Guidelines and Recommendations

Ola Hammarsten*, Janet V. Warner, Leo Lam, Peter Kavsak, Bertil Lindahl, Kristin M. Aakre, Paul Collinson, Allan S. Jaffe, Amy K. Saenger, Richard Body, Nicholas L. Mills, Torbjørn Omland, Jordi Ordonez-Llanos and Fred S. Apple

Antibody-mediated interferences affecting cardiac troponin assays: recommendations from the IFCC Committee on Clinical Applications of Cardiac Biomarkers

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Abstract: The International Federation of Clinical Chemistry Committee on Clinical Applications of Cardiac Biomarkers (IFCC C-CB) provides educational documents to facilitate the interpretation and use of cardiac biomarkers in clinical laboratories and practice. Our aim is to improve the understanding of certain key analytical and clinical aspects of cardiac biomarkers and how these may interplay. Measurements of cardiac troponin (cTn) have a prominent place in the clinical work-up of patients with suspected acute coronary syndrome. It is therefore important that clinical laboratories know how to recognize and assess analytical issues. Two emerging analytical issues resulting in falsely high cTn concentrations, often several fold higher than the upper reference limit (URL), are antibody-mediated assay interference due to long-lived cTn-antibody complexes, called macrotroponin, and crosslinking antibodies that are frequently referred to as heterophilic antibodies. We provide an overview of antibody-mediated cTn assay interference and provide recommendations on how to confirm the interference and interpret the results.

Keywords: assay interference; cardiac troponin; heterophile antibodies; immune complexes; macrotroponin; myocardial infarction.

***Corresponding author: Ola Hammarsten**, MD, PhD, Professor, Senior Physician, Institute of Biochemistry, Department of Clinical Chemistry and Transfusion Medicine, University of Gothenburg, Bruna stråket 16, Sahlgrenska Academy at the University of Gothenburg, 41345 Gothenburg, Sweden, Phone: +46 31 342 1000, Fax: +46 31 82 84 58, Mobile: +46 733 200834, E-mail: ola.hammarsten@clinchem.gu.se

Janet V. Warner, Faculty of Medicine, The University of Queensland, Saint Lucia, Australia

Leo Lam, Chemical Pathology, LabPlus, Auckland City Hospital, Auckland, New Zealand; and Biochemistry, Middlemore Hospital Laboratories, Auckland, New Zealand

Peter Kavsak, Department of Pathology and Molecular Medicine, McMaster University, Hamilton, ON, Canada

Bertil Lindahl, Department of Medical Sciences, Uppsala University and Uppsala Clinical Research Center, Uppsala University, Uppsala, Sweden

Kristin M. Aakre, Department of Medical Biochemistry and Pharmacology and Department of Heart Disease, Haukeland University Hospital, Bergen, Norway; and Department of Clinical Science, University of Bergen, Bergen, Norway. https://orcid.org/0000-0002-7340-6736

Paul Collinson, Departments of Clinical Blood Sciences and Cardiology, St George's University Hospitals NHS Foundation Trust, London, UK; and St George's University of London, London, UK. https://orcid.org/0000-0002-7000-5996

Allan S. Jaffe, Departments of Laboratory Medicine and Pathology and Cardiology, Mayo Clinic, Rochester, MN, USA

Amy K. Saenger and Fred S. Apple, Department of Laboratory Medicine and Pathology, Hennepin Healthcare/HCMC, Minneapolis, MN, USA; and Department of Laboratory Medicine and Pathology, University of Minnesota, Minneapolis, MN, USA

Richard Body, Emergency Department, Manchester University NHS Foundation Trust, Manchester Academic Health Science Centre, Manchester, UK; Division of Cardiovascular Sciences, The University of Manchester, Manchester, UK; and Healthcare Sciences Department, Manchester Metropolitan University, Manchester, UK

Nicholas L. Mills, BHF/University Centre for Cardiovascular Science and Usher Institute, University of Edinburgh, Edinburgh, UK

