An historical approach to the diagnostic biomarkers of acute coronary syndrome

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Abstract: Suspected acute myocardial infarction (AMI) is one of the leading causes of admission to the emergency departments in Western countries but also an increasing cause in many other nations. The diagnosis of AMI involves the evaluation of clinical signs and symptoms, electrocardiographic assessment, and measurement of cardiac circulating biomarkers. In the last sixty years, the use of laboratory markers has changed considerably. Early biomarkers assessment has entailed testing for total enzyme activity of aspartate aminotransferase (AST), lactate dehydrogenase (LDH) and creatine kinase (CK). Advances in electrophoresis allowed the identification of more cardio-specific isoenzymes of both CK and LDH, thus leading to the introduction of the CK-MB and LDH-1 activity assays. Soon thereafter, the development of immunoassays, as well as technical advances in automation, allowed the measurements of the CK-MB in mass rather than in activity and myoglobin. Currently, cardiac troponins have the highest sensitivity and specificity for myocardial necrosis and represent the biochemical gold standard for diagnosing AMI. This review provides a chronology of the major events which marked the evolution of cardiac biomarkers testing and the development of the relative assays from the first introduction of AST in the 1950s to the last high sensitivity troponin immunoassays in the 2010s.

Keywords: Acute coronary syndromes (ACS); acute myocardial infarction (AMI); cardiac biomarkers; cardiac troponin

Introduction

Despite the large efforts of the scientific community, cardiovascular disease (CVD) remains the main cause of death within industrialized nations as well as an increasing cause of death and morbidity in many developing countries (1,2). Between CVDs, the acute coronary syndrome (ACS) represents the most common cause of emergency hospital admission in Western countries (3,4), being associated with the highest mortality and morbidity (5).

Historically, ACS has included unstable angina (UA), non-ST-segment elevation myocardial infarction (NSTEMI), and ST-segment elevation myocardial infarction (STEMI) (5).

Since ACS requires immediate hospital admission and the prognosis is directly associated with timely initiation of revascularization, missed, misdiagnosis or late diagnosis may have unfavorable clinical implications. The triage and management of patients with chest pain should be based on clear and well-defined pathways. Moreover, early ACS diagnosis reduces complications and long-term risk of recurrence, finally decreasing the economic burden posed on the health care system as a whole (6,7).

Despite the great efforts, the biochemical diagnostic approach to ACS remains one of most difficult and controversial medical challenges (8). Ideally, a biochemical

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marker of myocardial ischemia should have a considerable concentration in the myocardium, absence from non-myocardial tissue and normal serum, rapid release into the blood at the time of ischemia, a relationship to the extent of injury and persistence in the blood for a sufficient length of time to provide a diagnostic window. In addition, the test should be rapid, easy to perform and relatively inexpensive (9). At present, cardiac troponins are the only accepted biomarkers for diagnosing myocardial injury and acute myocardial infarction (AMI) (10). The increasing focus on biochemical markers during the last five decades [from amino transferases and lactate dehydrogenase (LDH) to cardiac troponins] (11), has led to the identification of a near-perfect biochemical marker (12,13).

1954: aspartate aminotransferase (AST)

In 1954, serum glutamic oxaloacetic transaminase (SGOT), now called AST, has been identified as the very first biochemical marker for diagnosis of AMI (14,15). The first method was originally based on paper chromatography and was hence extremely time-consuming. In the same year, a medical student, Arthur Karmen, developed a more rapid and practical spectrophotometric method to measure enzyme activity (16).

Years later, Henry *et al*. (17) improved the technique originally introduced by Karmen. In the reaction, the oxaloacetate produced by the transaminase serves as substrate for malate dehydrogenase by which it is reduced to malate in the presence of dihydronicotinamide-adenine dinucleotide (NADH), which is simultaneously oxidized. The reaction was monitored by a spectrophotometer as decrease in light absorption at 340 nm. The AST method was then standardized and adapted for use on many automatic analyzers (18).

