

# CHAPTER 1

## Introduction

## **History**

*Clinical aspects.* Coronary heart disease (CHD) has a high incidence in the western world and may lead to serious complications: it is one of the main causes of death. To the spectrum of these heart diseases belong the acute coronary syndromes (ACS) ranging from (un)stable angina pectoris (UAP) to acute myocardial infarction (AMI). CHD is associated with well known risk factors, such as cholesterol blood level, hypertension, family history of heart disease and smoking.

The acute coronary syndromes are characterised by a process starting with the formation of an atherosclerotic plaque in the vessel wall. The next step in this process is plaque rupture. This plaque rupture will promote platelet aggregation and, subsequently, the formation of an intra-coronary thrombus. This thrombus will reduce the blood flow through the vessel. Finally, the vessel may occlude. This occlusion induces a sequence of events: starting with deprivation of oxygen followed by tissue necrosis. Subsequently, the cell loses its content and ultimately, this will reach the circulation.

The complaints of chest pain localised left precordial and radiating to the arm and neck during exercise are typically for the patient suffering ACS. Usually, the pain disappears at rest or with nitroglycerin treatment. This patient will be classified as having (stable) angina pectoris (AP). The World Health Organisation (WHO) defined AP as transient episodes of chest pain precipitated by exercise resulting in an increased myocardial oxygen demand. However, if the chest pain continues at rest, it will be classified as UAP. UAP together with a total occlusion of the coronary artery will result in AMI. Although both AMI and UAP have ischemia as provocative event, it is essential to establish the right diagnosis, because both diseases have to be treated different. Therefore, a demand has been grown for tools to make the right diagnosis. To these tools belong clinical investigations such as ECG-monitoring, scintigraphy and echocardiography, as well as biochemical blood testing. Besides ECG changes and the typical symptoms at presentation, elevations of biochemical markers are the third criterium to diagnose AMI according the WHO criteria (1). In the 1980's it became clear, that thrombolytic therapy improved the prognosis of the patient after an AMI. This prognosis was especially enhanced, if this therapy was started within six hours after the onset of symptoms, because the sooner the patient is treated with thrombolytics the less tissue will necrose ("time is tissue"). Therefore, it is very important to diagnose AMI as early as possible. Besides ischemia (hypoxia), other phenomena such as surgery, invasive treatments and contusion may cause myocardial damage and tissue necrosis. Subsequently, this tissue necrosis will result in leakage of the cell-content into the blood circulation and elevation of marker concentration. Furthermore, it was shown, that blood parameters might also be an important tool for risk stratification. Since these findings were reported, many studies were performed to look for an ideal cardiac marker and research in the field of the cardiac markers became of great importance.

*Biochemical aspects.* For several decades, elevation of biochemical markers in blood are used for the detection of myocardial cell necrosis. The use of aspartate aminotransferase (ASAT, EC 2.6.1.1) as a marker for the detection of acute myocardial infarction (AMI) was described by La Due (2) in 1954. Soon thereafter, the assessments of Creatine Kinase (3) (CK, EC 2.7.3.2) and Lactate Dehydrogenase (4) (LD, EC 1.1.1.27) were reported. Later, LD isoenzymes (either HBD or LD-1) were introduced as well as the measurement of the CKMB isoenzyme activity (5). An other conventional, non-enzymatic, marker is myoglobin. As enzyme activity measurements are easier to perform, they are more frequently used than myoglobin measurement. Although the CKMB-isoenzyme is not heart-specific, it has become the 'gold' (biochemical) standard for the

detection of myocardial tissue damage.

Since the mid 1980's, several other biochemical markers for the detection of myocardial tissue damage have been introduced. To this category belong myosin heavy and light chains, heart fatty acid binding protein, carbonic anhydrase III, glycogen phosphorylase BB, cardiac troponin I and cardiac troponin T. Most of these markers belong to skeletal as well as to myocardial tissue, but they have different concentrations in skeletal and myocardial tissues. Consequently, the ratio between the different markers has also been suggested to be of use for the detection of myocardial cell necrosis and for the discrimination between skeletal and myocardial tissue damage.

In the following section requirements for the ideal cardiac marker will be considered. Thereafter, clinical and analytical characteristics of some conventional markers will be reviewed as well as clinical, molecular, and analytical characteristics of the cardiac troponins. The other markers will not be considered. This is because of their limited applicability as a consequence of the non-heart specificity and because of the low commercial availability.

### **Requirements for the ideal cardiac marker**

Several requirements have been reported (6) which a marker should fulfill to be ideal: (a) a high concentration in the myocardium, (b) not present in other tissues, (c) rapid and complete release after myocardial injury, (d) homogeneous distribution within the myocardium, (e) release in direct proportion to the extent of the myocardial injury, (f) persistence in the plasma for several hours to provide a convenient diagnostic time window but not so long that recurrent injury might not be identified, and (g) suitable to analyse as stat parameter. These fulfilments result in several characteristics of these markers (6):

- *the size*; in general, the smaller the marker, the more rapidly it reaches the circulation (7).
- *the cellular localisation*; cytosolic proteins are released more rapidly than structural proteins after damage to sarcolemma. This results in earlier elevations of plasma levels of these cytosolic proteins (8).
- *the solubility*; macromolecules of low solubility move slowly out of the myocardium.
- *the release ratio*; Some macromolecules may undergo local degradation after release (9). Thus, the amount of marker depleted from the heart may be substantially greater than the amount that might be measured in plasma.
- *the clearance*; Smaller markers generally are cleared more rapidly than those of greater size (7,10).
- *the specificity for myocardium*; most macromolecules in the heart are also abundant in skeletal muscle or other organs and tissues.
- *the specificity for irreversible injury*; specificity for irreversible injury has been difficult to define for several reasons. The first is because of limitations in the detectability of marker proteins in plasma. This is related to the plasma levels normally present and the sensitivity of the assay. The second is due to limitations in the morphological detection of myocardial necrosis.
- *the detectability*; this requires accurate and easy-to-use assays with high sensitivity and low variability. The assumption that detection of markers by different assays is equally precise or that the results of activity and mass assays are equivalent is not necessarily valid (11).

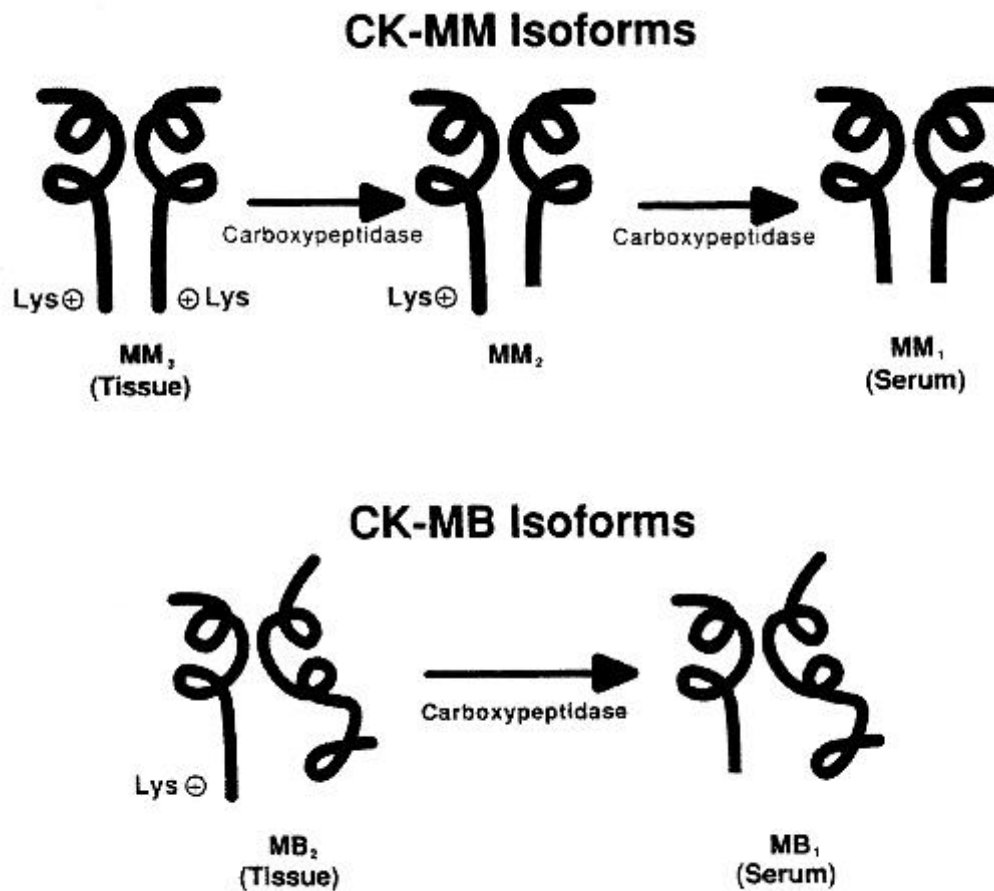
The search for the 'ideal marker' resulted besides the conventional parameters ASAT, LD, CK-total, and CKMB-activity, and more recently, in other markers such as myoglobin, CKMB-mass,

CK-isoforms, myosin heavy/light chains, heart fatty acid binding protein (hFABP), glycogen phosphorylase BB, carbonic anhydrase, enolase, and the troponins. From these markers the conventional CK (and CK related parameters), LD, and myoglobin and the new cardiac troponins will be considered.