Torbjørn Omland, Department of Cardiology, Akershus University Hospital, Lørenskog, Norway; and Institute of Clinical Medicine, University of Oslo, Oslo, Norway

Jordi Ordonez-Llanos, Servicio de Bioquímica Clínica, Hospital de Sant Pau, Barcelona, Spain; and Foundation for the Biochemistry and Molecular Pathology, Barcelona, Spain

Introduction

Cardiac troponin T (cTnT) and I (cTnI) are widely used biomarkers in the evaluation of patients with suspected acute coronary syndrome and are integral to the diagnosis of acute myocardial infarction (MI) [1]. The ability to measure very low cTn concentrations using high-sensitivity assays has improved diagnostic accuracy, allowed for more rapid triage of patients without increased admissions, and in some instances reduced hospital costs [2, 3]. However, there are many instances where the etiology of an increased cTn remains unclear. Some of these alternative etiologies are tabulated in Table 1. Clinically, chronic cTn increases portend a poor prognosis as underlying structural or coronary heart disease are associated with a higher risk of future cardiovascular events, especially when due to non-ischemic causes [4, 5], and one needs a diagnosis to determine optimal management. On the other hand, false cTn elevations may result in further examinations, including cardiac imaging and coronary angiography [6], that can be associated with some risk to the patient and unnecessary hospital costs [7, 8].

Table 1: Conditions associated with cTn elevations.

Cardiac causes
Myocardial infarction
Coronary revascularization procedure
Heart failure
Myocarditis
Cardiomyopathy
Takotsubo syndrome
Cardiac procedure other than revascularization
Catheter ablation
Defibrillator shocks
Cardiac contusion
Sustained tachyarrhythmia
Severe hypertension
General conditions affecting myocardium
Sepsis, infectious disease
Chronic kidney disease
Infiltrative diseases, e.g., amyloidosis, sarcoidosis
Chemotherapeutic agents
Critically ill patients
Strenuous exercise
Hypertrophy
Respiratory failure
Severe anaemia
Hypotension or shock
Stroke, subarachnoid haemorrhage
Pulmonary embolism, pulmonary hypertension

Modified from [1].

This was underlined by a recent study of patients admitted to hospital that indicated that those with evidence of cTn macrotroponin had lower mortality and a lower frequency of acute cardiac disease compared to patients with increased cTn but no evidence of interference [9].

All immunoassays, including those used to measure cTn are vulnerable to interferences that can result in erroneous measurement and thus imperfect clinical decisions. The ability to find analytical errors and interferences [10–12], and to establish a laboratory protocol to identify interferences if a clinician questions a cTn result, is the responsibility of the clinical laboratory [13]. The collected experience is that the vast majority of stable cTn increases that prompt referral to laboratories are due to a cTn-assay interference, suggesting that a clinical suspicion of interference can be quite specific. However, this may in part be due to limited awareness of cTn-assay interference amongst clinicians and under recognition in practice. The literature on immunoassay interference is complex but our collected experience is that interferences that result in stable falsely increased cTn concentrations are primarily mediated by IgG antibodies. This limits the type of evaluation protocols that we recommend should be available in the central laboratory to investigate stable cTn elevations that are questioned by the clinician. cTn interferences resulting in falsely low concentrations or by non-antibody mediated mechanisms are seldom identified clinically, although they do exist [14, 15].

The literature on troponin interference is limited and no guidance exists either for clinicians or laboratories on how to handle this problem, often leading to repetitive investigations and potential complications for the affected patients. The purpose of this article is to give an overview of the current literature and, based on that and our collective experience, provide guidance for clinical laboratories on how to identify interferences affecting cTnI and cTnT assays.

Mechanisms involved in cTn-assay interference

The most common cTn-assay anomalies are outliers, also called “fliers”, when the results between two successive analyses of cTn of the same sample vary significantly sometimes due to fibrin-clots [8]. However, the most common reason for false stable increases of cTn that prompt clinical suspicion of interference are mediated by patient IgG antibodies [16, 17]. It is therefore particularly important that the central laboratory, in addition to dealing with fliers, can identify and interpret IgG-mediated false cTn increases in a reliable and safe way.