AST increases in blood 3–4 hours after AMI, reaches the maximum value in blood in 15–28 hours and returns to normal values within 5 days (19). However, despite the high sensitivity for AMI, AST is a non-specific biomarker of cardiac tissue, wherein its activity can also increase in several other conditions including hepatic congestion secondary to congestive heart failure, myocarditis, electrical cardioversion, pericarditis, tachyarrhythmias, pulmonary embolism, and shock (20).

AST exists in human tissues as two distinct isoenzymes, one located in the cytoplasm (c-AST), and the other in mitochondria (m-AST), which differ in amino acid composition and immunochemical and kinetic properties (21).

In particular, m-AST is infrequently enhanced after myocardial injury (22,23), increases later and apparently provides different biological information compared to c-AST (24). Rabkin *et al*. observed in their study performed on 15 AMI patients evaluated with invasive hemodynamic measurements, that m-AST correlated significantly with the hemodynamic assessment of left ventricular dysfunction of myocardial necrosis (23).

1955: lactate dehydrogenase (LDH)

Hill and Levi were the first to demonstrate the presence of LDH in human blood serum (25), and one year later Wróblewski and LaDue observed an increase in LDH activity in serum of patients with AMI (26,27). Ulmer *et al*. confirmed this observation in a study population of 22 AMI patients (28).

Since LDH is present in nearly all human tissues, LDH isoenzymes, either as a-hydroxybutyrate dehydrogenase (HBD) or lactate dehydrogenase isoenzyme 1 activities (LDH-1), were described as possible biomarkers of AMI (29,30) by providing more organ specificity than total LDH activity (19). Moreover, LDH-1 activity can be corrected for *in vivo* or *in vitro* hemolysis by measuring the ratio of LDH isoenzymes 1 and 2: the ratio is over 1.0 in AMI patients, whereas it remains below 1.0 in samples of patients with hemolysis (31).

LDH and its isoenzyme LDH-1 increase in blood 5–10 hours after AMI, reach the maximum value in blood in 60–144 hours and return to normal values in 12 days (19).

1960: creatine kinase (CK) total enzyme activity

The first spectrophotometric method for assessment of creatine phosphokinase was developed in 1955 by Oliver (32). Tanzer *et al*. then developed an enzymatic method for creatine and CK determination, characterized by greater specificity and sensitivity than the previous (33).

The assay for CK total enzyme activity was finally optimized by Rosalki in 1967 (34), by modifying the Kornberg ATP assay (35). Interestingly, Rosalki developed this method during a dinner and wrote it on the back of the menu card. This method required the addition of creatine phosphate, ADP, and a thiol, and the combination of all reagents in individual gelatin capsules. The modern fully automated clinical chemistry analyzers use now the same basic reagents, only slightly modified and optimized (36).

It was only in 1960 that the CK activity was shown to be a potential biomarker of cardiac muscle injury (37).

Since CK appears in blood 3–9 hours after an AMI, reaches the maximum value in blood in 10–20 hours and returns to normal values in approximately 72 hours (19), the sensitivity of this biomarker is very high when blood is collected early after the onset of disease. Sorensen reported a sensitivity of 98% in the AMI diagnosis when blood was collected within 72 hours after the onset of disease (38). Moreover, he also demonstrated that patients with high CK activity measurement in the third day had a worse prognosis.

Years later, it was shown that total CK activity may be related to the extent of myocardial infarction and prognosis (39,40). On the other hand, this biomarker is characterized by low specificity, since its activity increases considerably in liver, biliary tract, kidneys and skeletal muscles diseases.

1972: creatine kinase MB isoenzyme (CK-MB) activity

The enzyme CK is present in humans in three isoenzymes BB, MM and MB, the name of which originates from the various combination of the M (i.e., muscle) and brain (i.e., brain) isoforms. The CK-MB isoenzyme, which is normally undetectable or very low in the blood, increases in both heart and skeletal diseases by showing highest concentration in cardiac muscle (~22% of the total CK content of myocardium compared to ~1–3% in the skeletal muscle) (41). Several studies confirmed that CK-MB subforms provide a reliable and specific diagnosis with high accuracy in the first hours of onset of cardiac symptoms (42-44).

In 1972, Roe *et al*. developed a zone electrophoresis method for the identification and quantitation in serum or plasma of the CK-MB isoenzyme (45). Successively this biomarker was measured by anion-exchange column chromatography (46) and in 1976, Roberts *et al*. developed a radioimmunoassay (RIA) for CK isoenzymes (47).

The assays for measuring the enzymatic activity of CK-MB isoenzyme represented important advances, especially in terms of improved specificity (48).

In 1979, the World Health Organization (WHO) included in the criteria for AMI diagnosis the demonstration of typical rise or fall patterns of CK, CK-MB, LDH, or AST activities (49).

However, several preanalytical or analytical variables (i.e., prolonged storage or inadequate preservation, inhibitors or interference from other enzymes or drugs, pH and ionic concentrations used in the analyses and assay temperature) may influence the CK-MB activity (50-54). Moreover, the evidence that the activity of CK-MB can be considerably enhanced in many skeletal muscle disorders and that its concentration is characterized by a relatively slow release from the injured muscle cell, lead to way to additional research aimed to identified more reliable biomarkers.

1978: myoglobin

Myoglobin is a small (17.8 kDa) globular oxygen-carrying protein found in heart and striated skeletal muscle, with an almost identical amino acid sequence (55). It is a cytoplasmatic protein with a low molecular size and it is rapidly released after myocardial injury. It appears in blood 1–3 hours after AMI, reaches the maximum value in blood in 4–7 hours and returns to normal values after 1–1.5 days (19,56). However, because of rapid clearance from blood, myoglobin may "miss" late-presenting patients, and it is less cardiospecific than CK-MB (57).

Myoglobin concentration increases in skeletal muscular dystrophy, trauma, inflammation (myositis) or in presence of acute or chronic renal failure. Moreover, increased myoglobin levels can occur after muscle injections or strenuous exercise and in presence of various toxins and drugs (58).

The first method to detect myoglobin in serum was a RIA developed in 1978 (59,60). However this method was time-consuming and not useful for STAT analysis. Following the development of latex-enhanced immunoassays (61), myoglobin was introduced in the emergency department setting for identification of AMI (62). An automated non-isotopic immunoassay was also successively developed (63).

Despite myoglobin has been for long considered as the best marker for ruling out AMI in the emergency room from 3 to 6 hours after the onset of cardiac symptoms, the negative predictive value (NPV) reaches only 89%, at best (64).

On the other hand, since myoglobin is rapidly cleared from plasma after coronary reperfusion, it has been demonstrated that this biomarker may allow the earliest and best discrimination between reperfusion or no reperfusion in patients treated with intravenous thrombolytic therapy (65,66). Moreover, rapid kinetic of myoglobin is important for detecting re-infarction in patients with post-infarction angina when troponins are still elevated, or lese during revascularization procedures (67).

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1985: CK-MB mass

The introduction of immunologic determination of CK-MB mass (i.e., protein concentration) was an important innovation, which virtually replaced the traditional enzymatic assay. The first "mass" immunoassay for CK-MB was developed in the 1985 (68) and was found to be much more sensitive than the measurement of enzymatic activity. One year later, Vaidya *et al*. developed a monoclonal antibody named "Conan MB" (in honor of a movie featuring the story of a barbarian warrior) directed against the CK-MB (69). This antibody was successively paired with an antibody to the B subunit of CK-MB. This two-site mass immunoassay is that currently used by all automated immunoassay instrumentation.

CK-MB mass measurement has the advantage to be more stable than the enzyme activity after storage and appears to be more sensitive, by increasing in plasma and serum more rapidly than CK or CK-MB activity (70,71). However, it is not sufficiently rapid when compared to myoglobin in the early diagnosis of AMI, mostly in the first 6 hours after symptom onset (72). As for the enzymatic activity, the mass value of CK-MB also increases in many conditions other than AMI (73).

In 1986, serum CK-MB mass measurement/total CK activity ratio was proposed to identify false-positive elevations of CK-MB arising from skeletal muscle (74). A ratio of less than 3 is consistent with a skeletal muscle source, while ratios greater than 5 are suggestive for a cardiac source. Ratios between 3 and 5 represent a gray zone.

