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Review

The role of antibody-based troponin detection in cardiovascular disease: A critical assessment

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ABSTRACT

Cardiovascular disease has remained the world's biggest killer for 30 years. To aid in the diagnosis and prognosis of patients suffering cardiovascular-related disease accurate detection methods are essential. For over 20 years, the cardiac-specific troponins, I (cTnI) and T (cTnT), have acted as sensitive and specific biomarkers to assist in the diagnosis of various types of heart diseases. Various cardiovascular complications were commonly detected in patients with COVID-19, where cTn elevation is detectable, which suggested potential prognostic value of cTn in COVID-19-infected patients. Detection of these biomarkers circulating in the bloodstream is generally facilitated by immunoassays employing cTnI- and/or cTnT-specific antibodies. While several anti-troponin assays are commercially available, there are still obstacles to overcome to achieve optimal troponin detection. Such obstacles include the proteolytic degradation of N and C terminals on cTnI, epitope occlusion of troponin binding-sites by the cTnI/cTnT complex, cross reactivity of antibodies with skeletal troponins or assay interference caused by human anti-species antibodies. Therefore, further research into multi-antibody based platforms, multi-epitope targeting and rigorous validation of immunoassays is required to ensure accurate measurements. Moreover, in combination with various technical advances (e.g. microfluidics), antibody-based troponin detection systems can be more sensitive and rapid for incorporation into portable biosensor systems to be used at point-of care.

1. Impact of heart diseases

Heart disease is a term used to describe many heart disorders. It can be divided into four main categories based on the pattern in which heart structure or function is affected. The primary categories are coronary artery and vascular disease, heart rhythm disorders, structural heart disease and heart failure. Coronary artery disease (CAD), also known as ischaemic heart disease (IHD), has remained the world's biggest killer for 30 years, causing approximately 40% of deaths in Europe and 25% in the US (Lelieveld et al., 2019; *WHO*, 2020).

Many heart diseases can be effectively controlled by a healthy lifestyle, with the WHO announcing that 80% of premature heart disease is preventable. Therefore, a manner by which heart disease can be accurately detected is imperative in ensuring the best treatment and prevention for patients suffering from the associated diseases. The accurate detection, and thereby diagnosis, of heart disease can potentially save millions of lives each year. There is considerable market interest in the development of diagnostic methods. A new report published by Grand View Research, Inc. indicated that the market of heart attack diagnosis is expected to achieve 15.4 billion USD by 2024, where rapid blood tests (e.g. troponin levels in blood) are predicted to gain major market share (*Grand View Research*, 2020). The ongoing investment into

Abbreviations: AA, amino acids; Abs, antibodies; CAD, Coronary artery disease; CK-MB, a dimer composed of M and B isoforms of creatine kinase; cTnAAbs, cardiac troponin–specific autoantibodies; cTnI/T, cardiac-specific troponins I/T; cTnI-T-C, cardiac-specific troponins IT and C complex; ELISA, enzyme-linked immunosorbent assay; Fab, fragment antigen binding unit; HAMA, human anti-mouse antibodies; H-FABP, heart-type fatty-acid-binding proteins; hs-cTnI/T, high sensitivity cTnI/T; IE, Infective Endocarditis; IHD, ischaemic heart disease; LOD, limit of detection; MI, myocardial infarction; mAb, monoclonal antibody; pAb, polyclonal antibody; POCT, point-of-care testing; QCM, quartz crystal microbalance; rAb, recombinant; SAW, surface acoustic wave; scAb, single chain antibody fragment; scFv, single chain fragment variable; URL, upper reference limit..

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diagnostic tests will aid in the development and improvement of new and existing troponin assays. This, in turn, could lead to improved healthcare for patients afflicted with the various forms of heart-disease. Within this sector, antibody-based strategies can play a vital role.

It is widely reported that patients with COVID-19 often present with various cardiovascular complications (e.g. acute coronary syndromes, unmasking occult underlying cardiovascular disease, arrhythmias, myocarditis, or as part of a systemic inflammatory syndrome) (Fried et al., 2020; Varga et al., 2020; Kotecha et al., 2021). COVID-19 impacts the cardiovascular system not only in the intermediate but also in long-term patterns. Acute cardiac injury is diagnosed in 8–62% of hospitalized COVID-19 patients and is associated with poor clinical outcomes (e.g. need for mechanical ventilation, and death) (Chung et al., 2021). Even though most people recover from COVID-19 within a few weeks, some of them (including those experiencing mild symptoms) continue to suffer "post-COVID-19 syndrome" symptoms after their initial recovery. These post-COVID-19 conditions usually persist for more than 4 weeks after COVID-19 diagnosis. Lasting damage to the heart muscle has been shown months after recovery from COVID-19, even in people who had mild symptoms (Abbasi, 2021; Chilazi et al., 2021; Dolgin, 2021). This may increase the risk of heart failure or other heart complications in the future.

2. Troponins in heart disease diagnosis and COVID-19

Troponins are a complex of proteins containing three subunits, troponin C, troponin I, and troponin T. Troponins are found in both cardiac and skeletal muscle tissue, where their role is to regulate muscle contraction. Within the troponin complex, only cardiac troponins I (cTnI) and T (cTnT) are considered cardiomyocyte-specific. Injury of the cardiac muscle (e.g. resulting from insufficient oxygen and nutrients) leads to the release of these troponins into the bloodstream (Park et al., 2017). For over 20 years, the cardiac-specific troponins, I and T, have acted as sensitive and specific biomarkers to assist in the diagnosis of various types of heart diseases, including MI, heart failure, arrhythmias and unstable angina. The European Society of Cardiology (ESC), American College of Cardiology (ACC), American Heart Association (AHA) and World Heart Federation (WHF) declared that myocardial injury can be effectively diagnosed by a measured increase of cardiac troponins in the bloodstream above the 99th percentile upper reference limit (URL) (which is also known as a positive troponin result) (Thygesen et al., 2018).