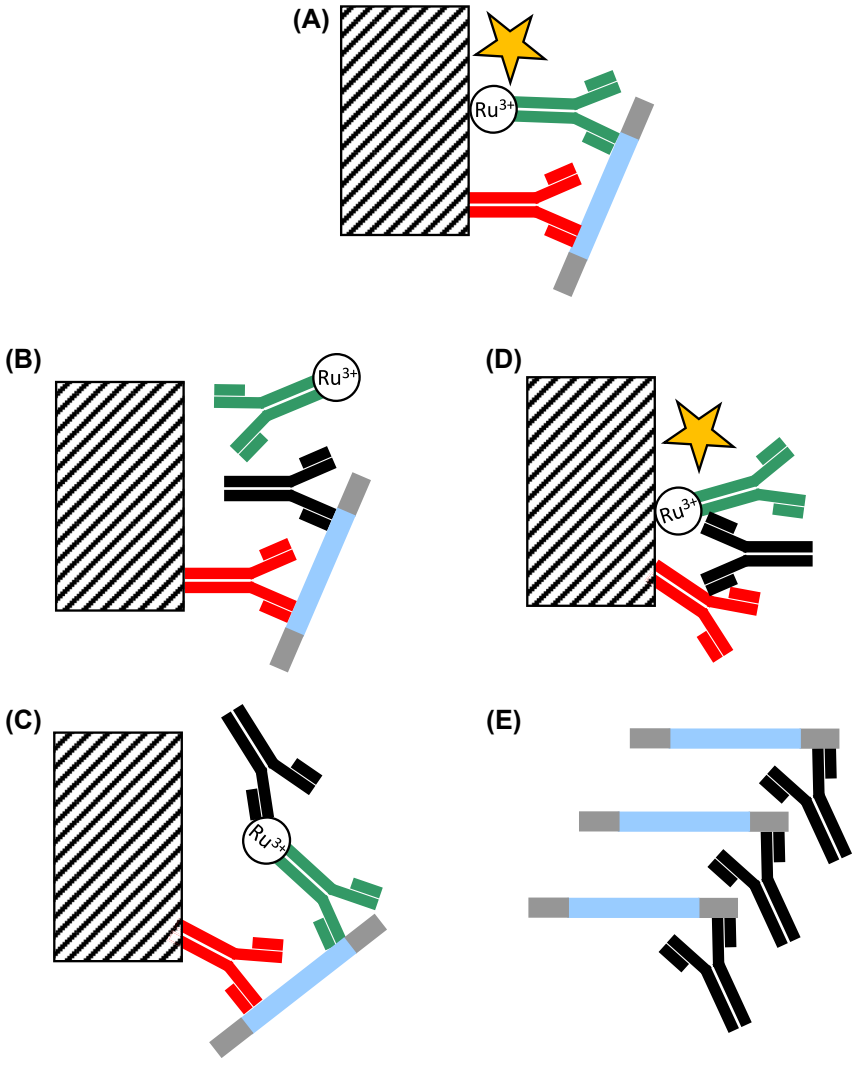

Figure 1: Possible mechanisms behind antibody-mediated cTn assay interference. (A) Typical immunoassay with one capture (red) and one Ruthenium labeled detection assay antibody (green) that colocalize on a cTn molecule (gray and blue bar) and generate a signal when the Ruthenium ion is brought in close proximity to an electrode. (B, C) Blocking anti-cTn antibodies. If patient anti-cTn antibodies (black) block assay antibodies from binding to cTn or generating a signal, the cTn assay may generate false negative results. (D) Heterophilic antibodies. Patient antibodies that crosslink assay antibodies can generate cTn-independent signals and result in false cTn-elevations. (E) Macrotroponin. Patient anti-cTn antibodies may form long lived antibody-cTn complexes, and result in a build-up of stable cTn increases that may not indicate an increased cTn release from the heart. (Figure based on the electrochemiluminiscent cTnT assay from Roche Diagnostics).