### **Creatine Kinase**

Creatine Kinase (CK) is an enzyme located in the cytosol and mitochondria of the cell. It is involved in cellular energy metabolism by catalysing the reversible transfer of a high energy phosphate group from adenosine triphosphate (ATP) to creatine, resulting in ADP and creatine phosphate. CK consists of 2 subunits M and B, each with a molecular weight of 41 kDa. So, three compositions of CK might occur: the iso-enzymes CKMM, CKMB and CKBB. Also a unique dimeric mitochondrial form exists. CKMM is most abundant in skeletal muscle (97-99%) and myocardial muscle (about 80%). CKMB is part of myocardial muscle (nearly 20%) and of skeletal muscle (1-3%). CKBB is most abundant in brain and intestines.

After necrosis of the cell, CK enters the circulation and is cleared by the lymphatic system (9). In the circulation the enzyme carboxypeptidase cleaves the carboxyterminal lysine from the M- and B-subunits (see figure 1). This phenomenon was firstly described by Wevers et al. in 1977 (12). As lysine is positively charged, high voltage electrophoresis on agar gel can discriminate between M-subunits with or without lysine. The CKMM-isoform with lysine at both subunits is called the tissue form (MM3), the isoform with lysine at one subunit the intermediate form (MM2) and the subform with lack of lysine at both subunits is called the plasma form (MM1). Isoforms are also detectable for the CKMB-isoenzyme. The isoform with lysine at the end of the M-subunit is called the tissue form (MB2). The one without lysine at the end of the M-subunit is called the plasma form (MB1). From experiments with



*Figure 1.* CKMM3 (tissue form) is transformed into CKMM2 (intermediate form) and into CKMM1 (serum form) by cleaving the carboxyterminal lysine from both M subunits. CKMB2 (tissue form) is transformed into CKMB1 (serum form) by cleaving the carboxyterminal lysine from the M subunit. The intermediate CKMB isoform (lysine present on the M-subunit, but absent from the B-subunit) is not recognisable in serum.

column chromatography and monoclonal antibodies directed against the M carboxyterminal lysine, it has become clear, that at least three CKMB isoforms circulate in vivo: first, the already mentioned tissue form, second, a form with a lysine on the M subunit but not on the B subunit, and third, a form with lysines absent from both carboxyterminal subunits (13,14). So, the cleavage of lysine from the B monomer seems to be favoured. Comigration during electrophoresis of the tissue isoform and the isoform with a lysine removed from the B monomer appears to account for the presence of only two species (13).

### **Creatine Kinase measurements**

*CK-total.* The approved recommendation on IFCC method concerning CK-total measurement was published in 1991 (15). Before 1991 different methodologies were accessible. Mostly, they were related to particular countries.

*CKMB-activity.* For CKMB activity measurements several methodologies have been described. Classical manual procedures used to separate and quantitate CKMB include ion-exchange chromatography and electrophoresis (16). These methods are usually very labour intensive and therefore are not available on a 24 hour basis. Moreover, carryover of CKMM into the CKMB fraction is a common problem with 'column' methods (17). This problem has been overcome by the immune-inhibition assay. This assay uses a monoclonal antibody against the CK-M subunit. After the inhibition of this subunit, the rest-activity of the CK-B subunit is measured. This is representative for the CKMB activity, if there is no CKBB in circulation. Thus, the presence of CKBB in circulation as a consequence of brain damage, pregnancy, carcinoma of the prostate, lung or gastrointestinal tract, tuberculosis or uterine abnormalities will result in falsely elevated CK-MB results. Up to 3.2% of hospitalised patients have detectable circulating levels of CKBB (18). Macro-CK, including immunoglobulin bound CK or aggregates of mitochondrial CK, has been another problem with analysis of CKMB-activity using immune-inhibition. As CK-M antibodies are interfered by macro-CK, falsely elevated CKMB levels may be measured in the presence of macro-CK. Assays based on monoclonal antibodies that measure CKMB-mass are rapid, highly sensitive and specific. Moreover, these assays are not interfered by the CKBB-isoenzyme and by macro-CK.

*CKMB-mass.* The CKMB-immuno assay has been introduced in 1985. This CKMB immunoassay overcomes the drawback of the CKMB-activity measurement, which includes co-measurement of CKBB, IgA- or IgG-bounded CK and mitochondrial CK. In contrast to the CKMB-activity measurement, which measures CK-activities (reported in U/L), and the CK-electrophoresis (reported in fractions or U/L), the CKMB immunoassay measures CKMB-mass and is reported in  $\mu\text{g/L}$ . The CKMB-mass assay has a better analytical specificity and sensitivity (less than 1  $\mu\text{g/L}$ ) than the CKMB-activity measurement (19). Furthermore, the CKMB-mass assay has been suited for stat analysis, because the assay procedure has been highly automated. However, the newly introduced immuno assay has been characterised for a long time by a lack of standardisation. Thus, different results were reported when using of reagents from various manufacturers. To overcome this standardisation problem, the AACC CKMB-mass assay standardisation Subcommittee was installed in 1992. This committee had to develop a reference material derived from human CKMB, so that the different results produced by the use of reagents from various manufacturers could be reduced. Ultimately, it took more than 10 years after the introduction of the CKMB-mass assay before CKMB-mass standardisation was reached (20).

*CK-isoforms.* Several methodologies have been described to determine the CK-isoforms: - electrophoresis using several kinds of gel (21) (cellulose acetate (22), agarose (23,24) polyacrylamide (25)), - isoelectric focusing (agarose (26-29)), - chromatofocusing (30-32), - immunoblot (33), - anion HPLC (34), - immunoassay (monoclonal anti-CKMM3 (35), monoclonal anti-CKMM1 (36), monoclonal anti-CKMB1 (37)). Major disadvantages of these methodologies have been the lengthy assay time, the lack of sensitivity in the very low CK range, and the inability to incorporate the technique easily into a clinical laboratory to be used on a 24-hours-a-day, 7-days-a-week-basis (38). The lack of a simple, rapid, quantitative methodology is a drawback for the use of CKMM and CKMB isoforms in clinical practice. However, the recently introduced CK-isoforms analysis by automated electrophoresis seems promising and it may have the potential for a

24-hours-a-day performance (39).

### **Lactate Dehydrogenase**

The enzyme Lactate Dehydrogenase (LD) catalyses the reversible transfer of 2 electrons and one hydrogen ion from lactate to NAD resulting in pyruvate and NADH. LD is a tetramer (MW 135.000 D) composed of M- (muscle; MW 34.000 D) and of H- (heart; MW 34.000 D) subunits. The two subunits are encoded by different genes. Because of the tetramer structure, LD consists of 5 isoenzymes LD-1 to 5. LD-1 contains four H subunits and is the predominant form in heart, but also occurs in erythrocytes, brain, pancreas, kidney and stomach. This heart isoenzyme is relatively heat-stable and it is active against the substrate hydroxybutyrate. After AMI, serum LD activities start to rise 12 to 18 hours after the onset of symptoms, peak at 48 to 72 hours, and return to normal by 6 to 10 days (40). LD-1 also increases within 10 to 12 hours, and returns to normal in approximately 10 days after AMI. Increased LD1 and the so-called flipped pattern in which the LD1/LD2 ratio is  $\leq 1.0$  have sensitivities and specificities of about 75 to 90% in patients suspected of AMI (41). The optimum interval for analysis of LD1 levels or the LD1/LD2 ratio is the 24- to 48-hour period after onset of chest pain. As the enzyme hydroxybutyrate dehydrogenase (HBD) acts as LD-1 activity, HBD is used in many laboratories as estimation of infarct size (42). LD-2 is composed of three H subunits and one M subunit, LD-3 of two H subunits and two M subunits, LD-4 of one H subunit and three M subunits and LD-5 of four M subunits. All LD isoenzymes are abundant in many tissues, whereas LD-5 is the predominant isoenzyme in skeletal muscle and in liver. LD is cleared by the reticuloendothelial system (43).