In 1990, rapid enzyme immunoassays for direct mass measurement of CK-MB mass as μg/L were developed (75,76).

In the same year Delanghe *et al*. suggested that these immunoassays were less vulnerable to analytical interference and that measurement of CK-MB mass concentration is better suited for infarct sizing than measurement of catalytic activity (77).

1963: the discovery of troponins

The identification, purification, and characterization of troponins should be almost entirely attributed to Professor Setsuro Ebashi, whose landmark contributions in the early 1960s established the molecular basis of the Ca^{2+}-regulation of muscle contraction. Its first contribution was the demonstration that calcium induced the contraction of actin and myosin filaments (78). Successively he showed

that the muscle relaxing component known at that time as the "Marsh factor" was actually made by vesicles (79), later named sarcoplasmic reticulum, which contained an enzyme that used ATP energy to remove calcium from the medium by transporting it to their lumen (80). In 1963, he also demonstrated the existence of a third factor (besides myosin and actin) which conferred calcium sensitivity to actomyosin (81). This factor, tentatively named "native tropomyosin" because of its similarity with tropomyosin, was later shown to be a complex of tropomyosin and a new complex of proteins named troponins (82). He proved that this complex is the Ca^{2+}-receptive site (83) and proposed the correct scheme for the molecular mechanism of regulation of contraction and relaxation (84). In the absence of Ca^{2+}, the contractile interaction between myosin and actin is suppressed by troponin-tropomyosin complex. On increasing Ca^{2+} concentrations, this suppression is removed by the binding of Ca^{2+} to the troponin complex which activates the contraction (85).

Shortly after the discovery of the troponin complex, Ohtsuky, a graduate student working in Ebashi laboratory, showed, by an electron microscopic study, that it is distributed along the thin filament at regular intervals of about 400 A (86), thus leading to the construction of a model of thin filament as an ordered assembly of troponin, tropomyosin and actin (85).

1971: troponin isoforms

In 1971, Greaser and Gergely demonstrated that the troponin complex actually consists of three components which were named TnC, TnI, and TnT on the light of their specific properties: Ca^{2+} binding capacity (TnC), inhibition of ATPase activity (TnI) and tropomyosin binding respectively (TnT) (87). The existence of the three troponin components and the above nomenclature was generally accepted in 1972 in occasion of the Cold Spring Harbor Symposium on muscle, a meeting which would become a hallmark in the history of muscle study. In the follow ten years, many researcher groups became interested in the study of troponins and the knowledge about these proteins increased rapidly. Once the amino acid sequences of troponin isoforms was finally determined (88,89), it became possible to search for the regions of functional significance (90). Such findings were then followed by a number of studies of fluorescence resonance energy transfer, nucleic magnetic resonance and X-ray diffraction which finally led to the

definition of the complete structure of troponin (91-93). In the meantime, gene expression studies showed that members of the TnC, TnI, and TnT gene families encode muscle-types specific isoforms differentially expressed in adult fast and slow skeletal muscles as well as in heart muscles. These include a fast skeletal and a slow skeletal-cardiac isoform of TnC (94-96), and a fast skeletal, a slow skeletal, and a cardiac isoform of both TnT and TnI (cTnT and cTnI). This exquisitely specific pattern of expression supported the use of cTnI and cTnT as biomarkers of cardiac injury.

Subsequent studies revealed that Mutations in the genes that encoding for two human cardiac Tn components, cTnI (TNNI3) and cTnT (TNNT2), are often responsible for cardiomyopathies (97-99).

1987: cTnI assays

In the 1980s, several research groups started to look at cardiac troponins as possibly specific cardiac biomarkers. Interest in TnI was prompted by the work of Cummins who developed the first RIA for the measurement of cTnI in serum in 1987 (100). This RIA methodology which was based on polyclonal rabbit antiserum, required two working days to be performed and had 10 ng/mL as the minimum detectable level. In his pioneer study Cummins showed that serum cTnI was elevated within 4 to 6 hours in patients with AMI, reached a mean peak level of 112 ng/mL (range, 20–550 ng/mL) at 18 hours, and remained above normal value for up to 8 days following myocardial injury. Three years later monoclonal antibodies directed against cTnI were described by two independent groups (101,102) one of which implemented an enzyme-linked immunoassay (ELISA) for quantification of serum cTnI. The assay developed by Bodor *et al*. had a detectable concentration of 1.9 µg/L and a working range of up to 100 µg/L. It required 3.5 hours to be performed (102). Such cTnI assay showed high specificity for cardiac injury even in the presence of acute muscle disease, chronic muscle disease, chronic renal failure, and after marathon running (103). During the following 20 years the cTnI immunoassay has been considerably optimized. Current generations of commercially available assays have an analytical sensitivity almost 100-fold higher (1 *vs*. 100 ng/L) than that of the experimental and commercial assays that were initially described. These assays were not fully standardized at this time and studies have documented substantial differences across methods (104,105). The main factors contributing

to the quantitative differences between the cTnI methods include the lack of commutable reference material and difference in the antibody immunoreactivity as well as in the antigen used as calibrators (106). The analytical characteristics of cTn assays currently on market have been recently described by Jarolim (107).

1989: cTnT assays

The first generation immunoassay has been developed by Katus and colleagues in 1989. It was based on an ELISA with two antibodies: the capture antibody conjugated to biotin (M7) and the detection antibody conjugated to horseradish peroxidase (lBIO) (108). This assay, automatized in 1992 by its incorporation onto the ES-analysers (Boehringer Mannheim TM) (109), had two problems. The first was due to the assay formulation which comprised a completely cardiac-specific capture antibody (with <0.5% cross-reaction to skeletal muscle) and a detection antibody that was only 78% cardiospecific. The 20% cross-reactivity of the second antibody resulted in falsely TnT levels in patients with massive skeletal muscle damage (rhabdomyolysis). Such problem was soon overcome in 1997 with the introduction of the so-called 'second generation' TnT antibodies (M11.7 as capture antibody and M7 as detection antibody), which completely abolished the non-specific binding to skeletal TnT (110). With this second generation assay, the normal range of cTnT was between 0 and 0.1 µg/L. The limit of detection (LoD) and linearity of this assay were <0.05 and 12 µg/L, respectively.

The second problem was related to the platform. Although the test on the ES-analyser had been fully automated, it was characterized by a turn-around time of over 90 minutes with assays run daily, which was hence inadequate to fulfil requirements for emergency testing. This problem was also overcome by the introduction of the Elecsys TM analyzers, on which the turn-around time of the cTnT test was comprised between 9 (Elecsys 1010) and 18 (Elecsys 2010) min. At variance with the methodology of the ES analyser the Elecsys analyzers is based on electrochemiluminescence immunoassay (ECLIA) technology and uses a ruthenium labelled component instead of the horseradish peroxidase on the detection antibody (111).

In 1999, the 'third generation' troponin T assay has been introduced. The difference between the second and the third generation is the use of human recombinant cTnT

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for calibration (third generation) instead of bovine cTnT (second generation), which considerably improved the assay linearity (112). The fourth-generation cTnT assay, introduced in 2007, used fragment antigen-binding (FAB) of two cTnT-specific mouse monoclonal antibodies in a sandwich format. The antibodies recognized two epitopes located in the central part of the cTnT molecule. The fourth-generation cTnT assay has a LoD of 10 ng/L and a 10% coefficient of variation (CV) at 30 ng/L (113).

The new high-sensitivity cTnT (hs-cTnT) assay is a modification of the fourth-generation assay, which was implemented in 2010 (114). In this fifth generation assay the biotinylated capture antibody was not changed, whereas the detection antibody was genetically re-engineered into a mouse-human chimeric detection antibody to reduce the susceptibility to interference by heterophilic antibodies. The analytical sensitivity was improved by increasing the sample volume from 15 to 50 μL, increasing the ruthenium concentration of the detection antibody, and lowering the background signal through buffer optimization. As a result, the analytic performance of the hs-cTnT assay had been significantly improved; the LoD was 5 ng/L, the 99th percentile cutoff point was 14 ng/L, and the CV was 10% at 13 ng/L.

