detection or capture rather than multiple. This epitope has a single amino acid substitution in horses compared to the human polypeptide, which likely affected assay performance [3]. This finding adds strength to the argument for species-specific analytical validation and highlights the potential limitations of published data derived from non-validated assays.

Pre-Analytical Storage Stability of Samples

No statistically significant difference in cTnI concentration was found between whole blood samples stored at 24°C or 4°C for 24 hours, and serum stored at -80°C. Similarly there was no difference in serum samples stored at -20°C for 20 days, and those stored at -80°C. Therefore, cTnI testing is feasible in the field in addition to its use in veterinary hospitals.

Cardiac Troponin I (cTnI) Release Curve

Hourly blood sampling of clinically healthy horses pre- and post-near maximal intensity exercise revealed elevations in cTnI concentration as early as 1 hour post-exercise, with peak concentrations present at 2-6 h [Chapter 3]. Concentrations returned to baseline or near-baseline within 24 hours. Detection and definition of an exercise-associated cTnI release curve in normal animals allows practitioners and researchers to sample horses at the optimal time post-exercise to detect elevations where they exist and to differentiate between clinically insignificant and significant elevations.

Impact of High-Sensitivity Assays
Use of a validated high-sensitivity cTnI assay allowed detection of cTnI concentrations for the majority of horses across all time points, which allowed a thorough investigation of the kinetics of cTnI release. Measurement by a contemporary cTnI assay detected cTnI concentrations at peak but failed to detect concentrations at other time points. Use of a high-sensitivity assay provides a longer window of opportunity to detect elevations post-exercise, in addition to allowing detection of small changes in concentration. The ability to detect concentrations in resting subjects allows for investigation of the natural history of cTnI release, and its associations with other variables of interest. In addition, subtle changes in cTnI concentrations among diseased or at-risk animals can be detected and followed appropriately. Therefore, clinicians and researchers should use high-sensitivity assays whenever possible.

Resting cTnI Concentrations and Associations with Demographic Variables

Resting concentrations of cTnI ranged from 0.22-37.61 ng/L with a mean of 2.21 ng/L. The distribution was right-skewed with a long right tail, indicating a wide variation in concentration within the clinically normal Standardbred racehorse population. Trotter had significantly higher resting cTnI concentrations versus pacers in the multivariate model. A positive association between increasing age and cTnI concentration was also identified.

Effect of Exercise on cTnI Concentration

cTnI concentrations increase to detectable levels in horses within 2 hours post-race. The change in concentration (delta cTnI) is highly variable between horses, with most horses experiencing mild elevations, while a small number show greater increases. The clinical implications of these mild elevations are unknown but may be due to episodes of transient
local ischemia, or normal myocyte turnover (4). However, the profound increases in cTnI concentration identified in a smaller number of horses are strongly suggestive of myocardial injury. Clinicians should be aware of the existence of these exercise-associated increases and that while clinical consequences are unclear, horses with profound elevations should continue to be monitored. Although the causal relationship between racing and cTnI release has not been fully elucidated, these findings suggest some level of myocardial damage is occurring in Standardbred racehorses. The need for further investigation and more intensive monitoring of these horses is clear.

Association between Race Variables and cTnI Concentration

There is a positive synergistic relationship between finishing distanced and ventricular arrhythmia, and post-race cTnI. The causal relationship and clinical significance of this association is unknown but may reflect myocardial injury. Greater monitoring of horses that finish distanced for arrhythmia and myocardial damage or dysfunction may reveal more about this relationship and prevent further issues.

Limitations:

Analytical Validation Substrate

Myocardium and serum harvested from a healthy horse were used to prepare samples and substrate for analytical validation. The benefit of this approach was that it ensured that the troponin I measured by the assays was the cardiac isoform. In addition, it eliminated the need to sample multiple horses with myocardial disease in order to procure the serum volume
needed for multiple validations. The limitations of this approach included the possibility that
serum spiked with isolated tropinin complex did not represent troponin release and
breakdown in vivo. The impact on validation was minimized by using western blot analysis
of the isolate to confirm the presence of cTnI.

**Lack of Gold Standard**

The lack of a gold standard method for measuring equine cTnI represented a limitation in this
project. Although analytical validation can be performed without a gold standard,
comparison of results between the assay of interest and an accurate standard would provide
greater certainty of its performance. In the absence of a gold standard, the researcher must
make some assumptions: i) the test substrate (in our case equine myocardium) contains the
analyte of interest but lacks cross-reactive substances, ii) the results given by the test of
interest reflect the true concentration in the sample, or are close. Some disparity between the
true and measured analyte concentration is acceptable, as long as the precision of the test is
high. Given the variance in relative recovery of the different assays investigated in chapter 2,
(at least some assays do not measure close to the true cTnI concentration. However, these
assays showed acceptable linearity and, in cases where precision was tested, good within-run
precision. These results indicate that the assays validated have clinically acceptable
performance.

**Sample Size**

A single donor horse was used for collection of substrates for analytical validation. Use of a
single horse was chosen in accordance with ethical and economic concerns. However, a
Subject Selection

Subject selection in Chapters 2-4 was done on a convenience basis as random sampling was not feasible. Chapter 2 required recruitment of a healthy horse ≤2 years old, that was undergoing elective euthanasia, in order to collect myocardium and serum. Owner willingness was therefore crucial and precluded use of random sampling.