LD activity measurements may be interfered by several causes. Haemolysis and thrombolysis (both in vivo and in vitro) will increase LD activities, because erythrocytes as well as thrombocytes have been an abundant source of LD-1 and LD-2. In patients with chronic muscle disease or recurrent skeletal muscle damage, LD-1 and LD-2 are reexpressed in skeletal muscle (44). LD activities may also be elevated in case of germ cell tumors, and diseases of the pancreas, stomach and kidney (45). LD macroenzymes (complexes of LD with immunoglobulins) may also cause elevations of total LD activities (46).

### **Lactate Dehydrogenase measurements**

Total LD activity is measured enzymatically. The IFCC recommended methodology was reported in 1994 (47). As M and H subunits are composed of different amino acids, LD isoenzymes are usually quantified after separation by electrophoresis (48). The isoenzymes (especially those rich in M subunits) are inactivated when chilled or frozen but are stable at room temperature for several days (49).

Immunoassays (50-53) and chemical assays (54,55) for the direct measurement of LD1 in serum have been developed, providing the following advantages over the LD1/LD2 ratio: more precise, simple to perform, minimal labor, and improved diagnostic marker for myocardial infarction.

The immunochemical assay for LD1 (Isomune LD<sup>TM</sup>) uses a goat anti-LD-M subunit antibody complexed with a donkey anti-goat antibody, selectively precipitating all LD isoenzymes containing the LD-M subunit (i.e. LD2-5) (50). The remaining LD isoenzyme activity, LD1, is then measured. Clinical studies comparing the accuracy of the Isomune-LD<sup>TM</sup> to electrophoresis have

demonstrated that LD1 offers increased sensitivity and diagnostic efficiency over electrophoretic methods, with similar specificities.

Two chemical inhibition assays for LD1 activity have also been reported. One is an automated assay for LD1 from Boehringer Mannheim™ and is based on the selective chemical inhibition of non-LD1 isoenzymes by guanidine thiocyanate (54). The other one is an assay manufactured by Abbott Laboratories™, and is based on measurement of LD1 activity after treatment of serum with sodium perchlorate in order to inhibit on a chemical-selective way all LD isoenzymes containing M subunits (55). Both chemical inhibition methods are comparable to the immunochemical assay in both analytic and clinical sensitivity and specificity.

## **Myoglobin**

Myoglobin is a cytoplasmic heme-protein with a molecular weight of 17.800 D. Although its function is not fully established, it is very likely that myoglobin plays a role in the oxygen diffusion in striated muscle fibres. Furthermore, it serves as source for oxygen storage within the muscle fibre. Myoglobin is not found in smooth muscle. The biological half-life time is approximately 10 minutes and it is cleared by the kidneys.

As early as in 1956 it was demonstrated that myoglobin was released after an acute myocardial infarction. But for methodological reasons (enzyme activity measurements were easier to perform), the cardiac enzymes CK and LD were more frequently used to confirm the diagnosis AMI. With the introduction of automated myoglobin analyses, it became possible to use myoglobin as an early marker for the detection of an AMI in daily practice. As the molecular weight of myoglobin is lower than that of the cardiac enzymes CK and LD, it is earlier elevated in blood after AMI than the aforementioned cardiac enzymes. However, myoglobin is not heart-specific: the concentration in skeletal muscle is comparable to that in myocardium. So, patients might be misclassified as experiencing an AMI, if elevated myoglobin concentrations in circulation are the result of only skeletal muscle damage or as a consequence of renal failure.

## **Myoglobin measurements**

Several methodologies have been described for the measurement of myoglobin. These methodologies include radioimmunoassay (RIA) (56,57), latex-agglutination (58-60), two site immunoassay (61,62), turbidimetric (60) and nephelometric (58) assays. The first quantitative measurement was reported in 1975 (56): a RIA-method using polyclonal antibodies. The analytical sensitivity was 0.5 µg/l and the intra- and interrun imprecision were 5% and 10%, respectively. Because of the long turnaround time of the assay, the RIA-methodology is not suited for the stat mode. Moreover, the use of radioactivity and the lack of automation are also disadvantages of this method.

With the introduction of antimyoglobin antibodies immobilised on latex particles, myoglobin could be measured semi-quantitatively. Subsequently, the immobilised latex particles were used in turbidimetric assays. Also nephelometric assays were introduced. Both methodologies concern quantitative assays with turnaround times of less than 20 minutes and are suited for stat analysis.

More recently, methods have been introduced using monoclonal antibodies (Mab's) in two-site immunoassays. These assays can be performed on serum as well as on plasma samples. In these



assays, two Mab's, which recognise different epitopes on the myoglobin molecule, are used. One Mab is immobilised on a surface, while the other is conjugated to a detectable label. The test specimen is incubated with the Mab's and the amount of bounded conjugate is measured. The manufacturers of the different immunoassays report that the intra- and inter-assay imprecision are between 3% and 5%.

## **The troponins**

### General description

Myofibrillar proteins are the building blocks of the contractile apparatus. These proteins comprise a thick filament, containing only myosin, and a thin filament containing actin, tropomyosin and troponin. Smooth muscle does not contain troponin. Consequently, the troponin complex is located only in the thin filament of the myocyte. It controls muscle contraction by regulating the calcium-dependent interaction between myosin and actin. It is a complex of 3 polypeptide chains. These isoforms are designated troponin C, troponin I and troponin T. The composition of the thin filament is shown in fig 2. The three troponin isoforms consist of subunits with different structures and functions:

- troponin C (TnC, molecular weight 18,000 D) is a  $\text{Ca}^{2+}$ -binding protein and contains four metal-binding sites. Two sites, located in the C-terminal domain, bind both  $\text{Ca}^{2+}$

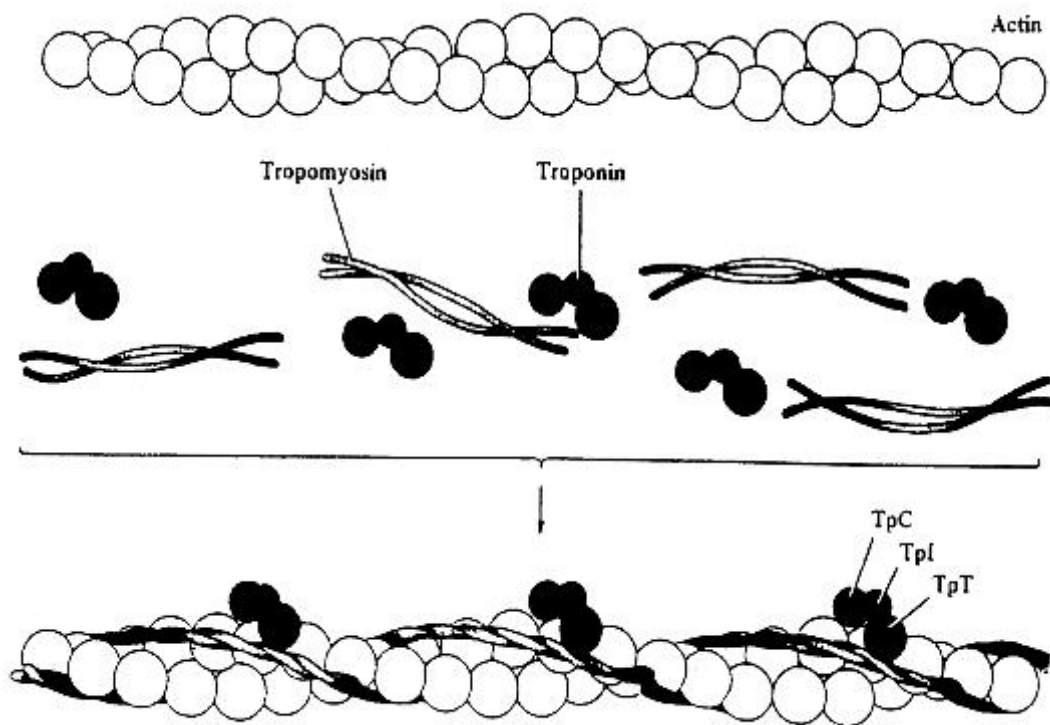


Figure 2. The composition of the thin filament of striated muscle. At each seventh actin position a troponin C-I-T complex is bound to tropomyosin. Abbreviations: TpC: troponin C; TpI: troponin I, and TpT: troponin T.

( $K_{\text{ass}}$  approximately  $10^7$  L/mol) and  $\text{Mg}^{2+}$  ( $K_{\text{ass}}$  approximately  $10^3$  L/mol, whereas two other sites located in the N-terminal domain specifically bind  $\text{Ca}^{2+}$  with a  $K_{\text{ass}}$  of about  $10^5$  L/mol (63-65).

- troponin I (TnI, molecular weight of 26,500 D) is a protein which inhibits actomyosin ATPase activity. This inhibition is reversed by the addition of  $\text{Ca}^{2+}$ -saturated TnC (63,64). TnI and TnC interact tightly with each other and the strength of their interaction depends on the saturation of  $\text{Ca}^{2+}$ -binding sites of TnC (63,64,66). In the presence of  $\text{Ca}^{2+}$ , the  $K_{\text{ass}}$  value for the TnI-TnC complex is approximately  $10^8$ - $10^9$  L/mol (66). Multiple sites of TnI-TnC interaction have been localised (67), and it is believed that in the presence of  $\text{Ca}^{2+}$ , TnI wraps around the central helix of TnC, forming contacts with both N- and C-terminal globular domains containing  $\text{Ca}^{2+}$ -binding sites (68).

- troponin T (TnT, molecular weight 39,000 D) binds the troponin C-I complex to the tropomyosin molecules. So, TnT provides proper fixation of TnC and TnI on the actin-tropomyosin filament (63,64). Although TnT interacts with both TnI and TnC, this type of interaction is not as tight as in the TnI-TnC complex (66). The interaction of the TnI-TnT binary complex with a  $K_{\text{ass}}$  of  $8.0 \times 10^6$  L/mol (69,70) is weaker than that of the TnI-TnC complex.

### Physiology of troponin

In resting striated muscle, the actin-myosin interaction is inhibited by troponin I-tropomyosin. Following excitation, intracellular stored calcium is released into the myofilament region, where it

binds to troponin C. This leads to a change in conformation of the whole troponin complex, allowing the troponin I to interact with troponin C and causing displacement of the tropomyosin strand. Inhibition of the actin-myosin interaction is eliminated and actin interacts with myosin, pulling the thin filament inwards and sliding it over the thick filament. The actin-myosin interaction is triggered by the consumption of ATP. As long as calcium and ATP are present, the actin-myosin interaction occurs repeatedly, resulting in active muscle contraction. When free calcium is no longer present to bind the troponin C molecule, conformational changes occur on troponin C. As a result, troponin I binds to actin, inhibits the ATPase activity of actin-myosin and causes muscle relaxation.

After tissue damage and cell necrosis several forms of troponin enter the circulation. The concentration of these isoforms is increased in blood for many days after AMI, because release from the structural elements requires degradation of the myofibril itself. Because of the very low to undetectable cardiac troponin I as well as troponin T values in the serum of healthy volunteers, cardiac troponin measurements permit the use of lower discrimination values compared to CK-MB. The forms of troponin released after cell necrosis include the complexes of troponin I, T, and C and the free subunits. In addition, cardiac TnI is released as both oxidised and reduced forms. The oxidation is the result of intramolecular disulfide formation of two cysteines (71). Human cardiac TnT contains no cysteine group; therefore, it is not capable of forming a disulfide bond.

### **Molecular aspects of troponin**

The different functions of the troponin subunits T, C and I are associated with distinct amino acid sequences encoded by separate genes (72). Troponin I exists in slow and fast twitch skeletal muscle, and cardiac muscle specific isoforms (73). In this respect TnI is similar to TnT but it is distinct from TnC. This troponin isoform has slow- and fast-twitch skeletal muscle specific isoforms but no cardiac specific isoform. The amino acid sequence of the two skeletal troponin I isoforms and the cardiac isoform has been solved. These three isoforms exhibit approximately 40% dissimilarity (74); the N-terminal of human cTnI has 31 additional amino acid residues that are not present on the skeletal isoforms. This results in the unique cardiac specificity of the cTnI molecule. The amino acid sequence of human cTnI has been determined after isolating cDNA of the cTnI (75). The gene of cTnI has been assigned to chromosome 19q13.3 (76), whereas the gene encoding human slow skeletal TnI isoform is carried on chromosome 1q12 (77).

Cardiac troponin I is not expressed during early fetal development in humans. Though the predominant fetal cardiac troponin I isoform is the slow skeletal TnI and complete transition to cardiac TnI takes place in humans only after birth (78), sufficient data exist to presume that cardiac TnI remains the only troponin expressed in the myocardium even during chronic disease processes (78,79).

Troponin T exists also in different forms depending on muscle types. Separate genes encode troponin T from cardiac muscle, and slow- and fast-twitch fibres. The gene for the slow skeletal muscle troponin T is thought to reside on chromosome 19 (80,81), whereas the gene encoding the fast skeletal TnT isoform is carried on chromosome 11p15.5 (82). A single gene encoding the human cardiac isoform of TnT is carried on chromosome 1q32 (83-85). Besides the major isotypes, there are several isoforms of troponin T within each striated muscle group in adult humans. Each of these is generated by alternative RNA splicing of a primary transcript from the same gene (85-87).

## **Troponin measurements**

Troponin I and troponin T are structural proteins that have no enzymatic activity and are measured by immunoassays. As already mentioned troponin I and troponin T from myocardial and from skeletal muscle have differences in their amino acid sequences. So, it has become possible to measure cardiac specific troponin I and troponin T immunologically. Human slow- and fast-twitch skeletal muscle troponin T has a rather high homology with cardiac troponin T. As a consequence, monoclonal antibodies must be carefully screened in order to obtain specificity toward only the cardiac isotype. Studies have shown that amino acid residues 98-258, the central part of the troponin T molecule, are highly antigenic as they contain high hydrophilicity and flexibility. Cross-reactivity by skeletal muscle troponin T can be minimised by using antibodies directed against amino acid residues 180-258.

In contrast to troponin I and troponin T, troponin C is identical in myocardial and skeletal muscle. For this reason, there is no (clinical) need for a commercial troponin C assay.

Of crucial relevance for assay development and calibration is: in what form are the troponins released into the bloodstream? For instance, cardiac TnI is highly susceptible to proteolysis, resulting in several questions and considerations: (1) what do differently configured immunoassays measure in serum (the whole molecule or various products of proteolytic degradation?); (2) what is the half-life of cardiac TnI in the bloodstream?; (3) cTnI has been shown to be phosphorylated by a cAMP-dependent protein kinase (88) and by  $\text{Ca}^{2+}$ -phospholipid-dependent protein kinase (protein kinase C) (89,90) at different sites. The phosphorylation of Ser-23 and Ser-24 changes the conformation of the TnI molecule and affects interaction of TnI with certain monoclonal antibodies (mAbs) (91,92), but it is unknown in what form cTnI (phospho or dephospho) is released into the bloodstream; (4) human cTnI contains two Cys residues (Cys-80 and Cys-97) (75), and oxidation of SH-groups of TnI affects its interaction with troponin components (93) and may also interfere with its binding to mAbs; (5) whether cTnI is released into the circulation in a free form (as an isolated protein) or as a complex with other troponin components and whether the mAbs are directed against the free or the complexed form determines the concentrations measured with commercial assays. This means that the sensitivity and the cutoff values for cardiac TnI will depend on the nature of the mAb used for detection of cardiac TnI. This should be realised, when comparing results of different commercial cardiac TnI assays. Without consensus about cardiac TnI standardisation, values from one assay to another can differ by a factor of 10 or more (94). First, these discrepancies are due to differences in the reference materials used in the assay calibration and second to the use of different antibodies. So, standardisation of the cardiac TnI assay is very important for comparison of the different assays. By using gel filtration chromatography, Wu et al. (95) examined the troponin T and I forms released after myocardial injury. They also characterised the immunoreactive response of different commercial cardiac TnI assays to purified troponin forms. They considered the following isoforms: -oxidised free cardiac TnI, -reduced free cardiac TnI, -ternary cardiac TnT-I-C complex, -binary cardiac TnI-C complex (oxidised cardiac TnI), -binary cardiac TnI-C complex (reduced cardiac TnI), -free cardiac TnT, -binary cardiac TnT-C complex, -binary cardiac TnT-I complex (oxidised cardiac TnI) and -binary cardiac TnT-I complex (reduced cardiac TnI). From this study they conclude that all cardiac troponin assays are well suited for the detection of myocardial injury, irrespective to the absolute values obtained.

## History of troponin measurements

The first radioimmunoassay (RIA) for the measurement of cTnI in serum used polyclonal rabbit antiserum and was described by Cummins et al (96). This RIA-methodology required 2 working days to perform and had 10 µg/l cTnI concentration as the minimum detectable level. In sera from patients with proven myocardial infarction, they showed that serum cTnI concentrations could be elevated above the normal range between 4 and 6 hours after the time of infarction. The cTnI concentrations remained elevated for up to 8 days after myocardial infarction (96). Since then, monoclonal antibodies directed against cTnI have been described by several investigators (97,98). With careful pairing of cardiac-specific monoclonal antibodies an enzymelinked immunoassay (ELISA) has been developed for quantisation of cTnI in human serum (97). This first monoclonal antibody based assay required 3.5 hours to perform. The minimum detectable concentration was 1.9 µg/l. The assay had a coefficient of variation of 10 to 20%. The working range of the assay was up to 100 µg/l.

Nowadays, various troponin I assays are commercially available: Opus Plus (Dade Behring<sup>TM</sup>), Access (Beckman<sup>TM</sup>), Stratus II, Stratus CS, Dimension RxL (Dade Behring<sup>TM</sup>), AxSYM (Abbott<sup>TM</sup>), Immuno I (Bayer<sup>TM</sup>), Immulite (DPC<sup>TM</sup>), ACS:180 (Bayer<sup>TM</sup>), Triage Cardiac System (Biosite Diagnostics<sup>TM</sup>), Vitros ECi (Ortho Clinical Diagnostics<sup>TM</sup>) and Alpha Dx (First Medical<sup>TM</sup>). They differ in results by the already mentioned lack of standardisation of both the calibrators and of the antibodies. For instance, the responses to the troponin IC-complex (the most predominant form of troponin I in serum after AMI) in relation to the free troponin I vary from a ratio of 6.41 (Stratus II), via 3.19 (Opus Plus), 1.40 (AxSYM), 1.23 (Access), to 1.07 (ACS:180) (99).

Several generations of enzyme immunoassays for the measurement of troponin T (100,101) have been developed by Katus and associates. The first generated commercial automated assay was incorporated onto the ES-analysers from Boehringer Mannheim<sup>TM</sup>. This test uses streptavidin-coated tubes and consists of several reaction steps. First, a biotinylated anti-troponin T (the capture antibody) bind to streptavidin and, subsequently, capture troponin T from the serum sample. After completion of this procedure, a second anti-troponin T antibody (the signal antibody) conjugated to horseradish peroxidase is added to label the captured troponin T. After a wash step, the substrate 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonate) is added and the absorbance is measured at 422 nm. Skeletal muscle troponin T was shown to have <0.5% and 20% cross-reactivity, respectively, for the capture and labeled antibodies used in this cardiac troponin T assay (101). The 20% cross-reactivity of the capture antibody results in falsely elevated troponin T levels in patients with massive skeletal muscle damage (rhabdomyolysis). This problem has been overcome with the introduction of the so-called 'second generation' troponin T antibodies (102). Especially, the capture antibody used in the second generation assay was modified and this resulted in fully heart specificity. Examination of healthy individuals showed that the normal range of cardiac troponin T is between 0 and 0.1 µg/l. The limit of detection and linearity of this assay are <0.05 and 12 µg/l, respectively.

Although the test on the ES-analyser had been fully automated, the turn-around time of over 90 minutes turned out to be too long to fulfil requirements for emergency testing. This problem has been overcome by the introduction of the Elecsys<sup>TM</sup> analysers. The turn-around time of the troponin T test on this analyser is 9 minutes (103). An other difference with the methodology of the ES analyser is the use of a ruthenium labelled component instead of the horseradish peroxidase on the signal

antibody. Recently, the so-called 'third generation' troponin T assay has been introduced. The difference between the second and the third generation is the use of human recombinant cardiac troponin T for calibration (third generation) instead of bovine cardiac troponin T (second generation). This calibrator change improves together with the one different amino acid in the sequence of the capture antibody M11.7 epitope the cardiac troponin T assay in two crucial properties: the linearity of the calibration curve and the achievement of a common mab-Bi plateau for samples and calibrators/controls. This generates a linear calibration curve which consequently improves precision and lot to lot variance. However, the change of the calibration curve also modifies the measuring range and causes an unlinear relationship to the second generation cardiac troponin T assay. The third generation assay was adjusted to the second generation in the low range from 0 to 0.2 µg/l. Starting at a level of 0.2 µg/l the deviation between the two generations enlarges nonlinearly, resulting in an upper limit of measuring range for the third generation assay of 25 µg/l, whereas this is 65 µg/l for the second generation assay (104).

### **Point of care testing of troponin**

With the need for a short turn-around-time for results of cardiac markers, point of care testing for these markers became a real requirement. Especially, the American Association for Clinical Chemistry made recommendations that the turn-around-time of cardiac marker testing should be within one hour. If central laboratory testing can not fulfill this requirement, point of care testing should be seriously considered (105).

The principles of point of care testing can be subdivided in qualitative and quantitative test methodologies. Qualitative measurement of troponin I became commercially available by Spectral Diagnostics<sup>TM</sup>. The antibodies from this test were comparable to those used in the Access<sup>TM</sup> Analyser (manufacturer Pasteur-Sanofi/Beckman<sup>TM</sup>). So, the cut-off value of this test is 0.1 µg/l.

With the introduction of the qualitative measurement of troponin T (the so-called troponin T-'strip'-test), it became possible to test troponin T near the patient and outside the laboratory. The turn-around time of this 'strip'-test is 20 minutes (106). The antibodies in this test are of course equal to those used on the analysers of Roche-Boehringer Mannheim<sup>TM</sup>. The cut-off value of this 'strip'-test is 0.1 µg/l. With the introduction of the afore mentioned second generation troponin T antibodies this 'strip-test' has also been modified. Later on, this test has further been modified because of the requirement of more accurate results for the point of care tests. By manufacturing a detection unit, it has become reality to report the results of the strip-test quantitatively in the range 0-3 µg/l (107).

Recently, quantitative outside laboratory measurement of cardiac troponin I has become reality with the introduction of the Stratus CS analyser (manufacturer Dade/Behring<sup>TM</sup>) and the Triage meter (manufacturer BioSite<sup>TM</sup>). The Stratus CS analyser measures cardiac troponin I, myoglobin and CKMB-mass from Li-heparin anticoagulated blood. The analyser itself separates the plasma from the cells by centrifugation and the tests are performed in the plasma. It takes fifteen minutes before the results are reported and it is up to the test performer whether cardiac troponin I, myoglobin and/or CKMB-mass is measured (108). This is in contrast to the Triage CP analyser. This analyser measures always all three components cardiac troponin I, myoglobin and CKMB-mass out of 250 µl Li-heparin anticoagulated blood. Within the analyser the plasma is separated from the cells by a membrane. It takes fifteen minutes to have all three test results reported with this analyser (109).

## **The scope of the thesis**

The aim of this thesis is to investigate the biochemical and clinical assessment of new biochemical markers in relation to conventional cardiac markers for the detection of different forms of myocardial tissue damage. These forms include ischemia in patients with acute chest pain, ischemia after major non-cardiac surgery, myocardial damage as a result of heart surgery, heart contusion after blunt trauma, and myocardial damage related to malignant diseases.

In chapter 2 the content and distribution within the human heart will be investigated first in order to answer the question if the biochemical markers are homogeneous distributed over the heart and second to investigate the person to person variety of the contents. The immuno-histochemical detection of both cardiac troponin I and cardiac troponin T are investigated in chapter 3 to investigate the assessment of these markers for the detection of AMI in postmortem patients and second in myocardial as well as in skeletal muscle tissue in order to determine the heart-specificity of these two markers. A technical evaluation of equipment to perform the CK-isoform determination as a stat analysis is described in chapter 4 as well as the results of a pilot study to investigate the clinical usefulness of this parameter in patients presenting with chest pain complaints at the emergency room. Further analytical evaluation together with the assessment of the clinical benefits of the CK-isoform analysis in relation to other biochemical markers are subject of investigation in chapter 5 in patients suffering acute chest pain (including UAP and AMI). The reliability and performance of point of care analysis of cardiac markers at the Coronary Care Unit is investigated in chapter 6. The detection of myocardial tissue damage after blunt trauma in patients with and without thoracic injuries is subject of the study, which is reported in chapter 7. The performance of biochemical markers for the detection of myocardial damage in patients undergoing major non-cardiac surgery is described in chapter 8. The release patterns of biochemical markers after several forms of heart surgery are reported in chapter 9 in patients with no complications after surgery. The performance of biochemical markers for the detection of myocardial damage related to malignant diseases is described in chapter 10. To this category belong lung cancer patients undergoing pleuro-pneumonectomy followed by intraoperative photodynamic therapy; and carcinoid patients, who may develop heart failure as a consequence of the carcinoid syndrome. Finally, in chapter 11 the current state as well as future perspectives for the clinical usefulness of the cardiac markers will be considered.

## references

1. World Health Organization. Report of the Joint International Society and Federation of Cardiology/World Health Organization Task Force on Standardization of Clinical Nomenclature and criteria for diagnosis of ischemic heart disease. *Circulation* 1979; 59: 607-9.
2. LaDue JS, Wroblewski F, Karmen A. Serum glutamic oxaloacetic transaminase activity in human acute transmural myocardial infarction. *Science* 1954; 120: 497-9.
3. Oliver IT. *Biochem J* 1955; 61: 116-22.
4. Wroblewski F, Rueggsegger P, Ladue. *Science* 1956; 123: 1122-3.
5. Gerhardt W, Waldenstrom J. *Clin Chem* 1979; 25: 1274-80.
6. Adams JE, Abendschein DR, Jaffe AS. Biochemical markers of myocardial injury is MB Creatine Kinase the choice for the 1990s? *Circulation* 1993; 88: 750-63.
7. Klocke FJ, Copley DP, Krawczyk JA, Reichlin M. Rapid renal clearance of immunoreactive canine plasma myoglobin. *Circulation* 1982; 65: 1522-8.
8. Samarel AM, Ferguson AG, Vander Heide RS, Davison R, Ganote CE. Release of unassembled rat cardiac myosin light chain I following the calcium paradox. *Circ Res* 1986; 58: 166-71.
9. Clark GL, Robinson AK, Gnepp DR, Sobel BE. Effects of lymphatic transport of enzyme on plasma creatine kinase time activity curves after myocardial infarction in dogs. *Circ Res* 1978; 43: 162-9.
10. Ellis AK. Serum protein measurements and the diagnosis of acute myocardial infarction. *Circulation* 1991; 83: 1107-9.
11. Kohse KP, Wisser H. Antibodies as a source of analytical errors. *J Clin Chem Clin Biochem* 1990; 28: 881-92.
12. Wevers RA, Delsing M, Klein Gebbink JA, Soons JBJ. Postsynthetic changes in creatine kinase isoenzymes. *Clin Chim Acta* 1978; 86: 323-7.
13. Prager NP, Suzuki T, Jaffe AS, Sobel BE, Abendschein DR. The nature and time course of generation of isoforms of MB creatine kinase in vivo. *J Am Coll Cardiol* 1992; 20: 414-9.
14. Billadello JJ, Fontanet HL, Strauss AW, Abendschein DR. Characterization of MB creatine kinase isoform conversion in vitro and in vivo in dogs. *J Clin Invest* 1989; 83: 1637-43.
15. Horder M, Elser RC, Gerhardt W, Mathieu M, Sampson EJ. Approved recommendation on IFCC methods for the measurement of catalytic concentration of enzymes: part 7. IFCC method for creatine kinase (ATP: creatine N-phosphotransferase, EC 2.7.3.2). *Eur J Clin Chem Clin Biochem* 1991; 29: 435-56.
16. Lang H. *Creatine Kinase Isoenzymes*. New York: Springer-Verlag, 1981.
17. Mercer DW. Separation of tissue and serum creatine kinase isoenzymes by ion-exchange column chromatography. *Clin Chem* 1974; 20: 36-40.
18. Apple FS, Greenspan NS, Dietzier DN. Elevation of creatine kinase BB CK in hospitalized patients. *Ann Clin Lab Sci* 1982; 12: 398-402.
19. Delanghe J, DeMol AM, Debruyzere ML, DeScheerder JK, and Wieme RJ. Mass concentration and activity concentration of creatine kinase isoenzyme MB compared in serum after acute myocardial infarction. *Clin Chem* 1990; 36: 149-53.
20. Christenson RH, Vaidya H, Landt Y, Bauer RS, Green SF, Apple FS, et al. Standardization of Creatine Kinase-MB (CK-MB) mass assays: The use of recombinant CK-MB as a reference material. *Clin Chem* 1999; 45: 1414-23.
21. Puleo PR, Guadagno PA, Roberts R, et al. Sensitive, rapid assay of subforms of creatine kinase



- MB in plasma. *Clin Chem* 1989; 35: 1452-5.
22. Yasmineh WG, Yamada MK, Cohn JN. Postsynthetic variants of CK-MM. *J Lab Clin Med* 1981; 98: 109-14.
  23. Apple FS, Sharkey SW, Werdick M, et al. Analysis of CK isoenzymes and isoforms in serum to detect reperfusion after acute myocardial infarction. *Clin Chem* 1987; 33: 507-10.
  24. Gerhardt W, Katus H, Ravkilde J, et al. S-Troponin T in suspected ischemic myocardial injury compared with mass catalytic concentrations of S-Creatine kinase isoenzyme MB. *Clin Chem* 1991; 37: 1405-11.
  25. Panteghini M. Serum isoforms of CK isoenzymes. *Clin Biochem* 1988; 21: 211-5.
  26. Abendschein DR, Morelli RL, Carlson CJ et al. CK-MM isoenzyme subforms in myocardial, lymph, and blood after coronary artery occlusion. *Cardiovasc Res* 1984; 18: 690-3.
  27. Sherkey SW, Elsperger KJ, Apple FS. Canine myocardial CK isoenzyme response to coronary artery occlusion. *Am J Physiol* 1989; 256: H508-14.
  28. Wu AHB, Gornet TG, Wu VH, et al. Early diagnosis of AMI by rapid analysis of CK-isoenzyme-3 subtypes. *Clin Chem* 1987; 33: 358-62.
  29. Apple FS, Hellsten Y, Clarkson PM. Early detection of skeletal injury by assay of CK-MM isoforms in serum after acute exercise. *Clin Chem* 1988; 34: 1102-5.
  30. Puleo PR, Perryman MB, Bresser MA, et al. CK isoform analysis in the detection and assessment of thrombolysis in man. *Circulation* 1987; 75: 1162-4.
  31. Clarkson PM, Apple FS, Byrnes WC, et al. Creatine kinase isoforms following isometric exercise. *Muscle and Nerve* 1987; 10: 41-5.
  32. Abendschein DR, Fontanet HL, Nohara R. Optimized preservation of isoforms of CK-MM isoenzyme in plasma specimens and their rapid quantification by chromatoferrin. *Clin Chem* 1990; 36: 723-7.
  33. Grace AM, Strauss AW, Sobel BE. Sensitive quantification of isoforms of canine MMCK with immunoblot procedure. *Analyt Biochem* 1985; 149: 209.
  34. Wu AHB, Gornet TG. Measurement of CK-MM sub-type by anion exchange liquid chromatography. *Clin Chem* 1985; 31: 1841-4.
  35. Panteghini M, Bonora R, Pagani F, et al. An immunochemical procedure for determination of CK-3 isoform in human serum. *Clin Biochem* 1990; 23: 225-8.
  36. Suzuki T, Shiraisi T, Tomita K, et al. Monoclonal antibody inhibitory CK-MM3 but not CK-MM1. *Clin Chem* 1990; 36: 153-6.
  37. Christenson RH, Russell ME, Pizzo CK. Specificity of an antibody for precipitating the MB1 isoform of creatine kinase MB. *Clin Chem* 1991; 37: 1023 (Abstr.).
  38. Apple FS. Acute myocardial infarction and coronary reperfusion. Serum cardiac markers for the 1990s. *Am J Clin Path* 1992; 97: 217-26.
  39. Swaanenburg JCJM, Pentinga M, DeJongste MJL, et al. Implications of automated creatine kinase (CK)-MM1,2,3/CK-MB1,2 isoform analysis as an early marker for the detection of myocardial tissue damage. *Scand J Clin Lab Invest* 1996; 56: 627-33.
  40. Wolf PL. LD isoenzymes in myocardial disease. *Clinic Lab Med* 1989; 9: 655-65.
  41. Lee TH, Goldman I. Serum enzyme assays in diagnosis of acute myocardial infarction. *Ann Int Med* 1986; 105: 221-33.
  42. Van der Laarse A, Vermeer F, Hermens WT, Willems GM, de Neef K, Simoons ML, et al. Effects of early intracoronary streptokinase on infarct size estimated from cumulative enzyme release

and on enzyme release rate: a randomized trial of 533 patients with acute myocardial infarction. *Am Heart J* 1986; 112: 672-81.

43. Smit MJ, Duursma AM, Bouma JMW, Gruber M. Receptor-mediated endocytosis of lactate dehydrogenase M by liver macrophages: a mechanism for elimination of enzymes from plasma. *J Biol Chem* 1987; 262: 13020-6.

44. Emery AEH. Electrophoretic pattern of lactic dehydrogenase in carriers and patients with Duchenne muscular dystrophy. *Nature* 1964; 201: 1044-5.

45. Von Eyben FE, Blaaberg O, Petersen PH, Horder M, Nielsen HV, Kruse-Anderson S, Parley E. Serum lactate dehydrogenase isoenzyme 1 as a marker of testicular germ cell tumor. *J Urol* 1988; 140: 986-90.

46. Pesce MA. The CK and LD macroenzymes. *Lab Management* 1984; 22: 29-41.

47. Bais R and Philcox M. Approved recommendation on IFCC methods for the measurement of catalytic concentration of enzymes. Part 8. IFCC method for lactate dehydrogenase(L-Lactate: NAD<sup>+</sup> Oxidoreductase, EC 1.1.1.27). International Federation of Clinical Chemistry (IFCC). *Eur J Clin Chem Clin Biochem* 1994; 32: 639-55.

48. Wieland T, Pfleiderer G. Chemical differences between multiple forms of lactic dehydrogenase. *Ann N Y Acad Sci U S A* 1961; 94: 691-700.

49. Kreutzer HH, Fennis WHS. Lactic dehydrogenase isoenzymes in blood serum after storage at different temperatures. *Clin Chim Acta* 1964; 9: 64-8.

50. Liu F, Belding R, Usatequi-Gomex M, et al. Immunochemical determination of LDH-1. *Am J Clin Pathol* 1981; 75: 701-7.

51. Ali M, Braun EV, Laraia S, et al. Immunochemical LD1 assay for myocardial infarction. *Am J Clin Pathol* 1981; 76: 426-9.

52. Bruns DE, Emerson JC, Intemann S, et al. LD isoenzyme-1: Changes during the first day after acute myocardial infarction. *Clin Chem* 1981; 27: 1821-3.

53. Adan J, Bernstein LH, Babb J. LD isoenzyme 1 total ratio: Accurate for determining the existence of myocardial infarction. *J Clin Chem* 1986; 32: 624-8.

54. Panteghini M, Bonara R, Pagani F. Evaluation of a new commercial kit for quantification of LD1 in serum. *J Clin Chem Clin Biochem* 1990; 28: 545-8.

55. Onigbinde TA, Wu AHB, Johnson M, et al. Enzyme kinetics of LD isoenzymes and the mechanism of chemical inhibition for the BMC LD-1 assay. *Clin Chem* 1990; 36: 1819-22.

56. Stone MJ, Wilterson JT, Gomez-Sanchez CE, et al. Radioimmunoassay of myoglobin in human serum. Results in patients with acute myocardial infarction. *J Clin Invest* 1975; 56: 1334-9.

57. Kubasik NP, Guiney W, Warren K, et al. Radioimmunoassay of serum myoglobin: evaluation of a commercial kit and assessment of its usefulness for detecting acute myocardial infarction. *Clin Chem* 1978; 24: 2047-9.

58. Massoubre C, Chivot I, Mainard F, et al. Immunonephelometric assay for myoglobin. *Clin Chim Acta* 1991; 201: 223-30.

59. Chapelle JP, Heusghem C. Semiquantitative estimation of serum myoglobin by a rapid agglutination method: an emergency screening test for acute myocardial infarction. *Clin Chim Acta* 1985; 145: 143-50.

60. Delanghe J, Chapelle JP, el Allaf M, et al. Quantitative turbidimetric assay for determining myoglobin evaluated. *Ann Clin Biochem* 1991; 28: 474-9.

61. Capelle JP, Lemache K, el Allaf D, et al. Fast determination of myoglobin in serum using a new

- radial partition immunoassay. *Clin Biochem* 1994; 27: 423-8.
62. Silva DP, Landt Y, Porter SE, et al. Development and application of monoclonal antibodies to human cardiac myoglobin in a rapid fluorescence immunoassay *Clin Chem* 1991; 37: 1356-64.
  63. Leavis P, Gregely J. Thin filament proteins and thin filament-linked regulation of vertebrate muscle contraction. *CRC Crit Rev Biochem* 1984; 16: 235-305.
  64. Zot AS, Potter JD. Structural aspects of troponin-tropomyosin regulation of skeletal muscle contraction. *Annu Rev Biophys Biophys Chem* 1987; 16: 535-59.
  65. Robertson SP, Johnson JD, Potter JD. The time-course of the  $Ca^{2+}$  exchange with calmodulin, troponin, parvalbumin and myosin in response to transient increase in  $Ca^{2+}$ . *Biophys J* 1981; 34: 559-68.
  66. Ingraham RH, Swenson CA. Binary interactions of troponin subunits. *J Biol Chem* 1984; 259: 9544-8.
  67. Farah CS, Miyamoto CA, Ramos CHI et al. Structural and regulatory functions of the  $NH_2$ - and  $COOH$ -terminal regions of skeletal muscle troponin I. *J Biol Chem* 1994; 269: 5230-40.
  68. Olah GA, Trehwella J. A model structure of the muscle protein complex  $4Ca^{2+}$ -troponin C-troponin I derived from small-angle scattering data: implication for regulation. *Biochemistry* 1994; 33: 12800-6.
  69. Malnic B, Farah CS. Regulatory properties of the  $NH_2$ - and  $COOH$ -terminal domains of troponin T. *J Biol Chem* 1998; 273: 10594-601.
  70. Ingraham RH, Swenson CA. Binary interactions of troponin subunits. *J Biol Chem* 1984; 259: 9544-8.
  71. Buechler KF, McPherson PH, applicants. Novel methods for the assay of troponin I and T and complexes of troponin I and T and selection of antibodies for use in immunoassays. International patent Application. PCT/US96/05476, 1995.
  72. Bucher EA, Maisonpierre PC, Konieczny SF, et al. Expression of the troponin complex genes: Transcriptional coactivation during myoblast differentiation and independent control in heart and skeletal muscles. *Mol Cell Biol* 1988; 8: 4134-42.
  73. Hartner KT, Pette D. Fast and slow isoforms of troponin I and troponin C. Distribution in normal rabbit muscles and effects of chronic stimulation. *Eur J Biochem* 1990; 188: 261-7.
  74. Wilkinson JM, Grand RJA. Comparison of amino acid sequence of troponin I from different striated muscles. *Nature* 1978; 271: 31-5.
  75. Vallins WJ, Brand NJ, Dabhade N et al. Molecular cloning of human cardiac troponin I using polymerase chain reaction. *FEBS Lett* 1990; 270: 57-61.
  76. Bermingham N, Hernandez D, Balfour A, et al. Mapping *TNNC1*, the gene that encodes cardiac troponin I in human and the mouse. *Genomics* 1995; 30: 620-2. 81. Sasse S, Brand NJ, Kyprianou P et al. Troponin I gene expression during human cardiac development and in end-stage heart failure. *Circ Res* 1993; 72: 932-8.
  77. Wade R, Eddy R, Shows TB, et al. cDNA sequence, tissue specific expression, and chromosomal mapping of the human slow-twitch skeletal muscle isoform of troponin I. *Genomics* 1990; 7: 346-57.
  78. Sasse S, Brand NJ, Kyprianou P, et al. Troponin I gene expression during human cardiac development and in end-stage heart failure. *Circ Res* 1993; 72: 932-8.
  79. Bodor GS, Oakley AE, Allen PD, et al. Troponin I in fetal and normal and failing adult human hearts. *Circulation* 1992; 86: (Suppl I): I-842.

80. Samson F, Lee JE, Hung WY, et al. Isolation and localization of a slow troponin (TnT) gene on chromosome 19 by subtraction hybridization of a cDNA muscle library using myotonic dystrophy muscle cDNA. *J Neurosci Res* 1990; 27: 441-51.
81. Samson F, De Jong PJ, Trask BJ, et al. Assignment of the human slow skeletal troponin T gene to 19q13.4 using somatic cell hybrids and fluorescence in situ hybridization analysis. *Genomics* 1992; 13: 1374-5.
82. Mao C, Baumgartner AP, Jha PK, et al. Assignment of the human fast skeletal troponin T gene (TNNT3) to chromosome 11p15.5: evidence for the presence of 11pter in a monochromosome 9 somatic cell hybrid in NIGMS mapping panel 2. *Genomics* 1996; 31: 385-8.
83. Townsend PJ, Farza H, MacGeoch C, et al. Human cardiac troponin T: identification of fetal isoforms and assignment of the TNNT2 locus to chromosome 1q. *Genomics* 1994; 21: 311-6.
84. Thierfelder I, Watkins H, MacRae C, et al. Alpha-Tropomyosin and cardiac troponin T mutations cause familial hypertrophic cardiomyopathy: a disease of the sarcomere. *Cell* 1994; 77: 701-12.
85. Mesnard L, Logeart D, Taviaux S, et al. Human cardiac troponin T: cloning and expression of new isoforms in the normal and failing heart. *Circ* 1995; 76: 687-92.
86. Gahlmann R, Troutt AB, Wade RP, et al. Alternative splicing generates variants in important functional domains of human slow skeletal troponin T. *J Biol Chem* 1987; 262: 16122-6.
87. Medford RM, Nguyen HT, Destree AT, et al. A novel mechanism of alternative RNA splicing for the developmentally regulated generation of troponin T isoforms from a single gene. *Cell* 1984; 38: 409-21.
88. Solaro RJ. Protein phosphorylation and the cardiac myofilaments. In: Solaro RJ, ed. *Protein phosphorylation in the heart muscle*. Boca Raton, FL: CRC Press, 1986:129-56.
89. Venema RC, Kuo JF. Protein kinase C-mediated phosphorylation of troponin I and C-protein in isolated myocardial cells is associated with inhibition of myofibrillar actomyosin MgATPase. *J Biol Chem* 1993; 268:2705-11.
90. Noland TA, Kuo JF. Protein kinase C phosphorylation of cardiac troponin I and troponin T inhibits Ca<sup>2+</sup> stimulated MgATPase activity in reconstituted actomyosin and isolated myofibrils and decreases actin-myosin interaction. *J Mol Cell Cardiol* 1993; 25: 53-65.
91. Katrukha A, Severina M, Bereznikova A, Esakova T, Gusev N, Petterson K, Iovgren T. Phosphorylation of human cardiac troponin I by protein kinase A affects its immunological activity. Cambridge, UK: International Congress of Clinical Enzymology, 1996: M12/P24.
92. Al-Hillawi E, Chiltom D, Trayer IP, Cummins P. Phosphorylation-specific antibodies for human cardiac troponin I. La Grande Motte: 25th European muscle congress, 1996: 6-15.
93. Ingraham RH, Hodges. Effects of Ca<sup>2+</sup> and subunit interaction on surface accessibility of cysteine residues of cardiac troponin. *Biochemistry* 1988; 27: 5891-8.
94. Panteghini M, Bonora R, Pagani F, et al. Rapid and highly sensitive immunoassay for determination of cardiac troponin I in patients with myocardial cell damage [Letter]. *Clin Chem* 1997; 43: 1464-5.
95. Wu AHB, Feng YJ, Moore R, et al. Characterization of cardiac troponin subunit release into serum after acute myocardial infarction and comparison of assays for troponin T and I. *Clin Chem* 1998; 44: 1198-208.
96. Cummins B, Auckland ML, Cummins P. Cardiac-specific troponin I radioimmunoassay in the diagnosis of acute myocardial infarction. *Am Heart J* 1987; 113: 1333-44.

97. Bodor GS, Porter S, Landt Y et al. Development of monoclonal antibodies for an assay of cardiac troponin I and preliminary results in suspected cases of myocardial infarction. *Clin Chem* 1992; 38: 2203-14
98. Larue C, Defacque-Lacquement H, Calzolari C et al. New monoclonal antibodies as probes for human cardiac troponin I: Epitopic analysis with synthetic peptides. *Mol Immunol* 1992; 29: 271-8.
99. Datta P, Foster K, Dasgupta A. Comparison of immunoreactivity of five human cardiac troponin I assays toward free and complexed forms of the antigen: implications for assay discordance. *Clin Chem* 1999; 45: 2266-9.
100. Katus HA, Remppis A, Looser S, Hallermeier K, Scheffold T, et al. Enzyme-linked immunoassay of cardiac troponin T for the detection of acute myocardial infarction in patients. *J Mol Cell Cardiol* 1989; 21: 1349-53.
101. Katus HA, Looser S, Hallermeyer K, et al. Development and in vitro characterization of a new immunoassay of cardiac troponin T. *Clin Chem* 1992; 38: 386-93.
102. Muller-Bardorf M, Hallermeyer K, Schroder A, et al. Improved Troponin T ELISA specific for cardiac Troponin T isoform: assay development and analytical and clinical validation. *Clin Chem* 1997; 43: 458-66.
103. Hoyle NR, Eckert B, Kraiss S. Electrochemiluminescence: leading-edge technology for automated immunoassay analyte detection. *Clin Chem* 1996; 42: 1576-8.
104. Klein G, Baum H, Gurr E, Ickert K, Junge W, Linder et al. Multicenter evaluation of two new assays for myoglobin and troponin T on the Elecsys 2010 and 1010 analyzers. *Clin Chem* 1999; 45 (suppl) A139.
105. Wu AHB, Apple FS, Gibler WB, Jesse RL, Warshaw MM, Valdes R Jr. National Academy of Clinical Biochemistry standards of laboratory practice: recommendations for the use of cardiac markers in coronary artery diseases. *Clin Chem* 1999; 45: 1104-21.
106. Muller-Bardorff M, Freitag H, Scheffold T, et al. Development and characterisation of a rapid assay for bedside determinations of cardiac troponin T. *Circulation* 1995; 92: 2869-75.
107. Muller Bardorff M, Rauscher T, Kampmann M, Schoolmann S, Laufenberg F, Mangold D et al. Quantitative bedside assay for cardiac troponin T: a complementary method to centralized laboratory testing. *Clin Chem* 1999; 45: 1002-8.
108. Heeschen C, Goldmann BU, Langenbrink L, Matschuck G, Hamm CW. Evaluation of a rapid whole blood ELISA for quantification of troponin I in patients with acute chest pain. *Clin Chem* 1999; 45: 1789-96.
109. Apple FS, Christenson RH, Valdes R Jr, et al. Simultaneous rapid measurement of whole blood myoglobin, creatine kinase MB, and cardiac troponin I by the Triage cardiac panel for detection of myocardial infarction. *Clin Chem* 1999; 45: 199-205.