cTn immunoassays have two or more antibodies that must bind to the same cTn molecule to generate a measurable signal (Figure 1A). The cTn concentration in a patient's sample will be underestimated if anti-cTn antibodies are present in the patient's blood and compete with the assay antibodies for binding to cTn, which results in blocking the signal generation (Figure 1B and C). Conversely, cTn concentrations will be overestimated if patient antibodies crosslink the cTn-assay antibodies independently of cTn binding (Figure 1D). These are referred to as heterophilic antibodies and include human anti-mouse antibodies (HAMA) and rheumatoid factors. A third type of interference is caused by the formation of immunocomplexes between patient anti-cTn antibodies and cTn, as shown in Figure 1E, and is known as macrotroponin. Immunoglobulins have a half-life over several weeks, whereas free cTn has a half-life of a few hours [18, 19]. Therefore, since the healthy heart constantly releases some cTn, circulating macrotroponin complexes persist and result in a higher-than-normal steady state level of cTn that may not indicate a stable increased cTn release from the heart due to injury. Consistent with this assumption, some studies have reported a lower risk of death and cardiac events among patients with cTn increases due to macrotroponin [9, 20].

As with most immunoassays, interference is often assay-dependent, likely because heterophile antibodies can be quite specific and may or may not bind to the cTn-assay antibodies (Figure 1D). Similarly, circulating cTn degradation products may or may not be involved in the macrotroponin complex or be measured by a given cTn-assay.

The frequency of cTn-assay interference

Different studies have reported very different prevalence of cTn-assay interference possibly because estimates are not only dependent on the cTn measured, but also on the cTn-assay design and population [17, 21]. When detected by

discordant results from two cTnI assays on 3,897 individuals and then further analyzed by removal of IgG by protein A, blocking of heterophile antibodies or gel filtration chromatography, the prevalence was 5% of patients with cTnI concentrations above sex-specific 99th percentile URLs [16]. Discordance for cTnI assays between one contemporary sensitive cTnI and a high-sensitive cTnI assay in 2,658 patients was 1.2% [22]. In one study interference was investigated in all samples received from primary care that showed cTnI concentrations above the URL for a cTnI assay. Evidence of interference was shown for 123/223 samples when analyzed before and after removal of IgG by protein A resin [17]. Of the 123 samples with evidence of cTn-assay interference in the first cTn-assay only 10 samples had evidence of interference in all of the six cTn-assay included in the study showing that cTn-assay interference is often assay specific [9]. Finally, cTnI assay interference was 17% of the analyses when 9 different assay platforms were tested on samples from 10 patients with autoimmune disease. In this study, cTnI was one of 5 analytes most commonly affected by assay interference among the 74 analytes included in the study [23]. Finally, the prevalence of cTnI-assay interference appears higher than for cTnT-assay interference [17].

In conclusion, although the true frequency of cTn assay interference is not known it likely constitutes several percent of all elevated cTn results on some cTn assay platforms.

Indications for cTn assay interference analysis

Interference testing and information about this problem with cTn-assays should be provided by the central laboratory. Current methods for identification of cTn assay interference are manual, labor intensive and time-consuming, potentially delaying necessary treatment of acute cardiac conditions. Interference testing should only be undertaken in consultation between laboratory and clinician if cTn assay interference is suspected, after other analytical causes such as fibrin-clots and pre-analytical factors such as hemolysis are ruled-out and where interpretative expertise and clinical information are available.

Scenarios in which cTn assay interference are likely to be present are listed in Table 2.

Recommendation #1: cTn-assay interference analysis should be done when there is a reasonable clinical suspicion that a cTn result is possibly incorrect or incongruent with the clinical findings.

Table 2: Findings indicating that cTn-assay interference may cause cTn elevation.

Non-acute conditions
Stable cTn elevation in the absence of a clear reason after clinical work-up.
cTn elevation to very different levels using different cTn assays in patients without acute myocardial infarction.
cTn levels show an unreasonable variation over time, after reanalysis of the same sample, after reanalysis in another sample type (plasma/serum) or after dilution of the patient sample.
Acute conditions
cTn elevation higher than expected during an acute cardiac event.
cTn levels linger on after an acute cardiac event.