For logistical reasons, Chapter 3 sampling was performed at a single training stable, which allowed researchers 24-hour access to study subjects and active participation from trainers, drivers, and staff. These logistical concerns made random selection of horses or barns unfeasible.
Data sampling in Chapter 4 was done in co-operation with Mohawk Racetrack in Ontario. Races, rather than individual horses, were chosen by track management as all horses in a race had to carry the same equipment during the race. Races were selected to allow for adequate set up time, and so as not to interfere with the racing schedule.

The limitation of convenience sampling is that the study population may not be representative of the target population. Care was taken in Chapters 3 & 4 to select a study population with demographics similar to the estimated true population in order to minimize the impact.

Sample Timing

The results of the post-exercise cTnI release curve revealed that peak cTnI concentrations occur at 2-6 hours post-exercise in clinically normal animals. In order to fully investigate the impact of racing on cTnI concentrations, blood samples should be taken at this peak. Unfortunately, because of the lack of on-track stabling at Mohawk racetrack, all horses are shipped in for the race and typically only stay 1 hour after to recover. Therefore, samples were taken before the peak release time, which may have biased results. Despite this potential bias, statistically significant elevations in cTnI concentration were found post-race.

Further Work:

Analytical Validation

The results of Chapter 2 demonstrate the need for analytical validation, as assays investigated did not all have clinically acceptable performance. Although efforts were made to include most contemporary cTnI assays available in Canada in the linearity and recovery
experiments, full validation was achieved for only 2 assays. Further studies should be performed on unvalidated assays and newly introduced assays.

In addition to the initial validation, quality control measures must be performed in these assays to ensure accurate laboratory performance. In cases where the assay is used in a single species (e.g., in racetrack laboratories), a species-specific standard material should be used. Development of such standards necessitates studies into a suitable medium that approximates in vivo samples.

Reference Intervals

Reference intervals were established using 2 validated assays in Standardbred racehorses 3-12 years old. Use of these reference intervals outside of this population, or using different assays, should be avoided as breed, age, and regional differences may have profound impacts on the reference interval. Laboratories, clinics, and researchers should take care to establish reference intervals in their population of interest using a validated assay.

Clinical Validation and Establishment of Diagnostic Cut-offs

Clinical validation of cTnI assays was outside the scope of this study. Further work investigating the use of cTnI assays in the detection and monitoring of myocardial injury is needed before these assays can be used with confidence in clinical practice. Care should be taken in these studies to select cases and controls free from skeletal muscle disease, as the degree of cross-reactively between cTnI and skeletal troponin I is unknown in horses.

The lack of a gold standard for detection or diagnosis of myocardial disease in horses will make clinical validation challenging. Confirming disease will require a combination of ECG
Diagnostic cut-offs for myocardial disease need to be established using the data from clinical validation studies. Examination of the diagnostic sensitivity and specificity at different cut points must be examined using ROC curves to determine the optimum diagnostic cut-off.

Cross-reactivity

Although numerous studies in the human literature [Chapter 1] have shown negligible cross-reactivity between cTnI and skeletal troponin I using contemporary assays, one study has found cross-reactivity in research assays (5). This cross-reactivity has not been examined in horses and with high-sensitivity assays. Cross-reactivity may have large impacts on the feasibility of using cTnI assays in horses. Horses, as performance animals, have a large percentage of their total body mass taken up by skeletal muscle. This fact, coupled with a high prevalence of skeletal muscle disease in horses, means that even a modest amount of cross-reactivity could have large impacts on assay results and subsequent diagnosis. Studies looking into the degree of cross-reactivity and its impact on clinical diagnosis in horses are therefore needed.

Resting cTnI and Demographic and Training Variables

The introduction of high-sensitivity cTnI assays has enabled measurement of resting cTnI concentrations in clinically healthy individuals. This greater sensitivity allows researchers to investigate the effects of demographic variables and performance variables on cTnI results. In human populations, these investigations have led to the establishment of sex-specific reference intervals, as well as a greater understanding of the impact of race intensity and
frequency, and of a range of demographic characteristics. In horses, investigation of these variables is important as target populations vary in respect to factors such as breed, age, sex, and performance level. The results presented in Chapter 4 demonstrate that age and gait influence resting cTnI concentrations. Larger studies with more heterogeneous populations are needed to further explore these associations and causal relationships.

**Associations between Racing, Arrhythmia, and cTnI Release**

Findings presented in Chapter 4 demonstrate a statistically significant increase in cTnI concentration after maximal-intensity exercise. Further associations were found between presence of ventricular arrhythmia in the recovery phase, finishing distanced, and post-race cTnI concentration. Unfortunately, because of the relatively short study period, the long-term clinical impact of these arrhythmias and periods of cTnI release could not be explored in this study. Longitudinal studies looking at future performance, and possible links between post-race cTnI concentration, arrhythmias, and exercise-induced pulmonary hemorrhage, and sudden death in these horses are needed to assess the cumulative effect of racing, and the need for further monitoring or intervention. Whenever possible, well standardized multicentre studies should be performed to broaden the internal and external validity of study results.

Establishing a causal relationship between possible myocardial injury (as evidenced by cTnI release) and exercise-associated arrhythmia represents a significant challenge, especially as the relationship may not be linear. As these events may occur close to each other in time, and since monitoring of myocardium during racing would require advanced imaging not available at this time, the question of an association between these events may not be clarified for some time. However, continued, intensive monitoring of racehorses throughout their careers may give some insight into the natural history of these possibly associated conditions.
References:


