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Recent advances in cardiac biomarkers detection: from commercial devices to emerging technologies

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Abstract: Although cardiac pathologies are the major cause of death in the world, it remains difficult to provide a reliable diagnosis to prevent heart attacks. Rapid patient care and management in emergencies are critical to prevent dramatic consequences. Thus, relevant biomarkers such as cardiac troponin and natriuretic peptides are currently targeted by commercialized Point-Of-Care immunoassays. Key points still to be addressed concern cost, lack of standardization, and poor specificity, which could limit the reliability of the assays. Consequently, alternatives are emerging to address these issues. New probe molecules such as aptamers or molecularly imprinted polymers should allow a reduction in cost of the assays and an increase in reproducibility. In addition, the assay specificity and reliability could be improved by enabling multiplexing through the detection of several molecular targets in a single device.

Highlights:

- Providing a rapid and reliable diagnosis of myocardial infarction is a major issue.
- Relevant biomarkers (troponins, natriuretic peptides) are widely used for diagnosis.
- Point-Of-Care immunoassays detect cardiac biomarkers but still need improvement.
- Alternatives are emerging for a better diagnosis: more sensitive, faster and multiplexed.

Keywords: biomarker; troponin; BNP; miRNA; myocardial infarction; point-of-care

1. INTRODUCTION

Cardiovascular diseases are the leading cause of mortality in the world [1–8], which represents 45% of all deaths [9]. In 2016, 17.9 millions of people died from cardiovascular diseases [10]. In France, 400 people die every day from such diseases [11]. According to the World Health Organization (WHO), 23.3 million people will die from cardiovascular diseases in 2030 [12]. Among them, acute myocardial infarction (AMI), acute coronary syndrome (ACS) and heart failure (HF) attract the most attention of scientists and clinicians [13]. Because of the very high frequency - one third of deaths [14,15] - of this pathology all over the world [2,7,8,12,16–21], AMI is one of the WHO's major priorities [22]. Worldwide, between 5% and 20% of patients presenting to emergency departments (ED) do so with symptoms of myocardial infarction [23,24], which is defined as "cardiomyocyte necrosis in a clinical setting consistent with acute myocardial ischemia", according to

the universal definition [25]. The main challenges include rapid intervention for at-risk, together with ruling-out of people who do not have any risk as reliably as possible in order to avoid overloading of emergency departments [24,26–28]. Indeed, the overcrowding of the ED is an important issue in most hospitals. Medical staff must triage patients as fast as possible in order to avoid overloading of the department. In particular, an earlier rule-out of patients with no risk of AMI would be appreciated. There is thus a pressing need to diagnose patients with suspected cardiovascular diseases as early as possible [2,5,8,17,19,20,29–31] to minimize the number of deaths worldwide [4,12,18,32] and reduce the likelihood of negative heart-related outcomes in the long term [14].

An initial diagnosis is typically by electrocardiography as soon as the patient arrives in the ED [25]. When the electrocardiogram presents a ST-segment elevation, which corresponds to an irregular heart rate and is thus a proof of a myocardial dysfunction, there is no need for a biomarker assay. Some patients, however, may be affected by a myocardial injury without persistent ST-segment elevation [7]. Indeed, an electrocardiogram allows the detection ACS in only 20-40% of all cases [33].

Experts regularly write guidelines in order to guide clinicians and standardize practices. The European Society of Cardiology taskforce - composed of experts in the field of cardiology - studied the management of acute coronary syndromes in patients presenting to ED with chest pain but without persistent ST-segment elevation on the electrocardiogram, resulting in the publication of guidelines [25]. In the USA, similar recommendations were published by both the American College of Cardiology and the American Heart Association [34]. The symptom of chest pain is not sufficient by itself to diagnose myocardial infarction [5,7,31,32,35,36]. Experts explain that such a diagnosis must be based on two major criteria: first, a variation of a cardiac biomarker concentration such as cardiac troponins due to their high specificity and sensitivity [7,8,32,34,35,37–39]; secondly, a clinical symptom among a defined list (e.g. ischemia, abnormal electrocardiogram, problems detected by imaging in the myocardial region etc.) [5,7,31,33,36,38]. Indeed, the blood concentration of several specific cardiac biomarkers evolves over time during a myocardial infarction. Hence, their measurements can be used to improve the diagnosis [3,7,12,20,26,31,40]. Concerning the biomarker assessment, one measurement value should be higher than the 99th percentile of the reference population to confirm the diagnosis [34,38,41]. Serial measurements may be also required to ascertain the temporal evolution of biomarker concentration. Suitable decision algorithms involving biomarkers testing are thus defined in order to guide patient care [42].

Most of the time, myocardial infarction is induced by the formation of atheromatous plaques in the inner layer of the wall of coronary arteries [43]. These plaques are composed of lipids and inflammatory cells. They do not produce symptoms when they stay inside the arterial wall. When a plaque ruptures, a blood clot obstructs the coronary artery and the downstream cardiac tissue begins to suffer hypoxia [2,5,15]. The myocardium thus enters into a state of ischemia [14,18,37], losing its capacity to pump and finally undergoes necrosis [20], corresponding to the acute coronary syndrome (ACS) [33,44–46]. As the delay between ischemia and total necrosis of myocardial cells is very short, (between 2 and 4 hours), a rapid and reliable diagnosis is imperative [47]. Therefore, for 60-80% of patients with chest pain and normal electrocardiogram [15,33], measurements of several biomarkers of myocardial injury have to be made to complement clinical assessment and electrocardiography. In 2000, the European Society of Cardiology and American College of Cardiology decided to add cardiac troponin level shifts to the universal definition of myocardial infarction [48,49]. In the version of this definition assessed in 2007, the troponin level represents one of the criteria for acute myocardial infarction in addition to clinical symptoms: “at least one value above the 99th percentile of the upper reference limit” [50,51]. Since then, troponin is by far the preferred biomarker [47] among clinicians as it allows a reliable diagnosis [40]. For patients with suspected non-ST-segment elevation ACS, this measurement is now mandatory, according to the 2015 European Society of Cardiology guidelines [9]. However, the time after symptom onset to reach a detectable concentration of troponin in blood can be several hours, depending on the sensitivity of the assay. Hence, troponin measurements at

the beginning of AMI are not reliable [12]. Other biomarkers, such as natriuretic peptides (NPs) whose concentration increases earlier in case of AMI [12], are also useful [14].

Plenty of cardiovascular biomarkers are used to diagnose cardiac issues. Several recent reviews that focused on cardiac biomarkers generally describe more than twenty relevant biomarkers [9,52,53]. However, clinicians have come to the consensus that both cardiac troponin and NPs are reliable quantitative biomarkers, which should be used to diagnose cardiac damages [54]. They have the characteristics of an ideal biomarker: they are measurable in blood, they are highly specific, and are rapidly detectable after the onset of first symptoms (example.g. chest pain) [9]. A reliable detection of at least one of these biomarkers is thus necessary to lead to the correct interpretation of symptoms.

However, current diagnoses are typically based on a single biomarker assay and lack reliability since no biomarker is perfect [12]. Therefore, multiplexed assays - combined detection of two or more biomarkers quantified at the same time - could give more reliable information than one biomarker alone [9,12,55,56] and accelerate AMI diagnosis [52]. This method also has the advantage of reducing the sample numbers and the assay cost [12]. A multiplexed detection should be the right approach to improve AMI diagnosis [53,57].

Furthermore, there exist limitations of the most frequently used biomarkers - cardiac troponins and NPs - such as an insufficient specificity or delayed elevation in blood. There is thus still a need for novel biomarkers [9,35,54]. For example, another category of biomolecules, which is much less known, namely microRNA (miRNA), has attracted the interest of researchers in the last few years [58]. Since myocytes release these nucleic acids into the blood stream during apoptosis, they can be used as biomarkers [14,35].

In general, biomarker assays are performed in the medical biology laboratories of hospitals. Firstly, the delay between the test order requested by the clinician and the results recovery is often long, at least 45 minutes in the best case scenario. Secondly, when there is no laboratory inside the hospital, the sample has to be sent to a central laboratory. In that case, the delay to obtain the test results is much longer, more than 1 hour [59]. Yet, international recommendations stipulate that ideally results must be available within less than 60 minutes [2,26,60]. Therefore, it is necessary to accelerate currently available assays. Several companies have developed, and continue to improve, point-of-care (POC) devices that are portable and capable of performing cardiac biomarker quantification measurements rapidly and with high sensitivity [14,26]. One of the main advantages of POC devices is that there is no need for sample transport, which allows for more rapid diagnosis [52]. Indeed, POC testing in ED decreases the waiting time by 35.5%, with a 22 % faster turnaround time [61]. POC devices have been shown to result in reduced patient length of stay in EDs [26,52,62]. Additionally, such devices can be used outside of a hospital setting, for example in an ambulance vehicle or directly in the field.

A lot of articles have already reviewed cardiac biomarkers [9,52–54,57,63–68]. This review will focus on the detection of three different cardiac biomarkers: the well-known cardiac troponins (cTn), natriuretic peptides (NPs), and emerging cardiac miRNAs. These three biomarkers belong to the three different categories of macromolecules: namely proteins for cTn, peptides for NPs, and nucleic acids for miRNAs. Due to the differences in composition and size of these biomarkers, the detection methodology strongly differs as we will see later. The first section will summarize information about each of these three biomarkers, the first two being currently used to for myocardial infarction diagnosis, the latter showing great potential to help in AMI diagnosis. Then, the second section will focus on current commercialised POC devices detecting cTn and NPs, and more particularly the methods used to detect these biomarkers. Finally, insights and prospects will be discussed in the last section concerning recent advances and future solutions on biomarker detection in order to improve myocardial infarction diagnosis. This layout is represented in figure 1.

