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The biochemical and clinical assessment of cardiac markers for the detection of various forms of myocardial tissue damage

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Jozef Cornelis Jacobus Maria Swaanenburg
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Promotores : Prof.dr. S. Poppema
Prof.dr. H.J.G.M. Crijns
Prof. F.S. Apple

Referent : Dr. M.J.L. de Jongste

Beoordelingscommissie:
Prof.dr. M.P. van Dieijen-Visser
Prof.dr. T. Ebels
Prof. D.B. Sacks

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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 losses 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

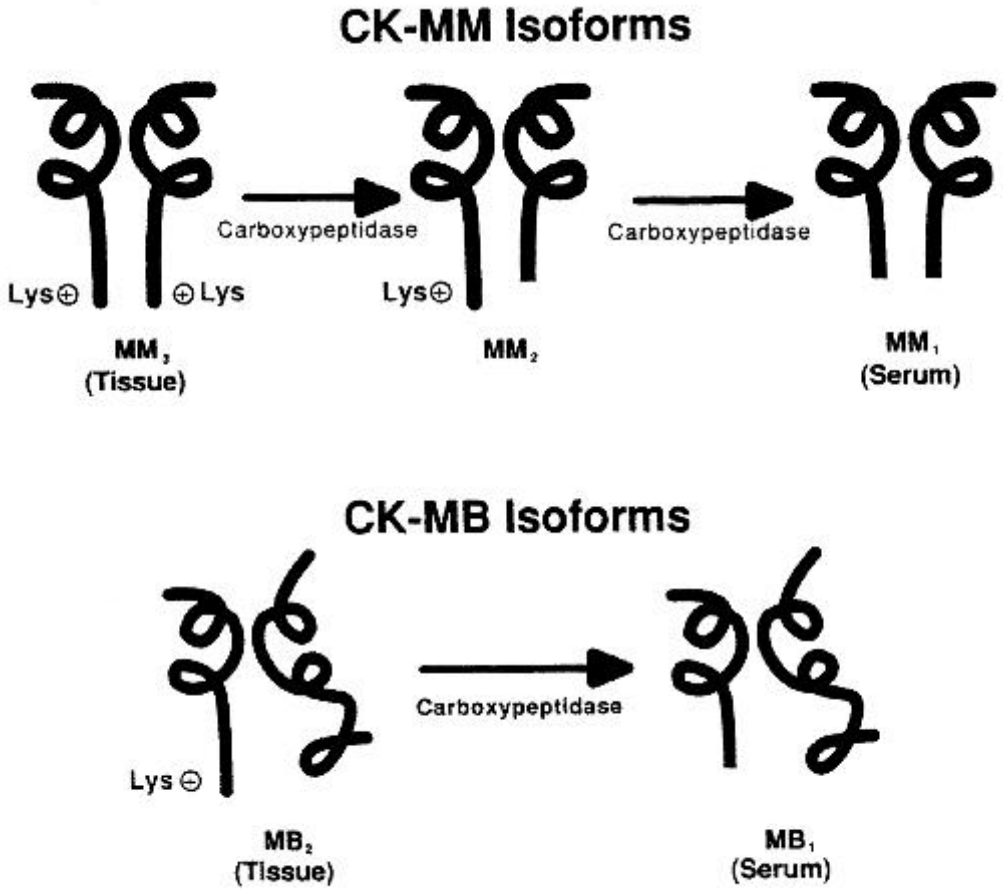

Figure 1. CKMM3 (tissue form) is transformed into CKMM2 (intermediate form) and into CKMM1 (serum form) by cleaving the carboxyterminal lysine from both M subunits. CKMB2 (tissue form) is transformed into CKMB1 (serum form) by cleaving the carboxy-terminal lysine from the M subunit. The intermediate 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 measurers 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 µg/L. The CKMB-mass assay has a better analytical specificity and sensitivity (less than 1 µg/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 $ 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™) 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™ 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 afore mentioned 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 epithopes 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 Ca^{2+}-binding protein and contains four metal-binding sites. Two sites, located in the C-terminal domain, bind both Ca^{2+}