Due to patent issues, cTnT assays are only available from one manufacturer (Roche Diagnostic). Therefore, in contrast to cTnI, standardization of the cTnT assay is not seen as a major problem. The only inconvenience is the current coexistence of the less sensitive fourth generation assay in the USA and the hs-cTnT assay in most other countries, since the hs-cTnT has not been licensed for use by the FDA so far.

2012: diagnostic value of cTn in AMI

According to the international consensus and task force definition of AMI established in 2012 (115), the diagnosis of AMI is based mainly on evidence of myocardial ischemia, along with an elevated value of cardiac biomarkers above the 99th percentile and demonstration of an increase or decrease over time.

The continuous improvement of the analytical sensitivity and assay precision at the low measuring range of cTn assays has ultimately led to the development of the so-called "hs" cTn assays which finally satisfy this criterion. In order to label a cTn assay as "hs", the IFCC task force suggested that cTn should be measurable in more than 50% of healthy subjects, and preferably in more than 95% (116).

The term ultra-sensitive is conventionally reserved to cTnI assay capable to quantify cTn at levels below the lowest concentrations seen in healthy subjects (117). The interest in this additional sensitivity goes beyond the management of patients with suspected MI and is limited to novel application of cTn assays such as measuring changes in cTn levels after exercise stress testing or after cardiotoxic chemotherapy (118). Nowadays, hs-cTn assays are considered the biomarkers of choice in the early diagnosis of AMI being able to detect cTn release at an earlier time point than the previous generations of cTn assays, especially in patients with a recent onset of chest pain (119-122). Most patients with an AMI, can be reliably identified within 3 h after admission, with nearly 100% sensitivity and 100% NPV using a hs-cTn assay, which indicates that observation time in the emergency department may be reduced for rule out of AMI (12). However, in patients with 3 h values unchanged, but in whom pre-test likelihood of AMI is high, additional subsequent sampling (e.g., at 2 or 3 h) may still be advisable.

As predictable, the improved sensitivity of the new generations immunoassays came along with a decreased specificity for AMI. Measurable troponin values can now be found in several non-ischemic cardiac conditions, including, among the other, atrial fibrillation, hypertension, renal and liver disorders, acute or chronic pulmonary disease (123) and even severe allergic reactions (124). Therefore, careful clinical assessment, serial testing and thoughtful differentiation are required to separate AMI from other acute and chronic disorders which can be associated with low-level and less harmful myocardial injury (125).

Conclusions

The relatively long history of AMI diagnostics has been marked by many milestones (*Table 1*). After more than 60 years of research we have now come to a point when hs-cTN immunoassays should be considered as "the best there is". But, with ongoing technological advances and increasing knowledge of the pathophysiology of myocardial ischemia, it seems premature to conclude that hs-cTn will also be "the best there will ever be". Many questions remain unanswered, mainly concerning the optima cut-offs and timing of serial sampling. Hopefully, further studies will help refine the clinical use of hs-cTn immunoassays in myocardial injury.

Table 1 Characteristics of AMI biomarkers

Biomarker	First assay development, year	Molecular weight, Da	Kinetics			Sensitivity for myocyte necrosis	Specificity for myocyte necrosis
			First detection, hours	Maximum value, hours	Return to normal values, days		
AST	1954	105,000	3–4	15–28	5	++	+
LDH	1955	140,000	5–10	60–144	12	++	+
CK total enzyme activity	1960	83,000	3–9	10–20	3	++	+
CK-MB activity	1972	83,000	3–8	10–20	3	++	++
Myoglobin	1978	17,800	1–3	4–7	1–1.5	+++	+
CK-MB mass	1985	83,000	3–12	12–18	2–3	+++	+++
cTnI	1987	23,900	3–7	10–20	10	++++	++++
cTnT	1989	37,000	3–8	15–120	14	++++	++++

AST, aspartate aminotransferase; LDH, lactate dehydrogenase; CK, creatine kinase; CK-MB, creatine kinase MB isoenzyme; cTnI, cardiac troponin I; cTnT, cardiac troponin T.

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