Cardiac TnI and cTnT are considered more specific and sensitive than other heart disease biomarkers, for example, a dimer composed of M and B isoforms of creatine kinase (CK-MB) and heart-type fatty-acid-binding proteins (H-FABP), which have been found in various organs. Cardiac Tns are also useful for determining disease severity as it is reported that the concentration of circulating troponin and the duration of elevation of cTn correlates to the infarct size (Hallén, 2012). Furthermore, an upregulation of cTn in blood can be detected in relatively early stages of disease, facilitating a timelier diagnosis when compared to alternative biomarkers. The above advantages enable troponins to play a crucial role in heart disease diagnosis. However, it is also important to acknowledge that troponin levels may also increase due to stroke, renal failure, hypoxia, strenuous exercise and sepsis, which are non-cardiac related issues. Therefore, it is crucial to consider other testing methods which complement troponin detection for more accurate diagnosis and better patient outcomes. Examples of tests which can be performed alongside troponin assays include D-dimer, ESC guidelines, blood levels of CK-MB and myoglobin, electrocardiogram, computerized tomography scan and magnetic resonance imaging.

Elevated serum troponin levels are commonly detected in hospitalized COVID-19 patients (Libby, 2020; Liu et al., 2020). High number of increases in cTn concentrations in COVID-19 patients likely reflect critical illness and possibly are evidence of ongoing localized inflammation (Nishiga et al., 2020; Kavsak et al., 2021). It was found that elevation of cTn predicts greater disease severity. Increases of cTn were relatively rare in COVID-19 survivors (1%–20%), highly common in severely ill patients (46%–100%), and nearly 100% in the critically ill and non-survivors (Guo et al., 2020; Sandoval et al., 2020; Shi et al., 2020). Habets et al. (2021) recommend measuring cTn levels in all COVID-19 patients admitted to hospital or who deteriorate during admission, as cTn levels can be used in risk stratification models and suggest how likely the patient will benefit from early medication. Nuzzi et al. (2021) studied the prevalence and prognostic impact of early serum troponin increases in a large Caucasian population admitted for severe COVID-19, in order to identify patients that might require a higher intensity of care. They identified that serial troponin measurements have great prognostic value in patients admitted for COVID-19. Therefore, specific and effective detection of cTn in patients with COVID-19 may play a crucial role in the present global pandemic by aiding the development of preventative and therapeutic solution for instance, in proactive measurement for prevention of serious cardiovascular diseases, as well as monitoring the possible long-term risk for cardiovascular outcomes in these COVID-19-infected patients.

2.1. Conventional and high sensitivity cTn assays

The assays for cTn (cTnI and cTnT) detection are typically available in conventional formats (usually detect blood cTn at ng/mL levels from 3 to 6 h following the onset of ischemic symptoms) or as high sensitivity (hs) hs-cTn (hs-cTnI and hs-cTnT) formats (usually detect blood cTn at pg/mL levels within 1–3 h following the onset of ischemic symptoms) (Diercks et al., 2012). The high sensitivity of hs-cTn assays enables the reliable measurement of cTn concentrations around the 99th percentile as well as in the normal range (Boeddinghaus et al., 2019; Boeddinghaus et al., 2020).

2.2. Troponins in coronary artery disease (CAD)

The elevation of cTn can be detected within 6 h in most patients with CAD, while negative troponin at this point effectively excludes the possibility of infarct in most patients. Elevated cTn levels in patients with stable angina is indicative of a worsening condition and increased risk of a heart attack. Contrastingly, unstable angina will not lead to troponin release. For myocardial infarction (MI) diagnosis, cTnIs are considered highly sensitive and specific, and are the preferred biomarkers. Upon MI, the heart muscle is damaged and, subsequently, degradation of cardiomyocyte tissue occurs, leading to an elevated cTn concentration in the blood. CTn levels may remain elevated for up to 2 weeks post cardiac event (Amsterdam et al., 2014). Levels of circulating cTn can be measured and utilised to differentiate between cardiac diseases causing chest pain such as unstable angina and MI, where no cTn release is observed in the former, while increased cTn release is expected in the latter. Troponin detection is widely used in clinical and research areas as a valuable biomarker for diagnosis of various CADs.

2.3. Troponins in heart failure prognosis

The elevation of cTn (either cTnI or cTnT) in blood is considered prognostic in heart failure as it is indicative of cardiomyocyte injury and necrosis of the cardiac tissue, particularly in acute heart failure. A worse prognosis is generally associated with higher concentrations of free cTn in the blood. Increased morbidity and mortality rates were widely observed in patients presenting with acute heart failure alongside an elevated cTn level (Wettersten and Maisel, 2015). Cardiac TnI and cTnT are proven valuable biomarkers for analysing patients with acute heart failure. Cardiac Tn measurements facilitate the assessment of acute coronary syndrome and to prognosticate the likelihood mortality and readmission, which aids in the long-term management of patients (Shah et al., 2018).

2.4. Troponins in other heart diseases

Alongside CAD and heart failure, blood troponin levels are shown to elevate in patients suffering from a range of other cardiovascular ailments. These include patients presenting with myocarditis, pericarditis and infective endocarditis. Since troponin appears to be a shared biomarker across many cardiovascular diseases (as cTn is a biomarker of cardiomyocyte necrosis), it is clear that the evaluation of blood cTnI and cTnT as prognostic and diagnostic biomarkers is essential.

Myocarditis, also known as inflammatory cardiomyopathy, is an inflammation of the heart muscle which can be caused by various etiologies. These include cardiotropic viruses, toxins, drugs and autoimmune processes (Janardhanan, 2016). Elevated cTnI is present in over one-third of patients with myocarditis, while increased cTnT was detected in 93% patients with myocarditis (Smith et al., 1997). However, an important factor to note is that a negative troponin result does not exclude the diagnosis of myocarditis (Assomull et al., 2007).

Troponin levels are implicated in another inflammation-related disease, pericarditis, described as an inflammation of the pericardium. It is widely observed that cTnI and cTnT levels are elevated in acute pericarditis, cTnI by 32.2% and cTnT by 60.9%. The troponin level is roughly related to the disease severity, which is probably biochemical evidence of myocardial inflammatory damage. However, unlike acute coronary syndromes, troponin is not a negative prognostic marker in viral or idiopathic acute pericarditis (Imazio et al., 2003; Gamaza-Chulián et al., 2014).

Infective endocarditis is an infection of heart valves or endocardium, which is usually caused by bacteria. The disease is associated with increased mortality and morbidity. The baseline levels of cTnT may be useful in predicting long-term survival rates in patients with IE, while cTnI may be useful as a potential prognostic marker in IE (Thoker et al., 2016).

A further cardiac injury thought to be associated with cTn measurements is myocardial contusion. This bruising of the heart muscle can occur with serious bodily injury. It is recommended to include blood cTnI and cTnT tests to aid the diagnosis of myocardial contusion after blunt chest trauma (Decavèle et al., 2018; Crewdson et al., 2019).

However, troponin is very likely a negative prognostic marker for pericarditis. The relatively modest cases never demonstrate elevated troponin levels even with high sensitivity diagnostic assays. Usually, increases in troponin cannot be detected except in patients suffering from extensive pericarditis, which is often associated with widespread involvement of the pericardium, severe pain and ST-segment and T-wave (ST-T) changes.

3. Antibody selection for use in troponin-related heart diseases diagnosis

Given the apparent role that cTns plays across many cardiovascular related ailments, it is clear that the accurate measurement of blood troponin levels is essential in the diagnosis of ACS and MI and/or prognosis of other cardiovascular ailments including myocarditis, pericarditis, infective endocarditis and myocardial contusion. To detect these circulating troponins, specific and sensitive antibodies are required. Therefore, a general introduction to the various types of antibodies available and issues to be considered for antibody selection, for specific and sensitive cTn analysis follows.

3.1. Types of antibody

Antibodies are available in three main types, namely, polyclonal (pAb), monoclonal (mAb) and recombinant (rAb). There are a number of detailed descriptions of antibodies and their applications (O'Kennedy and Murphy, 2017). A general comparison of advantages and disadvantages of pAb, mAb and rAbs is given in Table 1. Antibodies bind to specific regions on a target, termed the epitopes. Each of the antibody types has unique characteristics, and, therefore, the decision on which type of antibody to use is largely assay-dependant.

Table 1
Comparison of the advantages and disadvantages of polyclonal (pAb), monoclonal (mAb) and recombinant antibodies (rAb).

Types of antibody	pAb	mAb	rAb
Production cost	Low	Moderate	Low
Production time	Fast (around 1–3 months)	Moderate (around 3–6 months)	Slow (around 4–8 months)
Production difficulty	Low	Moderate	High
Storage stability	High	High	Moderate
Reproducibility	Low	High	High
Sensitivity	High	High	High
Antibody binding affinity	Variable	High	High
Cross reactivity	High	Low	Low
Genetic modification	Not Applicable	Difficult	Easy
Reduced animal usage	No	Yes	Yes

Polyclonal antibody (pAb) preparations consist of a mixture of antibodies (also known as immunoglobulins or Igs; Fig. 1A), secreted by various B lymphocytes which recognise different epitopes (antibody binding sites) on the same antigen (Ma and O'Kennedy, 2015). The generation of a pAb is relatively quick, inexpensive and easy. PAbs are usually very stable but may possess wide specificities to both the designated and other antigens and, therefore, can demonstrate high cross reactivity. Therefore, a pAb ideally should not be used in direct detection/diagnosis, but is instead widely used as a capture and/or secondary antibody (Hayes et al., 2018).

Monoclonal Abs are made by identical B lymphocytes and are produced by clones derived from a single parental cell. Monoclonal Abs are associated with high specificity as they bind to a singular epitope. They are also relatively stable as they have a complete immunoglobulin structure. These characteristics have garnered the interest of many researchers and the use of mAbs in detection/diagnosis and immunotherapy-based treatments is widespread (Ayyar et al., 2016). However, it is vital that the specificity is vigorously assessed as failure to do so has caused major problems with assays (O'Kennedy et al., 2017).

A recombinant antibody does not exist naturally but is assembled from DNA by combining antibody heavy chain and light chain gene sequences. Recombinant antibodies have several advantages (Table 1) and large quantities can be produced quickly and at low cost (Ma and O'Kennedy, 2017). There are many types of recombinant antibodies. For

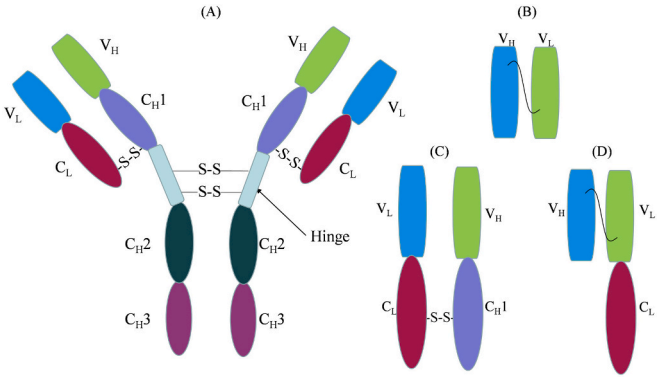

Fig. 1. Structure of a basic immunoglobulin (e.g. IgG) (A), scFv (B), Fab (C) and scAb (D). NH_2 = amino group; COOH = carboxylic acid group; V_H = variable region of an antibody heavy chain; V_L = variable region of an antibody light chain; C_L = constant region of an antibody light chain; $C_H1,2,3$ = constant domain one, two, and three, of an antibody heavy chain; S-S = disulphide bond.

instance, a single chain fragment variable (scFv) is 30 kDa and consists of V_H and V_L chains that are joined together by a flexible peptide linker, and is one of the most popular formats of recombinant antibodies (Fig. 1B). Other formats include a fragment antigen binding unit (Fab), which is of 60 kDa, consisting of V_H-C_H1 and V_L-C_L linked by disulfide bonds (Fig. 1C) and a single chain antibody fragment (scAb), which is of 40 kDa, consisting of V_H, V_L and light chain constant domains (Fig. 1D). Both formats are commonly used in assays (Zapatero-Rodríguez et al., 2018; Ma et al., 2019).

3.2. Critical issues to consider in antibody selection for cTn detection

The types of antibodies and their various characteristics make the selection of the appropriate antibody for cTn analysis an important, but solvable, challenge.

3.2.1. Selection of pAb, mAb and rAb in cTn detection

As described previously, pAbs which recognise multiple epitopes are usually not used for detection but may be incorporated for capture or for use as secondary labelled Abs for detection. Generally, mAbs and rAbs are more suitable options for both capture and detection Abs in cTn detection assays as their monovalency generally offers higher specificity.

Recombinant Abs have become more and more popular for biosensors due to the short generation time, convenient expression in hosts such as *Escherichia coli* (*E. coli*) and ease of protein engineering. Conroy et al. (2009) listed a large number of high quality rAb-based sensors used for various disease diagnoses and suggested that rAbs possess improved attributes for use in biosensor development. These include the capacity for enhancement of structure, stability, affinity, and specificity through multiple strategies, e.g. mutagenesis, and the addition of genetically coded tags that could aid directed immobilisation. Hyytiä et al. (2013) performed a comparison of capture antibodies including the formats of an intact mAb, $F(ab')_2$, Fab and cFab fragments in cTnI immunoassay. The results indicated that less rAbs were required for sufficient target capture due to their smaller size, and their incorporation into an assay may reduce the interference from matrix biomolecules, which may lead to increasing assay sensitivity. It was often believed that rAbs are not very stable due to their smaller size when compared to the full size antibodies (e.g. mAbs). However, this is not always the case. Ma et al. (2019, 2020) generated a very stable scAb against cTnI which retained its stability and specificity after storage at 37 °C for 6 months. Moreover, significantly improved stabilities have been reported after conversion of scFv to scAb or to Fab (Quintero-Hernández et al., 2007).

3.2.2. Factors to be considered due to the complex character of cTn

Another factor to consider, that is of crucial significance for troponin measurement, is the development of the antibody against the appropriate epitope(s). Due to the 'complicated' nature of cTn (especially cTnI), there are many factors that need to be considered for cTn detection. These include, but are not limited to, cross-reactivity of antibodies with skeletal isoforms of troponins, the occurrence of the cTn complex and associated exposure/potential occlusion of antigenic sites, proteolytic degradation which effects certain target epitopes on cTn, the choice of and need for the use of multiple epitopes in assay formats, phosphorylation and how it effects recognition, the presence of autoantibodies that might bind to potential antigenic sites, stability, and the presence of heterophilic antibodies (Zhang et al., 2011; Hytest, 2020). These factors are summarised in Table 2 and Fig. 2. Some potential solutions are also given.

Cardiac TnI, which is only expressed by heart tissue, is heart-specific, while cTnT is less specific as it can be expressed by some diseased skeletal muscles. However, given its instability, the majority of cTnI is found in patients' blood in a more stable complex alongside cTnC (cTnI-C), rather than in 'free' format (Gaze and Collinson, 2008). As such, detection of standalone cTnI is challenging due to the fact that it occurs at very low concentrations (Bates et al., 2010). Therefore, it is suggested to use the anti-cTnC or anti-cTn complex (cTnI-T-C) antibody as the capture Ab and a specific anti-cTnI antibody for detection (Hytest, 2020).

Proteolytic degradation is very common in cTnI and cTnT, especially at the N-terminal and C-terminal regions not bound (protected) by cTnC (Streng et al., 2016; Park et al., 2017). The cTn complex is believed to play a crucial role in protecting troponion from degradation. The most stable region of cTnI is between amino acid residues 30 and 110, the

Table 2
Factors affecting cTn detection and actual/potential solutions due to the 'complicated' nature of cTn.

Factors affecting cTn detection	Problems resulting	Actual/potential solutions
Cross-reactivity to skeletal isoforms of troponins	Elevated troponin levels measured in the absence of heart problems	Ensure use of antibodies that only detect cardiac forms of troponins
Potentially occluded epitopes as a result of complex formation between troponins I, T and C	Very hard to detect free format of cTnI as the majority of cTnI in a blood sample is in cTn complex format with blocked epitopes (Bates et al., 2010).	The anti-cTnC or anti-cTn complex antibody can be used as capture Ab while anti-cTnI antibody is used for detection (Hytest, 2020).
Proteolytic degradation	Very hard to detect the N-terminal and C-terminal of cTnI/T which usually undergo proteolytic degradation.	Antibodies against the cTnI epitopes located within the central region (protected from proteolytic degradation) should be included in a cTnI/T assay (Katrukha et al., 1998; Guy et al., 2013).
Use of multiple epitopes may provide more usable assay formats but these need to be fully characterised/validated	Obtaining various results by using different antibodies against different epitopes for the same sample and assay	Multi-Ab assay: two or more capture antibodies are applied with one or more detection antibodies (these antibodies are against various epitopes) (Guy et al., 2013; Hytest, 2020).
Phosphorylation	The structure of cTnI/T and the interaction within the cTn complex will be changed, which affects the binding of some anti-cTnI/T antibodies (Zhang et al., 2011; Hytest, 2020).	Need to use multiple antibodies to different epitopes to avoid such assay issues (Guy et al., 2013).
Autoantibodies	Significant underestimation of the blood cTn level due to presence of autoantibodies to certain epitopes e.g. the mid-fragment of cTnI, which is very stable and thus a very popular target for commercial cTnI assays, is also a highly targeted by autoantibodies (Adamczyk et al., 2010; Savukoski et al., 2014).	Antibody combinations which are against three or more carefully selected epitopes (cover the end and mid-fragments of cTn) should be considered for cTn detection (Savukoski et al., 2013).
Heterophile antibodies	False positive and false negative results (Tate and Ward, 2004; Warade, 2017); e.g. antibodies to mouse antibodies already present in patients' blood can interfere with assay functionality	Partly (e.g. antibody Fc or constant region replaced with a human Fc or constant region) or fully humanised (>95% of antibody sequence replaced with human sequence) antibodies should be included for cTn detection (Bolstad et al., 2013; Hytest, 2020).

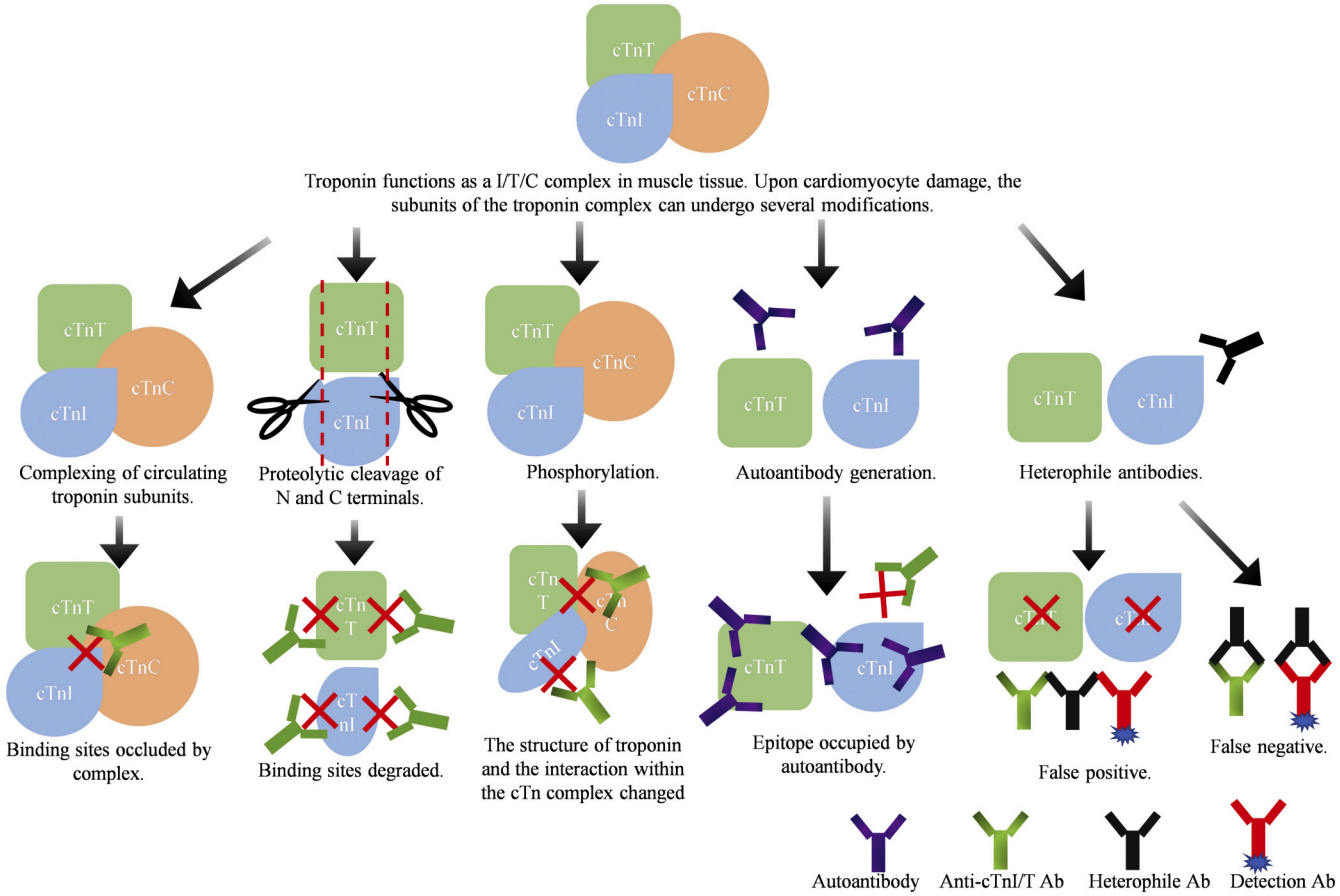

Fig. 2. Illustration of some factors to be considered for troponin detection assays due to the complex character of cTn. For example, the cTnI-C complex, proteolytic degradation of N- and C-terminal regions of cTnI, phosphorylation of cTnI regions, autoantibodies and heterophile antibodies will affect the detection of cTnI using anti-cTnI antibodies.

midfragment (Katrukha et al., 1998). For this reason, antibodies specific for epitopes on cTnI/T located within the central region should be included. This could limit, or ideally eliminate, the effect of proteolytic degradation on assay performance.

There are multiple epitopes of troponins that may be utilised in assay development. However, as aforementioned, not all epitopes may be suitable due to breakdown by proteolytic degradation or occlusion upon the formation of the troponin complex. Hence, the resultant concentrations recorded for a given sample may vary depending on the targeted epitopes and associated antibodies selected. This is indeed the case when different antibodies, assay formats and kits are used. However, it is generally agreed that use of multiple antibodies targeting different epitopes enables higher assay sensitivity and accuracy. In a multi-Ab assay, two or more capture antibodies are applied and one or more detection antibodies are used (Guy et al., 2013; Hytest, 2020). The use of such antibody panels should help to negate variation observed when fewer epitopes are targeted.

Another issue linked to epitope targeting of cTnI is the potential for phosphorylation of regions on the protein. For example, the amino acid residues S23, S24, S198 of cTnI and S179, S278, T287 of cTnT can be phosphorylated in vivo (Vahebi et al., 2005; Zhang et al., 2011; Kooij et al., 2013). The phosphorylated cTnI/T will then be released into the blood. The structure of cTnI/T and the interaction within the cTn complex will be changed due to the cTnI/T phosphorylation, which affects the binding of some anti-cTnI/T antibodies. In order to overcome the effect of troponin phosphorylation, use of multi-Ab assay formats mentioned above is necessary.

In addition to phosphorylation limiting epitope accessibility, the presence of autoantibodies can also pose a challenge in terms of available binding regions on the cTn molecule. Cardiac troponin–specific autoantibodies (cTnAAbs), present in a high percentage of individuals (5%–20%) with or without heart diseases, can lead to the significant underestimations of the blood cTn levels. This, in turn, can markedly affect the diagnosis of heart diseases (Adamczyk et al., 2010; Savukoski et al., 2014). Vylegzhanina et al. (2017) found that TnAAbs bind to the structural epitopes formed by cTnI and cTnT molecules in the troponin complex, and the concentrations of total cTnI at the early stage of AMI could be underestimated in approximately 5%–10% of patients. Interestingly enough, although autoantibodies to cTnT have been reported, no interferences in assay results have been noticed (Mair et al., 2018). Savukoski et al. (2013) reported that the midfragment of cTnI, which is very stable and thus very popular target for commercial cTnI assays, is also highly targeted by autoantibodies, while the epitopes around N-terminal and C-terminal are less targeted. Therefore, it was suggested that in order to limit such negative interference, a combination of antibodies against three or more carefully selected epitopes (covering the end and mid-portions of cTn) should be considered.

In addition to issues faced with detection of cTn, antibody cross-reactivity in the assay itself can also lead to inaccurate results. Heterophile antibodies are antibodies generated when patients have previously been exposed to various materials including certain animal–derived molecules. A major factor here is the existence of human anti-mouse antibodies (HAMA), as most diagnostic assays use antibodies generated in mice. HAMA can cause false positive and false negative results (Zaidi and Cowell, 2010; Warade, 2017). Various reagents and assays have been developed and tested in order to eliminate interferences of HAMA, including HAMA blocking reagents, but none of them is 100% effective (Tate and Ward, 2004; Emerson and Lai, 2013; Hu and Ho,

2020). Therefore, these inaccuracies caused by HAMA may be overcome by using partly or fully humanised antibodies (e.g. chimeric antibodies (Hytest, 2020)) or antibodies from other hosts. For the partly humanised antibodies, there are normally two options: a) the mAb Fc-region can be replaced with a human Fc or b) the antibody (mAb or Fab) constant domain can be replaced by a human constant domain. For the fully humanised antibodies (mAb, Fab or scFv), >95% of the antibody sequence can be replaced with a human sequence, only retaining the complementarity-determining region in the variable domain (Bolstad et al., 2013; Lu et al., 2020).

4. Heart disease diagnosis assays using anti-cTn antibody

The general cut-offs for cTnI and cTnT are 40 pg/mL and 100 pg/mL, respectively (Tanislav et al., 2016). A cTnI level above 40 pg/mL and/or cTnT above 100 pg/mL may indicate myocardial injury, and rising or falling patterns can be indicative of a heart attack. However, there is natural variation within the population, with some healthy individuals presenting atypical cTn measurements outside of the normal range. Moreover, circulating cTn levels are comparably low within the first 3 h after the onset of ischemic symptoms. Therefore, a sensitive and accurate cTn assay, with a limit of detection (LOD) lower than the 'normal' level is essential for early cTn detection and monitoring. This in turn will lead to more precise diagnosis. Increased sensitivity assays would also permit the confident measurement of elevated troponin in a shorter period of time, leading to quicker treatment of patients presenting with symptoms of cardiac damage.

4.1. Various cTn detection systems

The first generation cTn assays (usually optical assays) are less sensitive and can be time consuming, while the latest cTn assays (optical, electrochemical and acoustic assays) are generally more sensitive and rapid (Cummins et al., 1987; Katus et al., 1989; Cai et al., 2018; Christenson et al., 2020). Anti-cTn antibodies are the main biological reagents used for detection of troponin levels in blood. The following section provides a summary and comparison of cTn assays based on optical, electrochemical and acoustic techniques (Table 3). Moreover, for each type of assay, there are examples where a low LOD and run times were achieved, indicating that with proper optimisation (e.g. the application of microsphere technology, microfluidics and signal amplification system), almost any type of assay format can be suitable for sensitive/accurate sensors and/or point-of-care testing (POCT) system. Some studies developing assays used genuine clinical samples to support the value of the assay by having healthy and symptomatic patient blood samples (marked as 'clinical' under the 'setting' column in Table 3), while others used cTn standard solutions spiked into human blood to mimic clinical samples (marked as 'non-clinical' under the 'setting'column in Table 3).

4.1.1. Optical assays

Optical assays are the most widely used in clinical and research laboratories. Such assays include those based on absorbance (commonly referred to as colorimetric assays), fluorescence or chemiluminescence. The most popular colorimetric assay is the enzyme-linked immunosorbent assay (ELISA). Many different optical assays have been used for troponin detection. Generally, optical assays are easy to perform, cheap and stable but they are often less sensitive and more time consuming than other approaches. However, when used in combination with emerging technologies (e.g. microspheres, microfluidics and signal amplification systems using gold or other nanoparticles), optical assays can be highly sensitive and rapid (Zhang et al., 2014; Pawula et al., 2016; Ashaduzzaman et al., 2017; Fu et al., 2019; Fu et al., 2019; Christenson et al., 2020).

4.1.2. Electrochemical assays

In addition to optical sensing systems, numerous highly sensitive sensors for cTn detection are based on electrochemical approaches.

Table 3
Comparison of older cTn-detection assays and novel approaches, based on optical, electrochemical and acoustic techniques, using anti-cTn antibodies.

Type of assay	Advantage	Disadvantage and possible solutions	LOD	Time	Setting	Target
First generation cTn assays	Pioneer	Less sensitive and slow	10 ng/mL (Cummins et al., 1987)	N/A	Clinical	cTnI
			0.5 ng/mL (Katus et al., 1989)	90 min	Clinical	cTnT
Optimised optical assays	Easy to perform and cheap	Sensitivity can be improved by combination with microspheres, microfluidics and signal amplification systems.	0.016 ng/mL (Cai et al., 2018)	<15 min	Clinical	cTnI
			0.1 ng/mL (Zhang et al., 2014)	~1 h	Non-clinical	cTnI
			5 pg/mL (Fu et al., 2019)	7 h	Non-clinical	cTnI
			0.01 ng/mL (Christenson et al., 2020)	30 min	Non-clinical	cTnI
			0.5 ng/mL (Pawula et al., 2016)	~1 h	Non-clinical	cTnT
			7.6 fg/mL (Ashaduzzaman et al., 2017)	20 min	Non-clinical	cTnT
Electrochemical assays	Cheap, very sensitive, easy to use and fast	Narrow/limited temperature range and short/limited shelf-life.	5 pg/mL (Kim et al., 2016)	20 min	Non-clinical	cTnI
			10 ag/mL (Spain et al., 2018)	2 h	Non-clinical	cTnI
			0.033 ng/mL (Gomes-Filho et al., 2013)	~1 h	Non-clinical	cTnT
			0.1 pg/mL (Shanmugam et al., 2016)	<20 min	Non-clinical	cTnT
			0.187 fg/mL (Supraja et al., 2019)	10 min	Non-clinical	cTnT
Acoustic wave-based assays	Fast and sensitive	More expensive and complicated. Can be improved by applying microfluidics and multiple immobilisation techniques.	6.7 pg/mL (Lee et al., 2013)	8.5 min	Clinical	cTnI
			5 ng/mL (Wong-ek et al., 2010)	1 h	Non-clinical	cTnT
			0.008 ng/mL (Mattos et al., 2012)	~24 min	Clinical	cTnT

These include impedimetric, amperometric, potentiometric and conductance-based assays. Electrochemical sensors work on the principle of transducing the biochemical events (e.g. antibody-antigen interaction) to electrical signals, enabling 'real-time' detection. Advantages include high sensitivity, selectivity and reliability, cheap production, ease-of-use and their suitability for POCT (Gomes-Filho et al., 2013; Kim et al., 2016; Shanmugam et al., 2016; Spain et al., 2018; Supraja et al., 2019). However, some electrochemical sensors may have narrow/limited temperature range usability/stability.

4.1.3. Acoustic wave-based approaches

The principle of acoustic wave-based sensors is mass estimation using a piezoelectric crystal. The formation of an antigen–antibody complex on the surface of the electrode causes a mass change, which leads to a shift in the frequency of the crystal, generating a measurable electrical signal. It is fast and sensitive, but also expensive and sometimes complicated to use. However, microfluidics and multiple immobilisation techniques have been applied to resolve these problems (Wong-ek et al., 2010; Mattos et al., 2012; Lee et al., 2013). Therefore, while at present they are not as popular as optical and electrochemical sensors, their scope of use is increasing.

4.2. Point-of-care testing (POCT) using anti-cTn antibodies

Troponins are excellent biomarkers of various heart diseases, however, there is still a niche to be filled for rapid and available ways to test for a number of symptoms or elevated proteins at one time. POCT provides a solution to this problem by providing rapid diagnosis (usually showing results within minutes rather than hours). Ideally, POCT would be available at a patient's site-of-care, in an operating theatre or in a clinic (Yang and Zhou, 2006). POCT devices should be easy to use, or easy to be trained on, thus permitting a broader range of users. This could facilitate a whole new horizon for possible treatments and increase significantly the possibilities of early diagnosis and prevention of chronic and acute heart diseases (Luppa et al., 2011).

Large number of antibodies (including mAb as well as rAbs) have been successfully applied in cTn POCT (Hearty and O'Kennedy, 2011; Lin et al., 2020). The majority of cTn POCT devices used in the rapid detection of AMI are based on ELISA, fluorescence and chemiluminescence. LFIA-based detection is another key technology in the POCT, due to its simplicity, rapidity and ease-of-use. Electrochemical assays and acoustic wave assays also have promising potential for transition to POCT applications due to their high sensitivity and rapid result processing capabilities (Lee et al., 2013; Regan et al., 2018; Spain et al., 2018).

Lim et al. (2015) developed a rapid immunosensor for detecting high-sensitivity cardiac troponin I (hs-cTnI; <0.01 ng/mL in human serum) at POC. Two capture antibodies (recognising cTnI at amino acids (AA) 82–93 and AA 18–28) and two detection antibodies (recognising cTnI at AA 41–49 and AA 190–197) were employed. This hs-cTnI assay was completed within 25 min with a very low detection limit (0.003 ± 0.001 ng/mL cTnI), and the results showed a high correlation with those of the CentaurXP® reference system ($R(2) > 0.99$). This provided a much more cost-effective POCT for hs-cTnI detection when compared to the automated versions currently available.

Cai et al. (2018) reported a new POCT for the rapid (within 15 min) and early detection of cTnI in human plasma using a reliable lateral flow immunoassay. One detection antibody (recognises the epitope at AA 41–49) and two capture antibodies (recognising epitopes AA 18–28 and AA 82–93, respectively) were used. The immunoassay showed high sensitivity with a low detection limit at 0.016 ng/mL, a wide working range of 0–40 ng/mL, and a low coefficient of variations of 10%.

Katrukha et al. (2018) identified cTnI fragments (cTnI presents as an intact molecule with a repertoire of proteolytic fragments) and calculated their ratio in patient blood (serum) at different time points after AMI. The aim of the study was to distinguish the most stable part(s) of cTnI, in the hope of improving the accuracy of POCT. The ratio of the fragments in serial samples stayed stable in the period 1–36 h after AMI. It was suggested that AA 23–36 and AA 126–196 are the most stable parts of cTnI in the serum of patients within the first 36 h after AMI, which can be used as the detection target in AMI POCT.