Recommendation #2: Laboratories should provide a service for cTn-assay interference investigations.

Methods for detecting cTn assay interference

If there is clinical suspicion of cTn-assay interference we recommend repeat testing with the initial cTn assay utilized, following re-centrifugation, dilution or with an alternate specimen type (serum or plasma) [24–26]. If discrepant results persist the first-in-line method should be to retest specimen using a different cTn assay. If results are unclear other methods, like PEG precipitation, listed in Table 3 and in the flowchart (Figure 2) can be used if available. These methods are described in more detail below.

Repeat analysis with a different cTn-assay

Discordant results between different hs-cTn assays can be a sign of potential cTn assay analytical interference [17, 21].

Assay interference may be inferred if the clinical suspicion of cardiac disease is low, the increase in cTn remains stable over time, and either cTn concentrations are below the URL with an alternative assay or if cTn concentrations between two assays differ by more than 3–5 fold in conditions other than acute myocardial infarction [16, 22]. Our experience is that discordant troponin results between different assays are seen in approximately 50% of cases where analytical interferences are later confirmed. Some medical centers have implemented two different cTn assays to assess for the presence of analytical interferences

Table 3: Methods used in investigations of possible cTn-assay interference.

Routine lab methods				
Method	**Mode of action**	**Analysis time**	**Pros**	**Cons**
Alternative hs-cTn assay	Variable	<1 h	Easy. Fast. Inexpensive. Extensively used in clinical routine.	May miss cTn-assay interferences.
Polyethylene glycol (PEG) precipitation	Precipitation of large molecules including immunoglobulins	1 h	Easy. Fast. Inexpensive. Extensively used in clinical routine.	Exactly what is precipitated in patient sample not known. Different cTn assays are affected differently by presence of PEG in the sample.
Dilution	Unknown	<1 h	Easy. Fast. Inexpensive. Extensively used in clinical routine.	May miss cTn-assay interferences.
Reference lab methods				
Protein A/G spin column	Removal of IgG	1 h	Specific removal of IgG. Easy. Fast. Inexpensive.	Only IgG mediated interference will be detected. Not available in most labs.
Heterophile blocking reagent	Blocking of crosslinking antibodies	<1 h	Easy. Fast. Inexpensive. Extensively used in clinical routine.	Do not detect macrotroponin. Heterophile antibodies may be an uncommon cause of interference.
Gel filtration chromatography	Separation based on molecular weight	24 h	Will find any type of antibody mediated interference.	Slow and labor intensive. One sample at a time. Requires high laboratory skills. Requires special equipment such as chromatography equipment.
Sucrose gradient ultracentrifugation	Separation based on molecular weight	24 h	Will find any type of antibody mediated interference. Many samples can be run simultaneously.	Slow and labor intensive. Not an established technique in most labs. Requires high laboratory skills. Requires special equipment such as fluorometer and ultracentrifuge.

[16, 22, 27]. Furthermore, the laboratory may also collaborate with other local laboratories which utilize different cTn assays to aid in investigating potential interferences and possibly finding a cTn-assay without interference on individual patients.

On rare occasions, the interferent can involve both assays which may cause confusion. Importantly, truly discordant cTnI and cTnT concentrations may be present in acute MI when cTnI concentrations are often greater than cTnT during the first days [28]. In patients with chronic neuromuscular skeletal disease, cTnT assays have demonstrated real cTnT release from diseased skeletal muscle in approximately 50% of patients not representing myocardial injury, while cTnI assays are 100% myocardial tissue specific [29, 30].