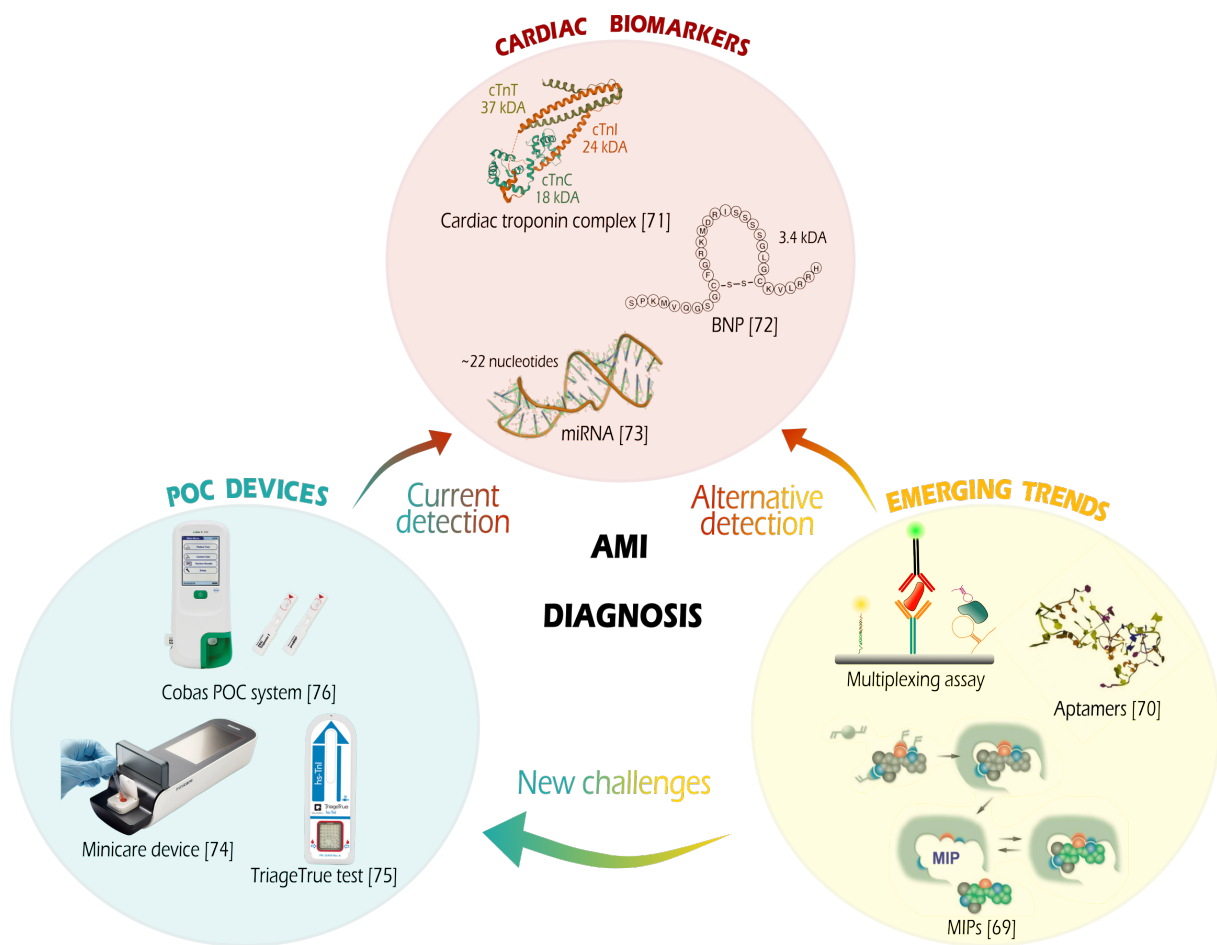


Figure 1: Graphical layout of the review.

2. CARDIAC BIOMARKERS

Recent reviews have described in details the most relevant biomarkers for cardiac diseases [9,52–54,57,63–68]. In this section, we will focus more particularly on three recent biomarkers namely troponin, natriuretic peptides and miRNAs due to their current use or recent interest as cardiac biomarkers. Furthermore, the comparison of the detection modes for those biomarkers, with different characteristics, allow us to give a representative description of the recent advances in the domain in the next two sections.

2.1 Cardiac troponin

A query on the database Scopus about articles containing in their title the following keywords: “troponin” & “cardiac” & “biomarker*” led to 131 results on April 9, 2020. Over the last 20 years, cardiac troponin I [8,14,15,19,35,38,46] and cardiac troponin T [15,35,36,38,46] have emerged as the preferred biomarkers for myocardial diagnosis. Indeed, they are the most sensitive and

specific biomarker of myocardial ischemia actually available. They are now the gold standard for AMI diagnosis [2,9,12,14,18,27,35,37,39,40,54,77–79].

Troponins are a complex of three proteins constituting the myocardial contractile system [19,27,29,33,38,40,45,80,81]. The three subunits are troponin C, troponin T, and troponin I [19,27,33,40,47,52,77]. Troponin C has a molecular weight of 18 kDa [14,47] and contains 116 amino acids. This protein binds calcium ions necessary for muscle contraction. As it is similarly expressed in the skeletal muscle and in myocardium [37,80], this protein is unspecific to heart damage and so presents no interest to cardiology [27]. Troponin T has a molecular weight of 37 kDa [21,37,47] and is present in two isoforms : one protein found in striated muscles and another one in myocardium [45]. It is also a marker of renal disease and an increase in its concentration can indicate skeletal muscle injury [29,46,52]. The last subunit is troponin I with a molecular weight of 24 kDa [37,47]. It is composed of 206 amino acids and exists in different isoforms in skeletal muscle and in myocardium. This latter protein prevents muscle fibers from contracting without calcium. Cardiac troponins regulate muscular contraction thanks to intracellular calcium. After myocardial infarction, damaged cells release cardiac troponin I (cTnI) into the blood for a period of 3 to 12 hours [2,9,15,37,40]. During AMI, the blood concentration of cTnI is lower than that of troponin T [80] but the kinetics of concentration increase is similar for both proteins [82]. According to the definition given in the “Biomarkers in heart failure: the past, current and future” review, a biomarker is “an objectively measured parameter that is an indicator of normal biological processes, pathogenic process or as a response to pharmacological therapy” [53]. Myocardial infarction corresponds to the necrosis of a part of the myocardium due to hypoxia caused by the interruption of blood flow in the coronary arteries. In that case, cardiac cells release troponins during their apoptosis [19,38,83]. Therefore, an increased troponin blood concentration means a cardiac muscle injury [2,15,29,50]. Thus, cardiac troponins are biomarkers of choice for myocardial infarction diagnosis [32,52,62,83,84].

According to recent guidelines, cardiac troponins should be quantified and recommend the following interpretation: “the higher the level [of cardiac troponin], the higher the likelihood for the presence of myocardial infarction” [25,31]. At present, troponins level measurements are an integral part of AMI diagnosis [24,26,37,38,40,51,54,84–86]. The first troponin assay described was performed in 1987 [87]. It required two days and the limit of detection was 10 µg/L [29]. Nowadays, cTn assays are marketed by several companies. These assays will be detailed in the next section. Most of them rely on a detection method based on the enzyme-linked immunosorbent assay (ELISA) principle using both a capture and a revelation antibody [2,16,37,39]. Since 2010, high-sensitivity troponin assays are available [88]. They allow a more rapid [35,38,49,78,89] and more accurate detection [27,31,35,38] compared to conventional assays, offering a reduction of the detection limit from µg/L to ng/L [27,37,47,50,89]. These highly-sensitive troponin measurements are very efficient at the first stage of the myocardial infarction because they allow the identification of early necrosis [29,50]. Myocardial infarction can thus be detected as early as 3 hours after symptom onset (Figure 2) [82]. To avoid false positive results, it is recommended [24,27,34] to monitor troponin kinetics [38,47] in at least two measurements [45], separated by 3 hours [35,90]. Currently, fast and high-sensitivity cardiac troponin assays are used almost everywhere because they are the best assays available for AMI diagnosis [91]. Very recently, in 2019, a scientific expert panel gave recommendations for transitioning to high-sensitivity troponin testing [41]. The report explained that these current measurements are now sufficiently sensitive that minor troponin concentration variations, such as a few ng/L, can be detected, allowing for a more accurate diagnosis [24,27,31,40,47,50,78]. Hence, an earlier risk stratification of AMI likelihood is now possible [27,31,35,40,78,89,92]. Thanks to these new assays, the delay between the two troponin measurements may be reduced to 1 hour [27,31,38,89,93]: the first measurement should be performed upon a patient’s admission to the ED, the second being taken one hour later. Such a protocol may be sufficient to rule-out patients with very low troponin level or rule-in patients with very high level or large increase after one hour [24,29]. However, different diagnostic protocols exist

[31,89,93] and there is still no consensus to define a unique cutoff allowing the exclusion of patients who have absolutely no risk of myocardial infarction [26,27,38,40]. Different factors like age or sex can influence troponin concentration [9,27,31,38,40] and thus should be taken into account for cutoff definition.

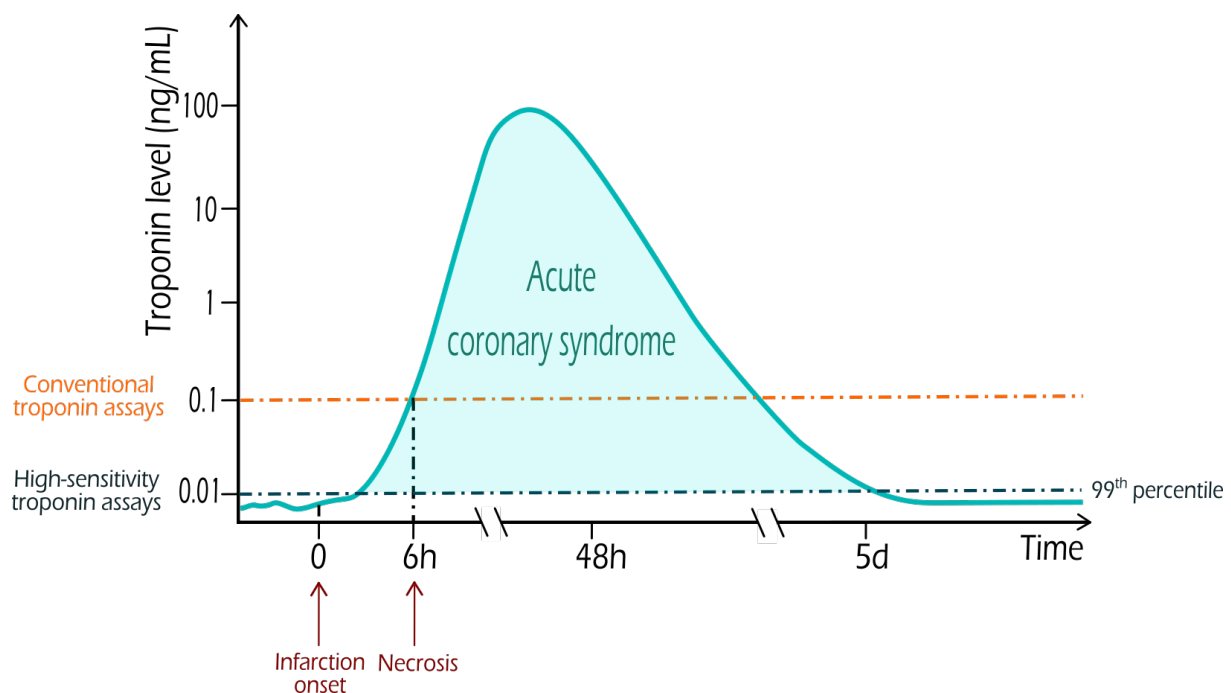


Figure 2: Evolution of troponin levels with time. Adapted from [94,95].

An additional point to highlight is the lack of specificity of troponin measurements in most assays for AMI diagnosis that can lead to false positive results [35,54,85]. Troponin increases can be caused by cardiac pathologies other than ACS [26,27,37,38,54] such as arrhythmia, anemia, pulmonary embolism, arterial hypertension... [25,31,35,37,45] Other parameters may also yield false positive or false negative results: failing antibodies [31], assay interference with blood components, calibration issues, lack of standardization, variable sensitivity, variability between lots, instrument problems [26,35,47,85]. Hence, a clinical assessment remains essential to make the right diagnosis [26,31,38,41,54].

Finally, a prognostic utility of cardiac troponin was demonstrated [27,35,89]. The troponin value gives information about the risk of death [25,31,34] and helps for risk stratification [37,38,47,93,96].

2.2 Natriuretic peptides

A bibliographic search was carried out on Scopus with the following search criteria: titles containing “natriuretic peptide” & “cardiac” & “biomarker*”. It led to only 37 publications as of April 9, 2020. This highlights the fact that these peptides are to date less well-known as cardiac biomarkers compared to cardiac troponins. They are nonetheless the most used cardiac biomarkers in the category of peptides [9,55,97].

NPs are hormones synthesized by several organs including the brain and the heart [53]. In the family of peptidic hormones, three peptides share the same ring structure composed of 17 amino acids. There are atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), and C-type natriuretic peptide (CNP). ANP and BNP amino acid sequences were identified in the 1980s [98,99]. These peptides are produced by myocardial tissue and maintain homeostasis in the cardiovascular system by binding NP receptors [100,101]. When the heart suffers from an atrial and ventricular dysfunction, the proBNP precursor is cleaved. The active BNP and the inactive N-terminal proBNP

(NT-proBNP) fragments are then secreted in the heart ventricles [53,102]. When any increase of the volume of fluid or pressure on the heart wall occurs, caused by heart rate dysregulation, concentrations of BNP and NT-proBNP in plasma increase [2,84,97,103–105]. The sensitivity of BNP is even higher than that of cTnI [12]. These peptides are thus appropriate biomarkers for diagnosing AMI very early [12,28,106] and are also considered as the gold standard biomarker for HF [2,26,53,55,57,58,83,84,100,104,107–109]. A dysregulation of BNP and NT-proBNP concentrations happens in case of ACS. Therefore, measurements of these biomarkers can be useful for AMI diagnosis. Cardiac dysfunction and HF can be diagnosed by the BNP and NT-proBNP biomarkers [12,28,55,97,100,101,104,108]. The American College of Cardiology Foundation, the American Heart Association and the European Society of Cardiology have no doubts about the validity and the reliability of these biomarkers in such cases [110]. Current guidelines from The European Society of Cardiology recommend that clinicians measure BNP and NT-proBNP plasma concentrations of patients with suspected acute HF [103]. They have set cutoff values of 35 pg/mL for BNP and 125 pg/mL for NT-proBNP for the diagnosis of chronic HF and these values are raised to 100 and 300 pg/mL respectively for acute HF [103]. As for cardiac troponin, cutoff values are multiparameter dependent (age, sex, obesity, kidney function) and should not be generic [2,27,40,54,55,77,103,105,108,110]. Furthermore, ELISA assays, used to quantify BNP, require several antibodies that bind different epitopes of the peptide [97,103,111]. So, the results vary from one technique to another [111]. Because of this divergence and the lack of international standards regarding NPs dosage, it is difficult to use a single cutoff value [77,97,103]. Moreover, the half-life of NT-proBNP is six times longer than the approximately twenty minutes half-life of BNP [111,112]. The dosage of NT-proBNP is thus preferred to BNP, especially for out-of-hospital clinical practice, since the stability of the first peptide is higher [111,113].

Furthermore, BNP and NT-proBNP specificity is not perfect, which prevents them being used alone as biomarkers for myocardial infarction. Pulmonary pathology, renal insufficiency, sepsis or diabetes can also induce an increase of BNP and NT-proBNP [12,54,57,77,102,103].

To improve the specificity of peptides assays, these NPs should be more deeply studied [100]. Actually, healthy people and patients presenting heart problems do not present these peptides in the same form (biologically active or inactive forms) [108]. Scientists and clinicians are mainly interested in BNP whereas ANP should be analyzed too [57,84,103,107]. Some useful information is obfuscated in results of the current assays, which do not detect every form of each peptide. Novel methods that detect and quantify the different forms of every peptide should be developed in order to evaluate with more accuracy the health of the patient [100].

In addition to their diagnostic function, NPs are also prognostic biomarkers for myocardial infarction [25,55,57,101,102,107,108,110,114]. During hospitalization for acute myocardial infarction, it is currently recommended to measure NT-proBNP levels to evaluate the subsequent risk of death [114]. Repetitive NP measurements could guide clinicians for treatment [115,116] and improve prognosis [117]. They can also be used to predict HF for patients affected by type 2 diabetes. These markers are widely used for risk stratification [9,26,28,54,83].

2.3 miRNAs

As previously explained, diagnosing cardiovascular diseases and particularly acute myocardial infarction is one of the most important health challenges. Detection of concentration variations of cardiac biomarkers are necessary for the diagnosis [43]. It is known that the cardiac troponin assay is the gold standard for such a diagnosis [13]. These cardiac proteins - released by cardiomyocytes - act as a good biomarker: they can be rapidly detected, precisely measured, and give useful and reliable information to help clinicians and improve patient care. Nevertheless, its lack of specificity for acute myocardial infarction encourage the research and discovery of novel biomarkers [13,43,118]. Circulating microRNAs (miRNAs) seem to be good candidates to that end [2,35,43,58,109,118–120].

miRNAs are endogenous, single stranded, highly conserved, and non-coding RNAs made of short nucleotidic oligomers (around 22 nucleotides in length) [5,14,18,30,35,43,44,58,109,118,119,121,122]. They were discovered in 2004 [123] and rapidly identified as biomarkers thanks to their stability in blood [124]. Since then, they have enjoyed increasing scientific interest [13].

miRNAs have a regulatory role in gene expression in cells [5,30,58,109,121], regulating about 66% of human protein-coding genes [44,58,109]. They bind messenger RNA to induce its degradation or prevent its translation into proteins [13,30,43,58,109,118,119,122]. Cells also release miRNA to communicate with each other [120] and to exchange genetic information [121]. These molecules are present in tissue and several bodily fluids including blood, urine and saliva [13,58,78,121,122]. They are very robust and chemically stable in body fluids [118,122]. Thus, they represent an interesting potential as biomarkers. Current detection methods to identify miRNAs are often performed on serum or plasma and are based on hybridization techniques, on quantitative polymerase chain reaction (qPCR) - which uses a normalization control [13]- or on next-generation sequencing (NGS) systems [121].

Some miRNAs called myomiRs are specifically expressed in cardiomyocytes [13]. This group is composed of miR-1, miR-133a, miR-208a/b and miR-499 [5,18,30,44]. Since their concentration increases in the case of cardiac infarction, they could be biomarkers for acute myocardial infarction [30,43,44,58]. However, it remains to be proven that they are able to improve the diagnosis of such disease compared to troponin biomarkers [13]. Their better sensitivity and specificity remain to be clearly demonstrated.

Many studies have been conducted in the last few years in order to test the suitability of such miRNAs as potential biomarkers for AMI. Among the group of cardiac biomarkers, it has been demonstrated that miR-208, which comprises miR-208a and miR-208b, is overexpressed in patients with MI and detectable one hour after symptom onset [118]. The performance of miR-208 as a biomarker for MI diagnosis compared to troponin is still a matter of debate: some studies conclude that miR-208 could detect AMI earlier than cTnI or cTnT, and that it is more specific [44,118], while others claim that even if miR-208 is cardio-specific it does not improve AMI diagnosis compared to troponin [125]. The performance of other myomiRs was tested: miR-499 shows high specificity to AMI and could be detected at the very beginning of symptom onset, (earlier than troponin [44]) while miR-221-3p has been also explored as a candidate for AMI diagnosis [43]. However, the reliability of the former study - focused on miR-221-3p - is limited due to biases [119]. Hence, scientists have not yet reached a consensus regarding the performance of these miRNAs as potential biomarker for cardiovascular diseases [30].

Furthermore, miRNA detection is hampered by intrinsic technical issues. Firstly, their concentration depends on biological parameters such as age and sex [13]. Secondly, there are as yet no established standard methods for their detection and measurement [121]. Many factors, especially in the pre-analytical steps, cause variability in the measurement results, including the presence of anticoagulant, storage conditions, temperature, centrifugation protocol, and the normalization method used [13,121]. There is thus a need to improve and standardize protocols in order to render miRNAs data sufficiently robust and reliable.

Cardiac miRNAs have a huge potential to become powerful biomarkers for myocardial infarction diagnosis but a lot of studies and standardization methods have yet to be implemented before their suitability can be established [30].

3. COMMERCIALIZED SOLUTIONS FOR CARDIAC BIOMARKERS DETECTION

The use of POC devices in ED represents a huge breakthrough for a rapid management of patients. Such systems can reduce the time needed to deliver a rapid diagnosis and consequently ED length of stay for the patient. It also opens up opportunities for on-site or decentralised detection. This is particularly the case for cardiac health issues and acute myocardial infarction (AMI) diagnosis. Usually, several specific cardiac biomarkers are detected in central laboratories according to well-defined procedures, as discussed above. Nevertheless, these procedures can take several hours to produce results and enable diagnosis, despite use in time-critical scenarios where rapid intervention is crucial to improve patient care. In this space, several companies have successfully commercialized POC devices, which can detect the most commonly used cardiac biomarkers, such as cTnI, cTnT and BNP, in just a few minutes. However, the main drawback of such devices is their sensitivity, which is lower than laboratory assays. It implies that POC devices need a highest cut-off point for an accurate detection [126].

Before describing widely used POC assays for cardiac biomarkers detection, let us define several terms that are used to quantitatively characterize the performances of a device:

- Limit of detection (LoD): The lowest concentration of the biomarker detectable by the device.
- Limit of Blank (LoB): The highest quantity value to be observed for a blank material containing no analyte.
- Limit of quantification (LoQ): The Lowest biomarker concentrations that can be reliably quantified by the device.
- Range of detection: Wide range of concentrations with a Coefficient of Variation (CV) lower than a specific percentage (generally 10%).
- Coefficient of Variation (CV): Indicate the dispersion of the values distribution around the mean value.
- Cut-off point: Reference decision limit generally defined at the 99th percentile of a normal reference population. Below this value, the test result is negative. Above, it is positive.

In order to optimize the detection and quantification of either troponins or BNP, several challenges must be overcome:

- The biomarker concentration in bodily fluids (typically blood or plasma) is, low especially for healthy patients (ng/L). Some amplification methodologies or labelling of probes will be implemented within the protocols of the POC devices to reach relevant LoD.
- Patient samples are generally complex (blood or plasma), containing many other analytes or interferences. Sample preparation and/or washing step may thus be required to avoid contaminations and reduce LoB.
- The sampling method (capillary blood) limits the amount of sample accessible for the assay.
- The affinity and selectivity of the capture probes is crucial for low LoD. High affinity and selectivity increase the signal-to-noise ratio because the probes capture only the molecule of interest.
- The time of detection is also essential. The objectives of the ED are to provide an accurate diagnosis as fast as possible in order to guarantee patient safety (especially in case of cardiac troubles), and to avoid the saturation of the ED.

In this section, several currently commercialized POC devices - detecting either troponins or BNP - will be presented, with a special focus on their detection methodologies.

3.1 Magnetic bead capture and frustrated total internal reflection detection

The Philips company has developed a POC system, called Minicare. It is based on a homogenous sandwich immunoassay [127,128] and a technology for the capture and concentration of the biomarker and its concentration through precise manipulation of magnetic nanoparticles [129]. This methodology aims to detect low levels of troponin I. One of the main characteristics of this system is the use of magnetic beads which are easy to control and useful during the washing step, thus reducing the loss of complexes and amplifying the signal. Moreover, the Minicare device can be handheld and used at the patient's bedside.

The detection process is as follows (Figure 3A). The system only needs a small amount of blood sample, approximately 30 μL . The blood cells are filtered through a membrane, which selectively draws only the plasma components into the reaction chamber using capillary forces. Only 0.25 μL of plasma are used for the reaction with antibodies. Magnetic beads are covalently functionalized with mouse monoclonal anti-cTnI antibodies that target the stable part of cTnI (amino acids (aa) 41-49). Thus, the antibodies on the beads capture the cTnI, then a magnetic field is applied in order to drive the beads onto the sensor surface. On this surface, 3 other types of antibodies are immobilized by physisorption: two anti-cTnI antibodies that recognize different cTnI epitopes (aa 20-100) on the troponin, and one antibody (anti-cTnC) that binds cTnC. The combination of these three antibodies permit an optimal detection of cTnI and cTnI-cTnC complexes. When the molecular sandwiches are formed, a magnetic pulse is generated in order to favour optimal bindings in the complexes. Then, a magnetic wash, created by a magnetic gradient, is performed in order to remove unbound beads [127,128]. This last step allows the minimization of the non-specific signal.

To quantify the concentration of cTnI present in the sample, a frustrated Total Internal Reflection (f-TIR) measurement is performed by the Minicare device [127] (Figure 3B). When an incident light, traveling through a medium such as air, hits a second medium at an incident angle greater than the critical angle, the light is totally reflected: that is total internal reflection. If a third medium with a higher refractive index than the second is added between the two first media, the energy of an evanescent wave is transferred from the second medium to the third one. This induces a decrease of the reflectivity of the interface. As the reflection is no longer total, this phenomenon is known as frustrated total internal reflection. Hence, this optical technology can be used to study biomolecular interactions at the interface between different media. Other optical techniques such as Bio-Layer Interferometry (BLI) or Surface Plasmon Resonance (SPR) are also able to detect interactions between a probe immobilized on a surface and a biomolecule of interest.

For the detection of troponin I, the Minicare device has the following performances: a level of blank (LoB) of 8.5 ng/L, a limit of detection (LoD) of 18 ng/L and a limit of quantification (LoQ) of 38 ng/L for a CV of 20% [128,130]. However, for the detection of troponin I, the Minicare device seems to be less accurate than other POC systems. Indeed, it has the highest LoD, LoQ and an important LoB compared to the others of the list displayed in Table 1.

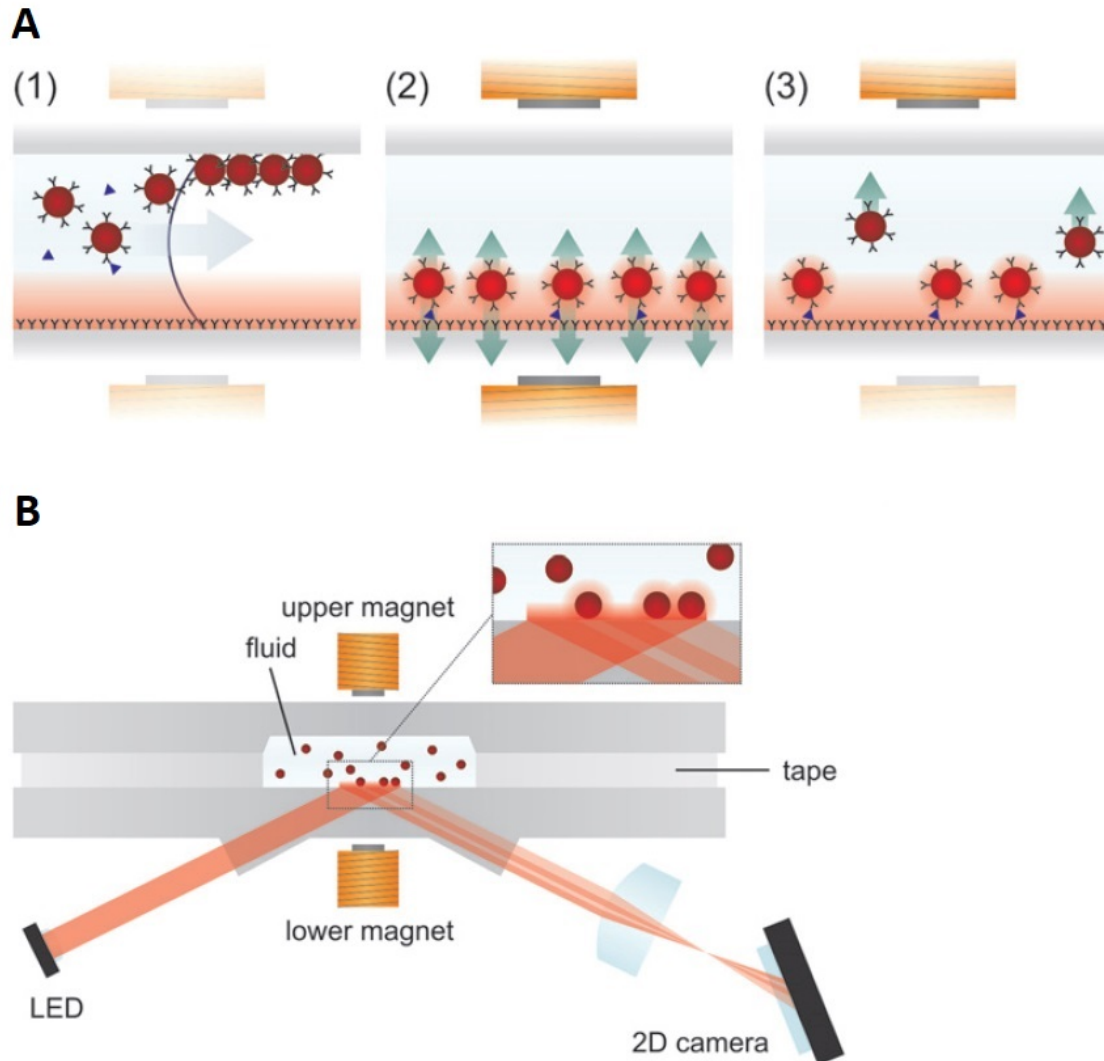


Figure 3: A. Immunoassay steps of Minicare (1) filling of the microchamber, magnetic beads capture the analytes; (2) magnetic pulsing to improve the binding process; (3) magnetic wash to remove unbound beads [127]. B. f-TIR scheme of magnetic nanoparticles bound to the sensor surface [127].

Using the same magnetic bead technology, the Minicare device is also capable of detecting BNP in blood samples. In this case, single epitope sandwich (SES) BNP antibodies (Hytest, Turku, Finland) are used to capture the analyte. A monoclonal capture antibody (mAB 24C5), specific to the stable fragment of the BNP molecule ring (sequence FGRKMDR), binds the BNP. A detection antibody (mAB Ab-BNP2) then recognizes the whole immune complex formed by the BNP and the capture antibody (Figure 4). The specificity of the detection of the BNP with this technology is very high because the detection antibody is specific to the whole immune complex [131–133]. For the detection of BNP in a blood sample, the Minicare device has the following performances: a LoB of 3.3 ng/L, a LoD of 5.8 ng/L and a LoQ lower than 9ng/L at a CV of 20% and lower than 30 ng/L at a CV of 10% [131].

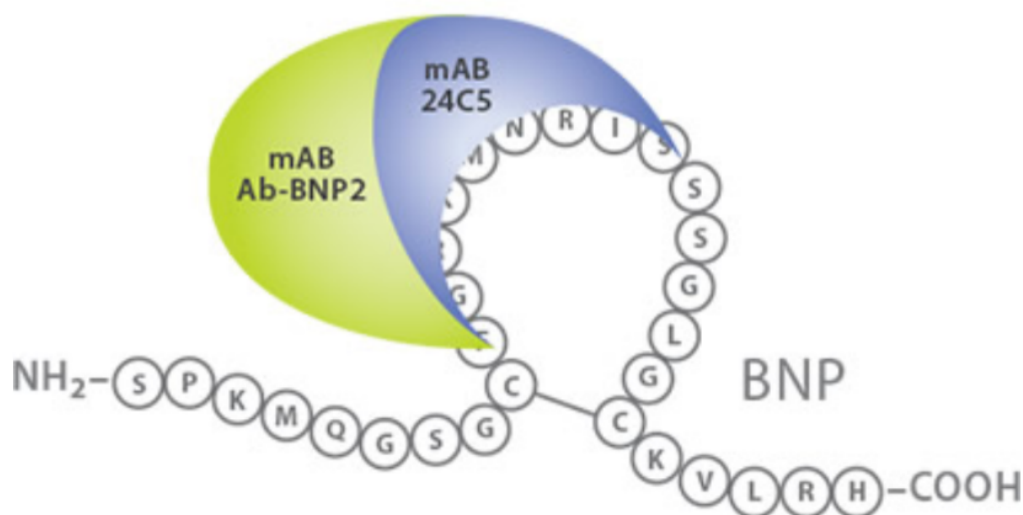


Figure 4: BNP immunoassay complex with SES [132, 133, 140].

3.2 Labelled antibodies and fluorescence detection

Another way to quantify the concentration of cardiac biomarkers in blood is through immunofluorescent labelling with antibodies.

Fluorescence is a well-known optical method used to quantify molecules and is easy to implement in a POC device. Moreover, the use of labelled antibodies improves the specificity of the detection and, in the same way, the precision as we can see in Table 1, Table 2 and Table 3.

The Radiometer company incorporates this method into the AQT90 FLEX system, using an immunoassay cartridge and a time-resolved fluorescence [134,135] detection of europium chelates (Eu chelate). Lyophilized antibodies specific to analytes of interest and, Eu chelate labelled antibodies are loaded in a 300 μ L chamber. The sample, which can be whole-blood or plasma, is injected into the chamber with a running buffer. The mixture is then incubated at 37°C for 7 to 15 minutes. Unbound molecules are removed in a washing step that reduces non-specific contribution to the signal. After drying of the chamber, the measurement is performed by exposing the complex to an excitation light source. The fluorescence emitted by the labels is measured, and finally compared to a calibration curve to obtain a quantitative measurement of the concentration of the analytes [135]. The same method is used for the detection of various cardiac biomarkers such as troponin I, T and NT-proBNP. The only differences are the lyophilized reagents, incubation times, and detection performances [136,137].

Fluorescence is also used in order to quantify the concentration of cardiac biomarkers by ET Healthcare's device known as Pylon. The system exploits labelled antibodies as the AQT90 Flex system, but with an additional fluorescence enhancement step[138]. The device is based on a probe tip which moves from wells to wells in the system. First, cTnI in the sample is captured *via* one of its epitopes (aa 27-40) on the probe tip which has been coated in purified goat polyclonal antibodies. After a 15 min incubation step, the probe tip is washed in a wash well. The probe tip is then put in contact with a secondary antibody - a mouse monoclonal biotinylated antibody - which recognizes another epitope of cTnI (aa 41-49) during a further incubation step of 60 seconds before washing to prevent non-specific interactions. The complex is incubated for 30 seconds with a matrix of polysaccharide conjugated with Cy5-streptavidin (Cy5-SA) and washed again. For a second time, the probe tip moves from the well containing the biotinylated antibody to the well with Cy5-SA in order to form a complex with a large quantity of Cy5-SA, which will amplify the fluorescent signal. Hence, the probe is covered by two layers of Cy5-SA. The measurement of the fluorescence takes only 2 seconds while the overall procedure takes approximately 20 min [139]. The fluorescence signal quality is improved thanks to branched polysaccharide, without amplifying the non-specific signal

[138]. Furthermore, the polysaccharide multivalency increases the number of sites for Cy5-SA grafting on the biopolymer chain, and thus increases the fluorescence signal **by increasing the number of fluorophores**. The different steps of the detection process are visible in figure 5.

For the detection of troponin I, the Pylon device reaches the following performances: a LoB

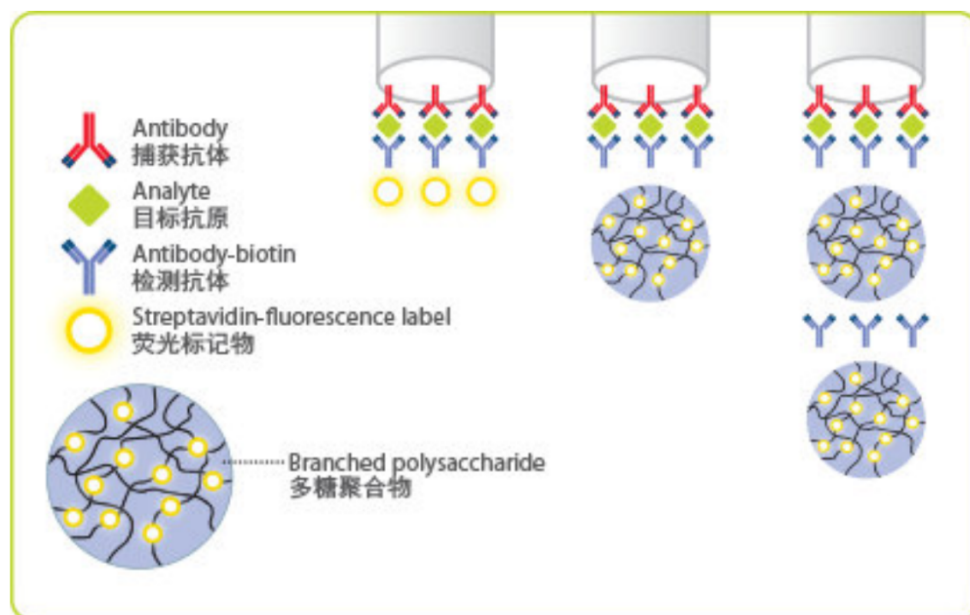


Figure 5: Pylon assay with two layers of Cy5-SA **branched** polysaccharide [138].

of 0.7-0.8 ng/L, a LoD of 1.2-1.4 ng/L and a LoQ of 2 ng/L for a 20% CV [139].

Besides, Pylon can also carry out a BNP immunoassay using a suitable cartridge. Within 14 minutes, it can detect the concentration of BNP in whole blood or plasma, using a Single Epitope Sandwich (SES) as described previously (Figure 4). In contrast to assays that are based on two antibodies recognizing two epitopes of the BNP target, it also detects a truncated form of BNP [140].

3.3 Electrochemistry-based devices

Electrochemistry is widely used for biosensing. In this domain, the measurement of the electric current, generated by electron transfer between a target molecule and an electrode, enables the quantification of the concentration of the target. Electrochemistry is characterized by its simplicity and can be easily miniaturized.

Abbott has developed and improved electrochemical devices to quantify troponin in a sample thanks to a two-site ELISA assay [141]. Initially, the troponin I is captured on a probe, which is fully covered by monoclonal anti-cTnI antibodies. The sample is then brought into contact with a second reagent composed of polyclonal anti-cTnI antibodies tagged with alkaline phosphatase enzyme (ALP). The complex formed is washed and brought into contact with an enzymatic substrate. The substrate is cleaved by the enzyme of the antibody, producing an electrochemical signal [142]. This procedure has been improved by using magnetic beads in order to capture the troponin and concentrate the beads on a magnet during a washing step [95] (Figure 6). The beads enhance the signal to noise ratio as the number of unbound analytes is reduced.

Two electrochemical sensors are used in this assay. Both, positioned side by side on a substrate, are composed of a gold microarray electrode. A third region on the substrate contains magnetic beads functionalized with capture antibodies. Antibody-enzyme conjugates that must then bind the analyte are immobilized on the first sensor. Below the second electrochemical sensor, a

high-field magnet - generating a field greater than about 0.1 T - attracts the magnetic beads onto the sensor following conjugation to cTnI [95].

Consequently, Abbott has combined the benefits of both magnetic beads and labelled antibodies: complexes formed are easier to manipulate and the signal is enhanced. Moreover, this device is portable and can accelerate AMI diagnosis (Table 1).

Abbott has also developed a BNP sandwich immunoassay, based on the same electrochemical approach, using a specific anti-BNP monoclonal antibody (specific to aa 5-13 BNP epitope) and a detection antibody (specific to aa 26-32 BNP epitope) [143].

However, it should be noticed that this device is less precise than all devices described here, with the highest 99th percentile and the highest LoB (Table 1).

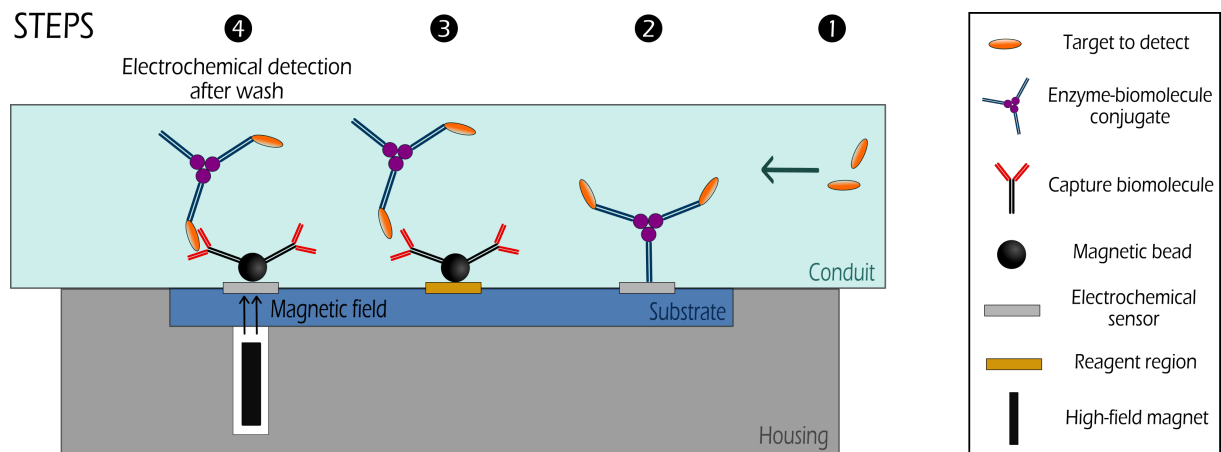


Figure 6: Detection procedure in the i-STAT of Abbott. Adapted from [95].

3.4 Chemiluminescence-based devices

Chemiluminescence refers to the emission of visible light, induced by a chemical reaction. This optical transduction mode may also be used for biosensing purposes, offering the same advantages as those use of fluorescence, namely a signal enhancement.

The device PATHFAST, commercialized by Mitsubishi, uses a chemiluminescence enzyme immunoassay (CLEIA) to quantify both troponin I and NT-proBNP, in 100 μ L of blood or plasma, within 17 minutes. Regarding troponin I detection, the sample is introduced in the device containing antibody-functionalized magnetic beads. ALP-labelled are then loaded in the device. Analytes of interest are captured in a molecular sandwich format, on one side by the antibody present on the surface of the magnetic beads and, and on the other side by the ALP labelled antibody. The magnetic beads are necessary to separate the analytes from the bulk sample. When the beads are separated, a chemiluminescent substrate is added in order to create a chemical reaction with the enzyme, and finally to enable detection thanks at a wavelength of 461 nm *via* a photomultiplier [144] (Figure 7).

PATHFAST can detect troponin I in the range 2.33 - 50,000 ng/L and has a LoD of 2.3 ng/L [145].

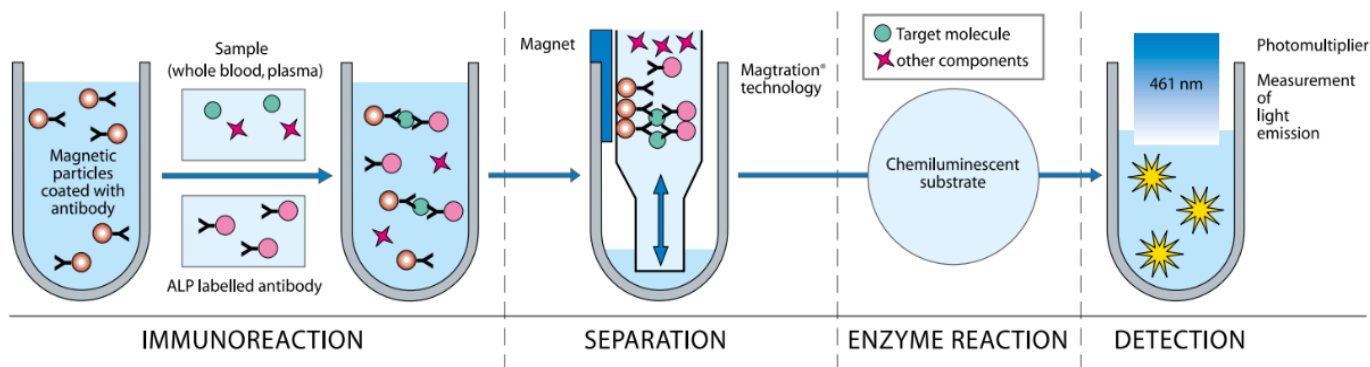


Figure 7: Pathfast test principle [144].

This device can also detect NT-proBNP, in whole blood or plasma, thanks to another immunochemiluminescent assay using a sandwich assay with 2 polyclonal antibodies. The first one is an alkaline phosphatase labelled anti NT-proBNP polyclonal sheep antibody (specific to aa 39-50) and the second is an anti NT-proBNP polyclonal sheep antibody linked to a magnetic bead (specific to aa 1-21). Then, a chemiluminescent substrate CDP-Star is added [146].

As in fluorescence devices, this system is large and thus cannot be easily used at the patient's bedside as it has not yet been miniaturised into a handheld device. Moreover, the time-to-result is longer than that of the devices using f-TIR or electrochemistry.

3.5 Lateral-flow based devices

Roche has developed many POC devices that detect cardiac biomarkers with the Cobas e [147] and Cobas h range of devices. Cobas h 232 is a handheld device, which can detect several cardiac biomarkers (e.g. troponin T, NT-proBNP) a variety of assays [76,148]. For example, for the detection of troponin T, two monoclonal antibodies specific to cTnT are used. One is immobilized on a nanoparticle while the other one is labelled with biotin. In combination they form a sandwich complex with cTnT. After erythrocyte isolation from plasma at the entry of the device, the resultant complex migrates to a detection zone where a coloured line appears if cTnT is detected in the sample, as is common in typical lateral flow assays. Finally, an optical system measures the intensity of the coloured line, which is proportional to the concentration of troponin T. Within 12 minutes and starting with 150 μL of sample, this immunoassay can detect the concentration of troponin T in a range from 40 to 2000 ng/L. It can also detect NT-proBNP within 12 minutes in a range of 60-9,000 pg/mL [76]. Moreover, Roche has improved its assays in the past few years, introducing a new monoclonal antibody which improves upon the measurement sensitivity [149]. The procedure to detect cTnT has also been improved in recent years, with, for example, the use of a tertiary amine in order to improve the efficacy [150].

Finally, the Quidel company has developed TriageTrue, a troponin I quantification lateral flow assay based on fluorescence. The analyte is sandwiched between a capture antibody and a detection antibody that is conjugated to a fluorescent label. The control is composed of a synthetic peptide, representative of a region of the analyte, coupled to human serum albumin carrier protein, which is immobilized on the surface of a solid phase (Figure 8). When the sample is dropped on the application zone of the device, the plasma is separated from the whole blood using a membrane [151], and migrates to the dried zone containing the reagents. It then migrates to a detection zone that contains a control zone and an assay zone [152].

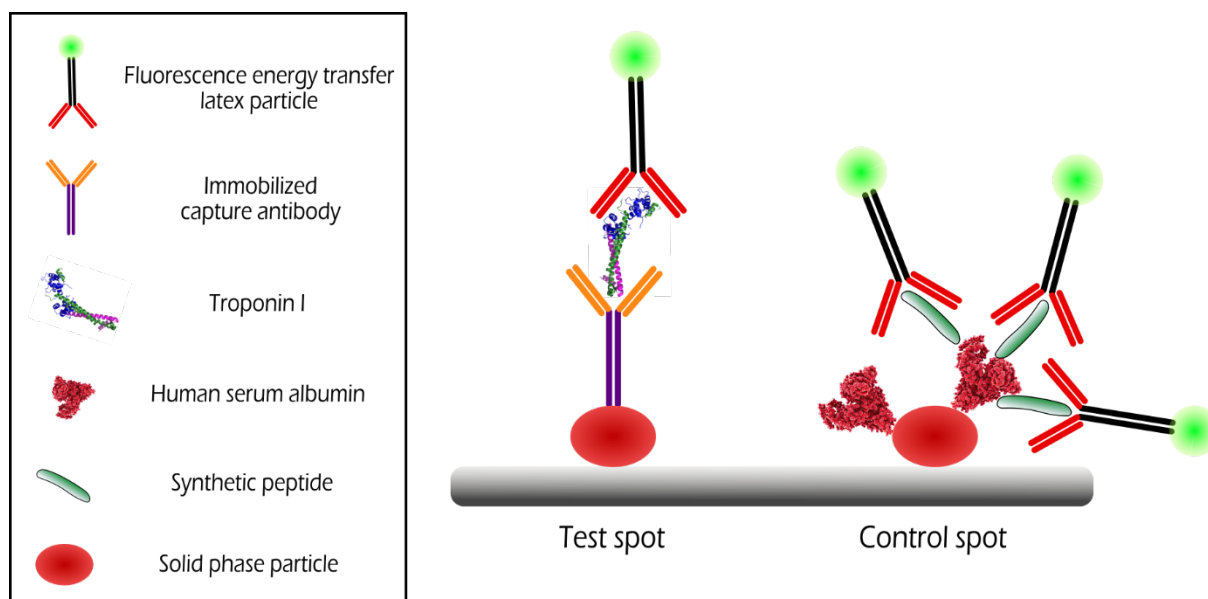


Figure 8: Scheme of an example assay used in Quidel devices. Adapted from [152].

Recently brought to market, this device is very competitive: troponin I can be detected at a concentration as low as 1,5 ng/L in whole blood [151].

3.6 Comparison of the analytical performances of the various commercial devices

Many detection methods exist to measure the concentration of cardiac biomarkers for diagnostic purposes. Several of them have been described in this review: f-TIR, fluorescence, chemiluminescence, and electrochemistry. They are the most used methodologies in POC devices for cTn, BNP and NT-proBNP detection.

The data collected and summarized in the below tables (Table 1, Table 2 and Table 3) highlight the fact that several companies have managed to develop competitive POC devices for cardiac biomarkers. Assays are usually performed in small volumes of complex samples. The LoD varies from 0.7 ng/L to 18 ng/L for cTnI, from 0.01 ng/L to 0.05 ng/L for cTnT and from 5 ng/L to almost 20 ng/L for BNP. These ranges of LoDs are wide. In each case the time-to-result meets requirements for a rapid diagnosis: all assays are carried out in 20 minutes or less. Companies have focused their research on improving sensitivity of POC devices. The probe's affinity for the target to be captured is crucial to improve the sensitivity of the device. Hence, some companies like Quidel are currently trying to develop peptidic probes that may exhibit comparable or even superior analyte-affinity compared to antibodies. Others (e.g. Abbott) rely on the use of magnetic beads to bind the target, reducing the loss during the washing step and thus improving the signal-to-noise ratio. Some publications comparing POC devices with laboratory-based bench assays confirm that their performances are equivalent [130,131,139,153–155].

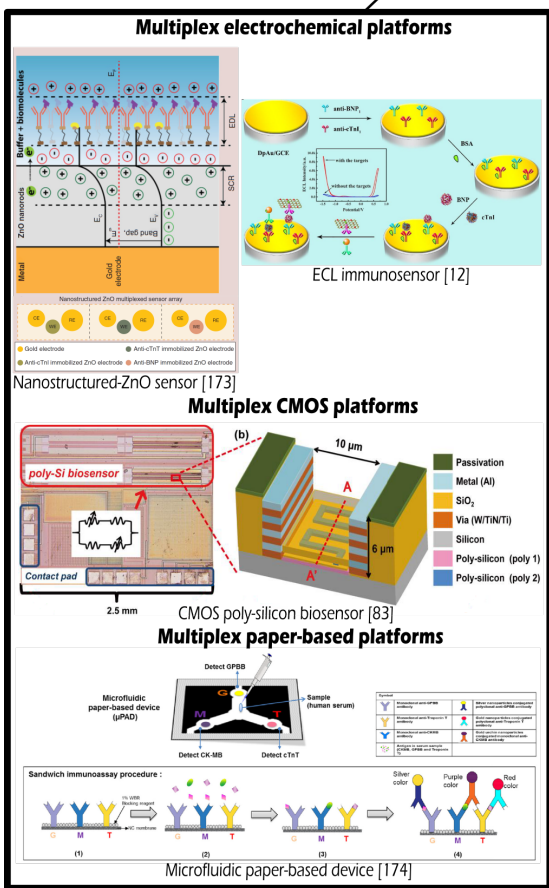
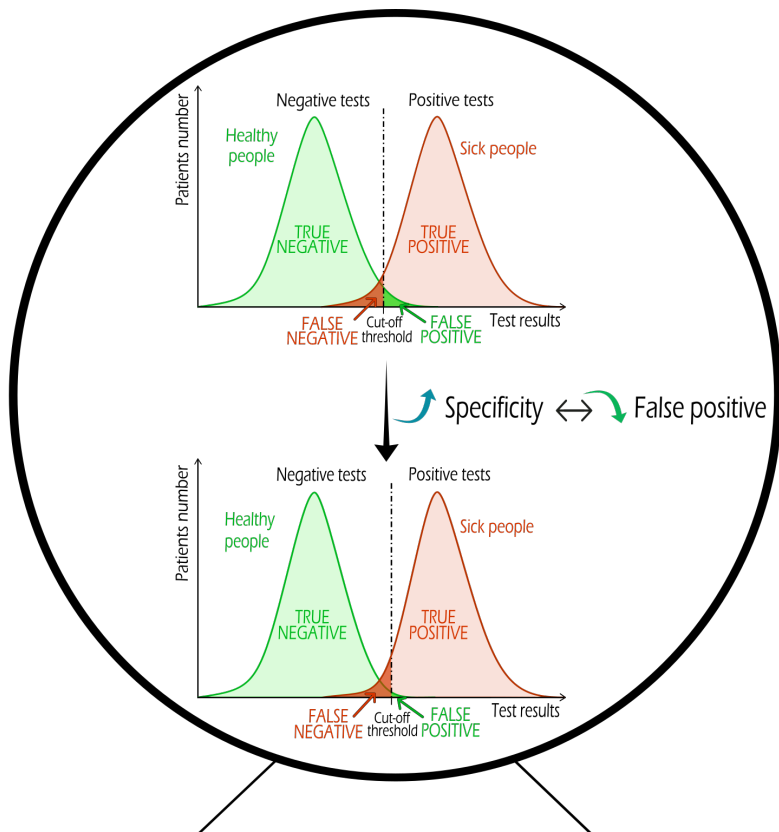
Although some devices like TriageTrue are efficient, the high LoQs show that it remains difficult to attain a quantitative measurement at very low biomarker blood concentrations. Furthermore, some compromises may be inevitable since no technology is perfect: designing a smaller device generally means a simpler and faster procedure but a loss of sensitivity (Table 1, Table 2, Table 3).

In addition, other limitations of the cited commercialized devices can be found. Indeed, many of them are not able to simultaneously perform the detection of several cardiac biomarkers, but usually require different cartridges. Finally, some of them have a better sensitivity if the analysis is

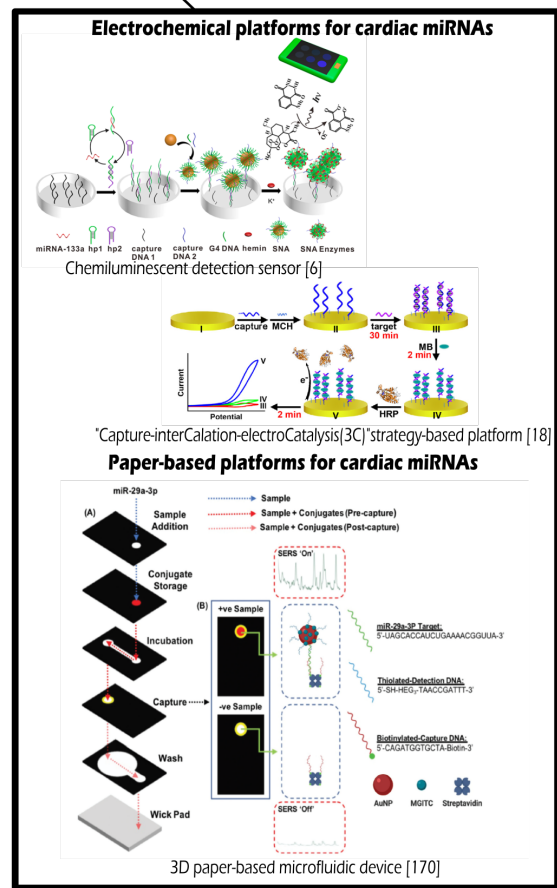
performed 3 hours or 6 hours after symptom onset, which can be an issue for a rapid diagnosis of AMI.

Another important point is the choice of the cut-off point and its medical impact. Researchers have developed high-sensitivity assays in order to lower the cut-off point and improve both the range of detection and the sensitivity of POC devices [126]. This cut-off point is defined according to the third universal definition of myocardial infarction, which recommends that it must be set at the 99th percentile of cardiac troponin in a normal reference population, in order to deliver a relevant diagnosis [153]. The cut-off value is crucial as it defines the amount of false positive and false negative tests expected in a standard population. Since distributions of healthy patients and people suffering from AMI depend on age and sex, it may be relevant to set up different cut-off points by categories. The minimal cut-off value is also greatly dependent on the sensitivity and on the limit of detection of the device. A low value is generally expected to limit the number of false negative results (corresponding to rule-out of patients while they effectively suffer from AMI) with the detrimental effect of an increase of the number of false positive results that potentially induces a saturation of ED. With higher cut-off values, potentially induced by high LOD of POC devices, the amount of false negative tests is larger, as it can be seen on the curves of figure 9. It leads to the dramatic effect of the discharge of patients effectively suffering from AMI. Since many POC systems have reproducibility issues and thus difficulties in reaching CV lower than 10% near the 99th percentile, the cut-off values are not necessarily relevant [62,154]. Comparisons of current commercially devices presented in many publications [155–158] highlight the lack of standardization of such devices. Since results depend on the device itself, no universal cut-off can be defined. Therefore, as the cut-off point is defined for each platform with its own reference population, comparisons are of limited value when made between datasets that have been acquired using different POC devices. Since this value is very important to minimize false positive and false negative results, clinicians usually focus on the use of a single, common platform to avoid confusion.

Scientists are still working on solutions to improve current POC devices for a better MI diagnosis. They are summarised in figure 9 and discussed in the following part.



MULTIPLEX ASSAYS FOR SEVERAL CARDIAC BIOMARKERS DETECTION



CARDIAC microRNAs DETECTION

Figure 9: Multiplex platforms for cardiac biomarkers detection and biosensors for cardiac miRNAs detection. Both categories could improve the specificity of actual AMI POC devices.

Biomarker	Platform / Company	LOD	LoB	LoQ	99 th percentile	Range of detection	Time of detection (min)	Detection method	Sample
cTnI	Minitcare / Philips [128,159] 	18 ng/L	8.5 ng/L	38 ng/L	43 ng/L	NA	10	f-TIR	Blood / Plasma 30 µL
	AQT90 FLEX / Radiometer [137,159] (460 x 480 x 450 mm; 35 kg) 	10 ng/L	NA	NA	23 ng/L	NA	<19	Fluorescence	Blood 2 mL
	Pylon / ET Healthcare [139,159]  Pylon 1	1.2-1.4 ng/L	0.7-0.8 ng/L	2 ng/L	26 ng/L	NA	20	Fluorescence	Blood / Plasma 40 µL
	TriageTrue / Quidel [151,159] (8.5" x 6.25" x 2.74" ; 1.5 pounds) 	0.7-1.6 ng/L	Plasma: 0.0-0.4 ng/L Whole blood: 0.5-0.8 ng/L	Plasma: 2.1 ng/L Whole blood: 1.7 ng/L	20.5 ng/L	0.1-1000 ng/L	NA	Fluorescence	Blood / Plasma 175 µL
	I-STAT cTnI / Abbott [141,159] (209 x 52 x 64 mm; 520 g) 	NA	20 ng/L	NA	80 ng/L	0.00-50.00 ng/mL	<10	Electrochemistry	Blood / Plasma 17 µL
	PATHFAST / LSI Medience [145,159] (343 x 569 x 475 mm; 28 kg) 	2.3 ng/L	1.23 ng/L	15 ng/L (for CV at 10%)	Woman: 24.2 ng/L Man: 27 ng/L	2.33-50,000 ng/L	17	Chemiluminescence	Blood / Plasma 100 µL

Table 1: Specifications of cTnI detection assays.


Biomarker	Platform / Company	LoD	LoB	LoQ	99 th percentile	Range of detection	Time of detection (min)	Detection method	Sample
cTnT	Cobas h232/ Roche [76,160] (244 x 105 x 51mm; 526 g) 	0.05 ng/L	NA	NA	NA	40 – 2000 ng/L	12	Electrochemiluminescence	Blood 150 µL
	AQT990 FLEX / Radiometer [160]	0.01 ng/L	NA	NA	17 ng/L	NA	12	Fluorescence	Blood / Plasma

Table 3: Specifications of cTnT assays.

Biomarker	Platform / Company	LoD (ng/L)	URL (ng/L)	%CV at URL	Time of detection (min)	Method of detection
BNP, proBNP, NT-proBNP	Pylon / ET Healthcare [140,161]	10	100	10%	14	Fluorescence
	AQT90 FLEX / Radiometer [136,161]	≤20	133 (95 th)	≤7.2%	<11	Fluorescence
	Minicare / Philips [131,161]	5.8	NA	NA	NA	f-TIR
	PATHFAST / LSI Medience [161,162]	5	125 (<75y) 450 (75y)	4.7% (125 ng/L) 3.4% (450 ng/L)	NA	Chemiluminescence
	i-STAT / Abbott [143,161]	NA	50 (95 th)	NA	NA	Electrochemistry
	Cobas h232 / Roche [76,161]	NA	NA	NA	12	Electrochemiluminescence

Table 2: Specifications of BNP and NT-proBNP detection assays

4. CHALLENGES AND EMERGING TRENDS IN CARDIAC BIOMARKERS DETECTION

As discussed above, there is an urgent need for new POC systems capable of assisting clinicians in myocardial infarction diagnosis using accurate cardiac biomarkers detection [6,32,105,163]. Several solutions are already used to diagnose myocardial infarction with high reliability. Numerous robust POC systems that are able to detect cardiac troponins and BNP with high affinity are already commercialized. The latest assays achieve very high sensitivity in order to avoid false negatives as much as possible. However, they still have drawbacks such as high cost, long processing times [3,105], and a lack of standardization [164]. Researchers are thus working on solutions to provide better sensitivity, user-friendliness, and which can be competitive with previous solutions in terms of size and cost. Other transduction methods, such as [165–168] or turbidity measurements [169] allow the quantification of analytes in a complex medium but they are not as widely used as the previous ones. Indeed, these latter techniques are generally not sensitive enough and are more difficult to implement in a POC or handheld device for emergency use. An increasing number of devices are becoming available that rely on common wireless standards. Smartphones, for example, enable the control and automation of the detection performed in a portable device. Both the device and the smartphone can be connected by Bluetooth [170]. Such platforms are low-cost and thus could be used in developing countries. POC devices are often based on microfluidics to handle sample and reagents. Therefore, researchers are working on new designs to achieve better performances, in particular with regard to processing time. For instance, more and more experimental devices are using a disc shape and the centrifugal force in order to sequence the reactions [171]. Lutz et al. have developed a microfluidic platform that is able to detect cTnT in only 30 μ L of sample and within 11 minutes, with better performances (LoD of 7.5 ng/L with a CV < 10%) than some laboratory analysers (Elecsys 2010: 14 ng/L and CV at 10% [172]).

Besides this, two serious issues remain. First, the lack of specificity of actual cardiac biomarkers that can induce false positive results and thus misdiagnosis [18,78], and secondly, the use of antibodies, which have numerous disadvantages (variability, production cost and time, stability). Different solutions are emerging to address these problems.

4.1 Multiplexing assays for a better specificity

In order to improve the specificity of actual assays for AMI diagnosis, the first solution is the combination of multiple cardiac biomarkers analysis. Indeed, multiplexed assays provide more information than measurement of a single biomarker, and thus can substantially help for diagnosis [3,4,12,16,83,164]. For instance, a study conducted by Wang *et al.*, shows that parallel measurements of high-sensitivity cTnI (hs-cTnI) and NT-proBNP are more reliable for AMI diagnosis than a hs-cTnI assay alone [28]. Such assays have other significant advantages including lower sample volume, economical use of reagents, short time-to-result, and can be less expensive [4]. Therefore, different multiplexed assays based on various detection methods for cTn and BNP detection are under investigation [4,173].

In the early 2010s, good performance was demonstrated in a multiplexed immunoassay for the detection of BNP, cTnI and C-reactive protein (CRP) based on photonic suspension array showed [4]. Instead of classical 2D sensors substrates, the assay relies on crystal beads as solid supports for the immobilization of probe molecules. This strategy improves the diffusion of molecules and thus accelerates the reaction. It also reduces the sample consumption and consequently the cost.

Different electrochemical platforms for the detection of multiple cardiac biomarkers have also been developed. Shanmugan *et al.* demonstrated the possible parallel detection of cTnT, cTnI and BNP in human serum with a limit of detection close to the relevant concentration for an application in a clinical setting [173]. Thanks to nanostructuring of the sensing surfaces, and thus an increased surface area, they succeeded in amplifying the signal so that the sensitivity was enhanced. Grabrowska *et al.* managed to develop another electrochemical sensor that can quantify

cTnI and BNP without any labelling step. The technology of screen-printed electrodes has allowed the parallel sensitive detection of cTnI and BNP at the low level of 1 pg/mL in serum [105].

Yang *et al.* have built the first biosensor based on electrogenerated chemiluminescence (ECL) for multiplex detection of AMI biomarkers. This device can simultaneously detect myoglobin, cTnI and cTnT with detection limits of 31 pg/mL, 0.79 pg/mL and 0.30 ng/mL, respectively [16]. This assay has the benefits of being sensitive, stable in storage and requires small samples volumes to process the analysis. Two years later, Zhao *et al.* have developed another ECL immunoassay for the detection of BNP and cTnI on the same electrode surface. This assay reached low detection limits: 3.3 pg/mL for cTnI and 3.8 pg/mL for BNP [12].

Lee and coworkers have published a study demonstrating for the first time the capacity of a field-effect complementary-metal-oxide-semiconductor (CMOS) biosensor to detect several cardiac biomarkers in clinical settings. This device enables the quantification of cTnI, BNP and interleukin-6 (IL-6) with the limits of detection of 32 pM for the first two biomarkers and 45 pM for IL-6, and could thus be implemented for AMI diagnosis [83]. Another example based on a Field-Effect Transistor (FET) was described as a multiplexing biosensor technology for cardiac biomarkers. This technology has several advantages including rapidity, high sensitivity, reliability, small size and low-energy consumption that make it ideal for POC diagnosis. This portable system, based on a microfluidic platform and a FET sensor, can process an automated detection of cTnI, NT-proBNP, CRP and fibrinogen in 4 μ L of clinical samples in 5 min [3].

Finally, paper-based biosensors have recently attracted the interest of the scientific community. Indeed, this low-cost technology allows the development of affordable and environmentally friendly lab-on-chip devices that could be used as diagnostic tools for many diseases including AMI [39]. Lim *et al.* team have recently developed two microfluidic paper-based biosensors to detect multiple cardiac biomarkers. The first is based on a colorimetric sandwich immunoassay using gold nanoparticles for glycogen phosphorylase isoenzyme BB (GPBB), creatine kinase-MB (CK-MB) and cTnT multiplex analysis [174]. The sensitivity of this low-cost sensor, requiring only a smartphone camera, has since been improved by utilizing Surface Enhanced Raman Scattering (SERS). The multiplex quantification of the same three biomarkers thus reached limits of detection that are below the classical cut-off values [175]. Go *et al.* have created another paper-based biosensor for the simultaneous and rapid detection of heat-type fatty acid binding protein (FABP), myoglobin and cTnI. This portable fluorescence immunoassay uses zinc oxide nanowires to improve the fluorescence signal and so increase the sensitivity [176]. We can also cite the work of Li *et al.* based on chemiluminescence [177], the paper-based lateral flow assay of Han *et al.* [178], and the technology of Guo's team [179], each of which use paper technology for multiplexed analysis of cardiac biomarkers.

4.2 miRNAs: novel biomarkers for a better care

Another solution to improve the AMI diagnosis sensitivity is the use of novel biomarkers [5]. As mentioned previously, miRNAs might be promising biomarkers to enable specific and rapid diagnosis of AMI [78,122]. Their stability, specificity and early rise in serum and plasma after AMI are arguments in favor of their emerging use in AMI diagnosis [5].

Shi, Sun and Mi have worked on chemiluminescent detection of cardiac miRNA. Thanks to an amplification assay performed by a spherical nucleic acid enzyme (SNAzyme) and a smartphone for the detection, they proposed a portable biosensor capable of optically detecting a miRNA concentration variation of 10 fM in human serum [6,17]. They have recently improved the sensitivity of the assay by a factor of 10 by using G-quadruplex DNAzymes on a metal-organic framework [180].

Chen *et al.* have set themselves the aim of reducing the miRNA detection time in order to accelerate AMI diagnosis. To achieve this, they have developed the "Capture-interCalation-

electroCatalysis 3C” strategy that requires 35 minutes instead of several hours as is typical for such a test [18]. This electrochemical method is based on the miRNA capture by a DNA probe, followed by the incorporation of an active probe in the helix, and finally a final amplification based on horseradish peroxidase (HRP).

A new technique for miRNA detection, chip-based digital PCR, has been described recently. This method allows the direct and absolute quantification of miRNA with more accuracy and reproducibility in patient samples than classical quantitative real-time PCR [122].

Finally, Mabbott *et al.* have combined the technologies of paper-based microfluidics, colorimetry and SERS to detect a miRNA. This strategy meets the REASSURED (i.e. Real-time connectivity, Ease of specimen collection and environmental friendliness, Affordable, Sensitive, Specific, User-friendly, Rapid and robust, Equipment-free, and Deliverable to end users) standards of POC according to the WHO definition, but quantification of analytes is not yet possible with this method [163].

4.3 Aptamers: a relevant alternative to antibodies

All these examples, summarised in figure 9, show promising results towards increasing performance of POC devices for AMI diagnosis. However, the vast majority of the assays are using antibodies as probes for capturing target biomarkers. These recognition proteins present several drawbacks. Their stability is low [32,36] resulting in a short storage life, they are fragile and subject to denaturation as a result of temperature variations [7]. In addition, they cannot be synthetically produced [16], leading to high costs [7,32,36] and batch-to-batch variability [20]. They could thus be responsible for the divergence in measured cTnI values between assays of actual POC devices [20]. Therefore, alternative methods to immunoassays are emerging.

One such exciting alternative is the use of aptamers [2]. These short nucleic-acid sequences (either RNA or DNA) are selected through the famous systematic evolution of ligands by exponential enrichment (SELEX) procedure to be specific to a target molecule [8,20,79]. They can bind proteins, nucleic acids, small molecules, and ions with an affinity usually comparable to antibodies. They are cheaply and rapidly chemically synthesized [7], and can be easily modified or functionalized. This synthetic method of production allows for high reproducibility [3] and good yields [105]. Compared to antibodies, they are more stable over time and less sensitive to high temperature. The reversibility of their denaturation - enabling the recovery of their folding shape afterward - is also a big advantage [3,79]. Their storage, transport, and manipulation are thus easier [14]. Hence, an increasing number of aptamers are used as specific and sensitive probes to replace unstable and expensive antibodies in biosensors known as ‘aptasensors’ [3,8,16,79].

Negahdary *et al.* have worked on the development of electrochemical aptasensors for the detection of cTnI [19]. Another example is the aptamer-based POC system designed by Jo *et al.* A sandwich of two aptamers is able to detect cTnI with good performances [181]. Another biosensor based on fluorescent troponin aptamer was designed and tested by Liu *et al.* [8]. First, the binding between graphene oxide platform and fluorescent aptamer leads to the quenching of the aptamer fluorescence. Then, when cTnI binds the specific aptamer, their high affinity results in the release of the aptamer from graphene oxide and consequently the recovery of its fluorescence. The combination of graphene oxide and fluorescent aptamers shows good performance for detection of troponin with high sensitivity. Qiao and co-workers have reported the development of another novel electrochemical aptasensor using the semiconducting nanomaterial molybdenum disulphide (MoS₂). A limit of detection of cTnI in human blood serum below 1 pM was achieved [79]. In 2020, Negahdary *et al.* reviewed the use of nanostructures - nanoparticles with optimized materials - in aptasensors that allow for a troponin detection below 1 pg/mL. Such a remarkably low LoD is due to the increased detection surface area of immobilized aptamer probes that the nanostructuring induces

[14]. With such sensitivity, these sensors could be efficient tools for cardiac biomarkers analysis. Therefore, they can be expected to be shortly integrated into commercial POC devices [14].

Multiplex technologies that have been cited above may also utilize aptamers as biorecognition elements. For instance, the electrochemical biosensors of Grabrowska *et al.* is in fact an aptasensor for BNP and cTnI [105]. It is also the case for the ECL sensor able to detect cTnI, cTnT and myoglobin [16]. A further notable example is that of the FET sensor of Sinha *et al.* that uses four aptamers to bind four cardiac biomarkers [3].

All these examples, summarized in figure 10, support the conclusion that aptamers seem well-positioned as appropriate affinity reagents for cardiac biomarkers assays and then constitute powerful alternatives to antibodies.

4.4 Molecularly imprinted polymers: a future AMI diagnostic tool?

A final alternative to immunosensors is the use of molecularly imprinted polymers (MIPs). These new molecular probes have recently experienced increasing interest in the domain of biomimetic sensors [164]. In contrast to the biosensors presented above, MIPs are based on artificial recognition polymeric elements. They consist of synthetic functional monomers that polymerize in the presence of the target analyte. When the latter is removed, the polymer contains specific cavities, which have the size and shape matching the properties of the depleted analyte. Such structure can thus selectively bind the same target. The synthesis process is easy [21] and the polymers produced are stable, at low cost and can even be reused [32,36,182].

Several MIPs have been designed in recent years to selectively detect cardiac troponin. Scarano, Minunni and Palladino have been working to develop antibody-free optical biosensors for cTnT and cTnI based on such MIPs. They first managed to prove the specific detection of the protein with a polydopamine-based MIP and SPR transduction, but the limit of detection was above cut-off values [32]. Through the exploration of another material called norepinephrine that was never used before for MIP biosensors applications, they recently reported promising results for the detection of cTnI in human serum [182].

Two other examples of MIP-based biosensors for cTnT have been developed. Unlike the previous platforms using the optical technique SPR, both MIP-based biosensors use electrochemical detection on screen-printed electrodes (SPE). The first such sensor, proposed by Silva *et al.*, utilizes a nanostructured-MIP. Pyrrole monomers, electropolymerized on a reduced graphene oxide SPE, enable high conductivity and increase the number of available biomimetic sites. This strategy allowed a limit of detection of 6 pg/mL [21]. The second sensor, developed more recently, uses multi-walled carbon nanotubes (MWCNTs) in addition to MIP in order to increase both the surface area of the electrode and its electrical conductivity. In that way, a limit of detection of 0.04 pg/mL was reached [36].

Although the application of MIPs for cardiac biomarkers detection is not yet widespread, the proofs-of-concept cited above are very encouraging for the use of MIPs as suitable alternatives to antibodies in POC devices for AMI diagnosis.

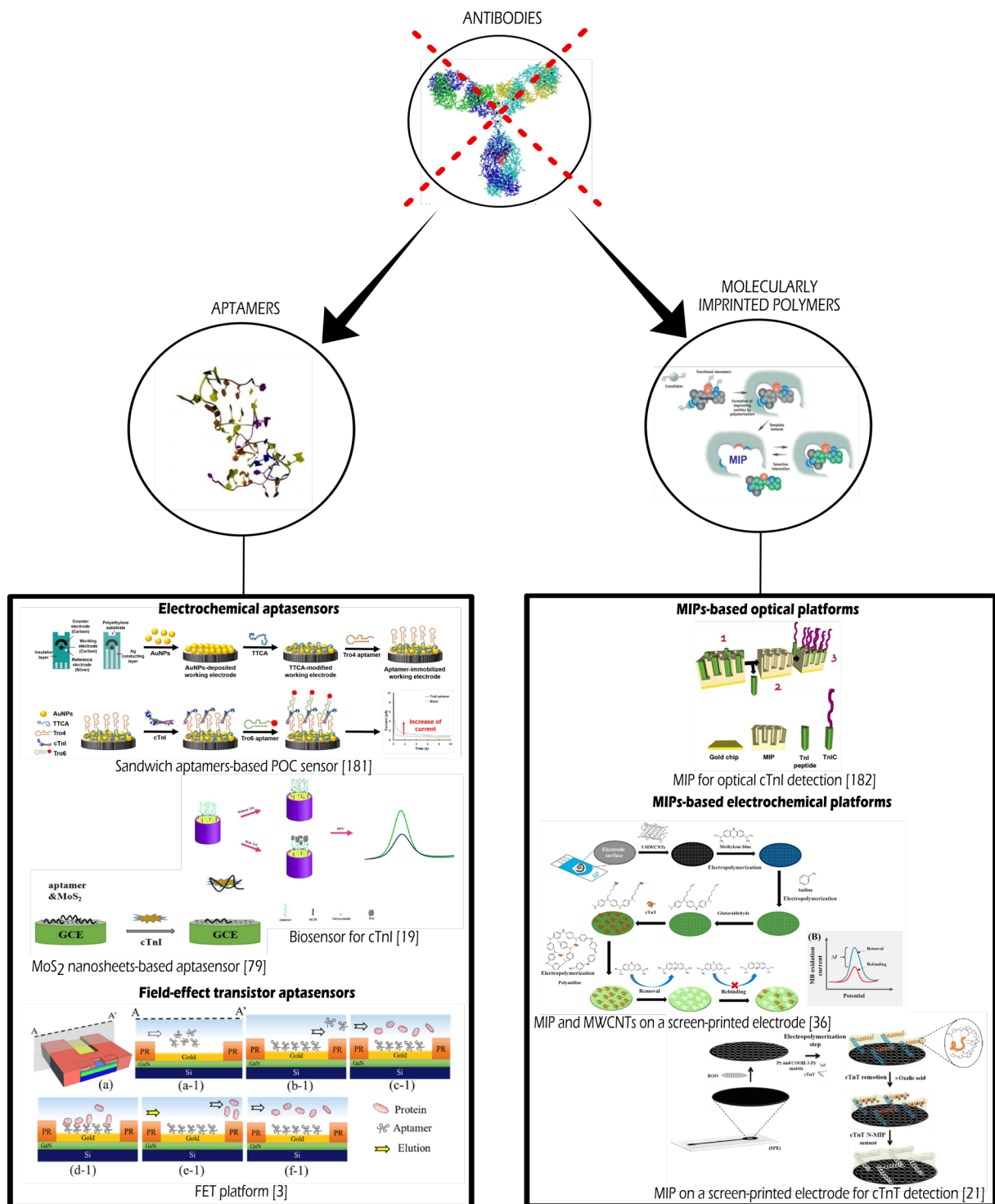


Figure 10: Platforms using two different alternatives to antibodies: aptamers and MIPs [69,70].

5. CONCLUSIONS AND PERSPECTIVES

As myocardial infarction is one of the major causes of death worldwide, public authorities encourage clinicians to improve and accelerate AMI diagnosis. This has motivated extensive research in recent years in both academia and industry, resulting in a range of innovations, successfully commercialized products, and avenues for further investigation. Thus, this review aims at describing the most promising advances of cardiac biomarker detection for AMI diagnosis.

Two biomarkers for AMI, namely cardiac troponin I and brain natriuretic peptides are the most widely used. Emerging biomarkers, such as miRNAs, are not yet exploited in commercial POC devices but present a huge potential. Shorter analysis time, better sensitivity, and the demand for portable POC devices are the requirements for a better diagnosis and an earlier triage of patients in the ED. It appears that several currently available commercial products are already capable to meet some of these criteria. Some limitations of these methods could be overcome using new advanced technologies. It is clear that they represent a promising potential innovation in clinical care. These methods are currently under development and still at an experimental stage. They seem to show that multiplexed analysis of several cardiac biomarkers and implementation of novel ones – such as miRNAs - are required to improve AMI diagnosis and patient care. In addition, it is important to note that alternatives to antibodies – such as aptamers or MIPs - are almost essential to improve the performances, reduce the cost, and allow the use of the current POC devices, especially in resource-limited settings.

Author Contributions:

Maud Savonnet: conceptualization, writing - original draft and editing, revision. Tristan Rolland: writing – original draft and revision. Myriam Cubizolles: conceptualization, funding acquisition, supervision, revision. Yoann Roupioz: revision. Arnaud Buhot: conceptualization, funding acquisition, supervision, revision.

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Conflicts of Interest:

The authors declare no conflict of interest.

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