The above examples identify various cTn detection technologies that have transferred well to POCT, ranging from simple and cost effective paper-based assays such as LFIA, to more sophisticated immunosensor systems. The transition to POCT is important as it facilitates diagnosis at bedside which, in turn, reduces the time until results turnaround. Overall, this can lead to improved patient care by shortening the length of time it takes for an appropriate course of treatment to be determined.

One crucial element for successful POCT is validation of such an approach using whole blood in settings whereby the end-point system is highly user friendly and does not require operation by individuals with clinical diagnostic laboratory expertise. Presently, most POCT validations are performed by researchers, who have laboratory analysis backgrounds, and testing is achieved using serum or plasma, but not whole blood. However, for the successful deployment of troponin POCT, use is required by individuals without specialized training. Additionally, this testing would ideally be performed using whole blood, rather than serum or plasma, with all processing requirements incorporated within the system and not requiring operator intervention. Use of either serum or plasma would require additional steps, limiting use by non-trained individuals (Tuck et al., 2009; Thermo Fisher, 2021) One of the main drawbacks in the preparation of serum or plasma from whole blood is the centrifugation step, however, techniques are available which could eliminate these lab-based steps (e.g. centrifugation). For example, Lee (2018) reported a blood separation device, SerumSTAT collection tube (SerumDPT Bioscience; Cambridge, MA), that successfully eliminates centrifugation and reduces preanalytical errors and turnaround times. The SerumSTAT enables serum or plasma (depending on whether anticoagulant is used) separation from whole blood while the blood is being drawn from the patient's arm into an evacuated tube. It delivers 250 μL of serum or plasma within 1 min, which completely removed the need for centrifugation before clinical testing. A further system, the Vivid™ Plasma Separation Membrane (PALL Corporation, USA, 2021), enables highly efficient one-step plasma separation (from whole blood) in the absence of centrifugation steps. These preparatory steps could be incorporated along the POCT workflow to provide rapid methods with which to harvest serum or plasma. Additionally, validation of these techniques must be performed in a suitable setting, for example, outside of an analytical laboratory and performed by individuals that do not necessarily have extensive clinical diagnostic assay expertise.

5. Conclusion

It is clear that troponins, specifically the cardiac associated cTnI and cTnT, play a crucial role in heart disease diagnosis. Elevated troponin levels are implicated in a broad range of cardiovascular disease and are proven as highly valuable biomarkers for both diagnostics and prognostics. However, further research is required for certain cardiovascular ailments to truly discern the diagnostic and prognostic value of troponin level measurements. It is suggested that other testing methods (e.g. D-dimer, ESC guidelines, blood levels of CK-MB and myoglobin, electrocardiogram, computerized tomography scan and magnetic resonance imaging) should be employed parallel to troponin assays as troponin levels may also increase due to non-cardiac related issues.

Nonetheless, the continued development and improvement of new and existing cTn assays will benefit the field of cardiovascular and COVID-19 treatment and prognosis. In order to facilitate assay optimisation it is important that thorough investigation is performed into antibody characteristics to optimise their performance. MAbs and rAbs are generally the antibody types used in such assays, due to their monovalency which ensures specific detection of cardiac-tissue associated troponins. But this specificity needs to be fully validated. The

employment of rAbs offers multiple additional benefits, including capacity for enhancement of structure, affinity and specificity, thus providing significant potential improvements for biosensor-based and other assay systems. Recombinant Abs also have increased capacity for better orientation due to the addition of tags, and this, linked to their smaller size can improve assay performance. Moreover, humanised rAbs (e.g. Fab and scFv) can be used in overcoming issues caused by HAMA (Bolstad et al., 2013; Lu et al., 2020). Lack of stability is often cited as a disadvantage of rAbs, but this can be successfully overcome by engineering and other strategies (Quintero-Hernández et al., 2007; Ma et al., 2020).

These anti-cTn specific antibodies, in conjunction with sensitive and rapid assays, may enable timely diagnosis and aid prognosis of MI or ACS, thus, saving more lives and reducing the testing and care burden in clinical settings.

Comparisons of cTn-detection systems based on optical, electrochemical and acoustic techniques, indicate that each have associated advantages and disadvantages. Some advantages include the introduction of systems incorporating microfluidics, various antibody immobilisation techniques and signal amplification approaches using gold nanoparticles, detailed in references of Section 4. However, disadvantages do exist in that some of the developed systems can be time consuming and offer low sensitivity. Therefore, continued efforts for the design of cost-effective, sensitive and specific systems are essential.

Moreover, the employment of multi-Ab assays is necessary for more sensitive and accurate cTn detection. The targeted detection of epitopes which span the length of the cTn molecule can limit negative interferences caused by proteolytic degradation, phosphorylation and other factors described. For the multi-antibody assay formats, an ideal assay configuration would include three or more antibodies against various cTn epitopes, covering both the N and C terminal ends of cTn, as well as the central regions. Another important consideration is to include a capture antibody against cTnC or the cTn complex since the majority of cTn exists in complex with these other troponin sub-units. To address current issues with non-specific signals in the assays used for cTn detection, partly or fully humanised antibodies (e.g. Fab and chimeric antibodies) are suggested. Their employment could minimise the effect of naturally occurring human anti-species antibodies, for example HAMA, which can cause inaccuracies in the results.

Furthermore, the continued incorporation of antibodies into portable devices for use in POC can enable improved patient treatment by providing rapid result readouts, thus leading to better patient outcomes. The specific, sensitive and rapid antibody-based troponin detection system would reduce the time from symptom onset and presentation at emergency room to diagnosis and treatment, leading to superior patient outcomes (including for COVID-19-infected patients).

Declaration of competing interest

Conflict of Interest: none declared.

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