Polyethylene glycol precipitation

An alternative method for the detection of cTn assay interferences is polyethylene glycol (PEG) precipitation using a protocol that precipitates most plasma proteins, including immunoglobulins [21]. The cTn concentration is measured before and after PEG precipitation. If the decrease in cTn-concentration is more than 80% (recovery <20%) or if the decrease is significantly different from routine control samples, the stable cTn increase is likely due to patient antibody mediated cTn assay interference. This method is simple to perform, and most laboratories have experience with PEG precipitation procedures, as it is also used to investigate macroprolactin. There are published detailed protocols for interpretation in the context of other information such as serial dilutions and patient clinical data [31]. However, interpretation of results may still be convoluted [32]. PEG precipitation protocols promote non-specific precipitation of most proteins in the sample rather than those of a target single-antibody immune complex, thus PEG precipitation does not assess the origin of the patient's cTn assay interference. In addition, different cTn assays are affected by the presence of PEG in the sample to varying degrees, which may confound interpretation [31].

Blocking agents for heterophilic antibodies

Patient specimens can be treated with commercially available heterophilic antibodies blocking reagent (e.g., HBR, Scantibodies Laboratory Inc., Santee, CA).

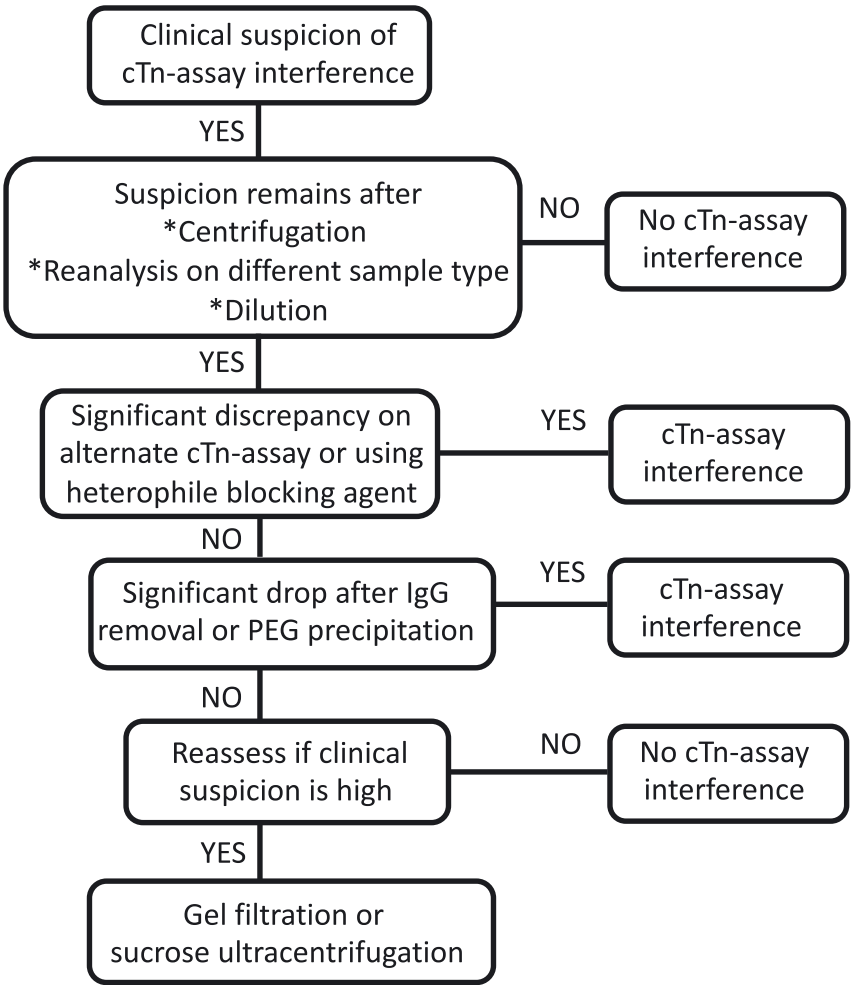

Figure 2: Suggested algorithm for investigation of suspected cTn-assay interference.

A significant decrease in the cTn concentration indicates a falsely increased cTn due to the presence of crosslinking antibodies. While the definition of a significant decrease is presently not clearly defined, among 24 case reports the median decrease in cTn concentration was 96% (IQR 73–97%) following HBR treatment when interference were present [33]. Studies that have systematically evaluated the effect of HBR treatment demonstrate that only a subset of suspected cTn-interferences due to discordant results between assays were identified with this method [34]. HBR is not formulated to react with macrotroponin, therefore this finding is not unexpected. As the composition of HBR is proprietary information, any interference detected by this method cannot be definitively explained.

Immunoglobulin removal

The vast majority of falsely increased cTn concentrations reported in the literature are due to interferences from circulating IgG. The most direct way to investigate a potential false cTn increase is to analyze cTn concentrations before and after removal of IgG by protein A [16, 17] or protein G [27] resins. Currently there are no automated methods for IgG removal, so spin columns with protein A and/or G resins, or beads alone are often used, sometimes as a kit [6]. Concurrent analysis of TSH [35] or ferritin [27] may be used to compensate for non-specific losses but that assessment is not always performed [16]. Complete removal of IgG can be determined but overloading the protein A or protein G spin columns is not a problem using published protocols [17, 27].

Patient antibody mediated cTn assay interference may be present if the cTn concentrations decrease more than 60% after removal of IgG (<40% recovery) [17] or if the decrease in cTn concentration is significantly different from routine control samples with true cTn elevations. Often, the decrease in cTn concentration is over 5-fold when the patient cTn increase is due to antibody interference and the interpretation is straight-forward as reported in around 70% of the

cases [27]. However, the within-sample variability in recovery of the protein G spin column method is over 10% which may add confusion [27]. Finally, rheumatoid factors are crosslinking IgM antibodies, according to most rheumatoid factor assays [36], and may be missed by the protein A/G method that mainly remove IgG. The extent of this potential problem is unclear [34, 37, 38] but caution should be used when interpreting results from the protein A/G method on patients with high rheumatoid factor titers. In these cases PEG precipitation, anti-IgM or anti-IgA resins may be used as a complementary method.

Molecular weight methods

Most circulating cTn are degradation products with a molecular weight below 40 kDa [27], whereas the antibody-cTn complexes that cause macrotroponin and interfering antibodies have a molecular weight around 150 kDa. It is therefore possible to detect all types of patient antibody mediated cTn assay interference by methods that determine the native molecular weight of the measured cTn and could be viewed as reference methods. Methods that have been used in clinical practice to determine the molecular weight of cTn in circulation include gel filtration chromatography [16, 17, 39] and sucrose gradient ultracentrifugation [27]. Native gel electrophoresis can also separate proteins by weight [40].

Methods that determine native molecular weights are laborious and should not be the first-line method. However, sometimes results remain inconclusive after removal of immunoglobulins by protein A/G resins or PEG precipitation. In these situations, additional analysis of the native molecular weight of the measured cTn often aids in the interpretation and at our sites the results from molecular weight methods are always conclusive.

If available, specialized laboratories with gel filtration chromatography or sucrose gradient ultracentrifugation experience could be consulted to analyze cTn-interference in this manner, providing a valuable resource if PEG and/or protein A/G findings are inconclusive. Macrotroponin specimens are stable for over a week at +4 °C [27] and can be stored frozen for extended periods of time [35], allowing for this analysis by reference laboratories.

Reporting of cTn assay interference

It is important in the post-analytical phase that residual cTn concentrations after removal of IgG or PEG precipitation are not automatically regarded as the patient's baseline cTn levels. Results from a cTn assay interference analysis should be interpreted in the context of the individual patient and not simply reported as a before-and-after result in the patient's health record without comment or interpretation. Consultation with a laboratory professional is essential to explain these findings to the clinician and ensure that the finding is permanently recorded and easily accessible in the patient health record so that the clinicians will be alerted that future cTn results from this patient may be unreliable. The lab may also suggest how this patient's samples may be analyzed in the future, perhaps with an alternate cTn-assay.

Recommendation #3: The residual cTn concentrations after removal of IgG or after PEG precipitation should not be reported as the patient's cTn concentration in their healthcare record.

Recommendation #4: cTn-assay interference analysis should be accompanied with an interpretation of the results and generate a permanent, easily accessible entry in the patient's healthcare record.

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