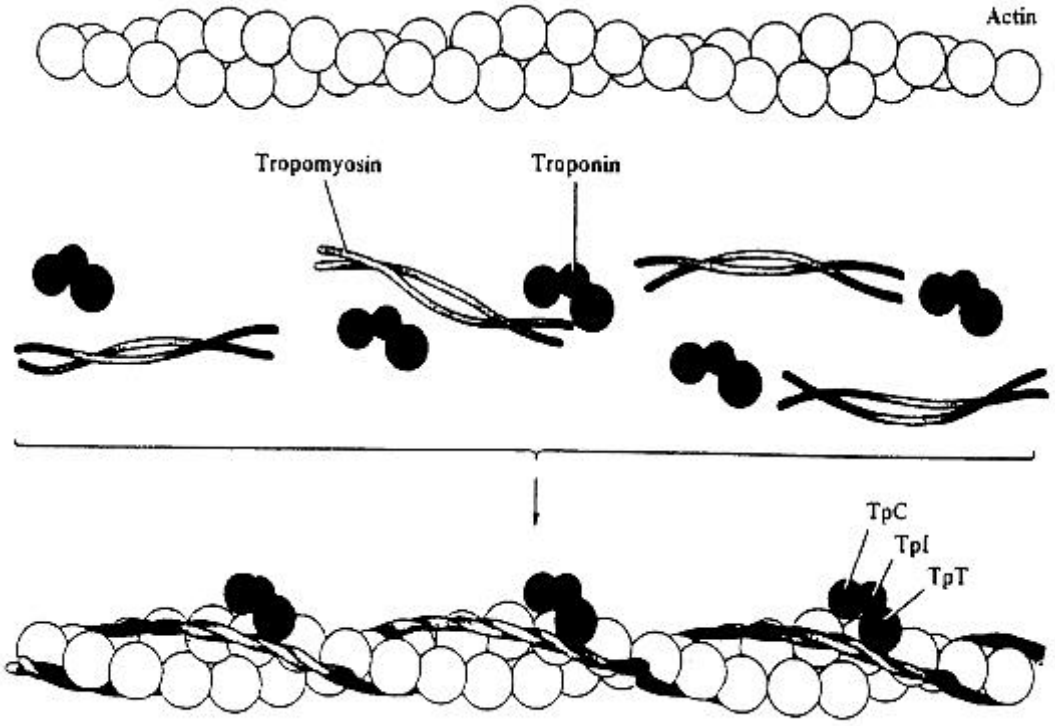

Figure 2. The composition of the thin filament of striated muscle. At each seventh actin position a troponin C-I-T complex is bound to tropomyosin. Abbreviations: TpC: troponin C; TpI: troponin I, and TpT: troponin T.

(K_{ass} approximately 10^7 L/mol) and Mg^{2+} (K_{ass} approximately 10^3 L/mol, whereas two other sites located in the N-terminal domain specifically bind Ca^{2+} with a K_{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 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 Ca^{2+}-binding sites of TnC (63,64,66). In the presence of Ca^{2+}, the K_{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 Ca^{2+}, TnI wraps around the central helix of TnC, forming contacts with both N- and C-terminal globular domains containing 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_{ass} of 8.0×10^6 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 susceptable 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 Ca^{2+}-phospholipid-dependent protein kinase (protein kinase C) (89,90) at different sites. The phosphorylation of Ser-23 and Ser-24 changes the confirmation 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™), Access (Beckman™), Stratus II, Stratus CS, Dimension RxL (Dade Behring™), AxSYM (Abbott™), Immuno I (Bayer™), Immulite (DPC™), ACS:180 (Bayer™), Triage Cardiac System (Biosite Diagnostics™), Vitros ECi (Ortho Clinical Diagnostics™) and Alpha Dx (First Medical™). 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™. 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 ™ 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 nonlinearily, 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™. The antibodies from this test were comparable to those used in the Access™ Analyser (manufacturer Pasteur-Sanofi/Beckman™). 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™. 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™) and the Triage meter (manufacturer BioSite™). 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 ul 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.

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CHAPTER 2

The content and distribution of troponin I, troponin T, myoglobin and "-Hydroxy-Butyric acid Dehydrogenase (HBD) in the human heart.

Joost CJM Swaanenburg [1], Petra J Visser-Van Brummen [1], Mike JL De Jongste [2], Anton THM Tiebosch [1].

[1] Department of Pathology and Laboratory Medicine,
[2] Deparment of Cardiology, University Hospital Groningen.

Summary
We investigated the content and distribution of the new heart-specific markers troponin I (measured with two methodologies), troponin T in relation to the conventional non-heart specific myoglobin and HBD in the hearts of 35 postmortem patients. Tissue was obtained from the right ventricle (RV), from the left ventricle (LV) areas posterior, lateral, anterior wall as well as the interventricular septum, from the right atrium (RA) and from the left atrium (LA). For troponin I ^{AxSYM} we found median values in µg per gram wet weight tissue (and per gram protein) of 2765 (50.0), 2453 (64.1), 1291 (33.9), and 1264 (31.4) respectively for the RV, LV, RA and LA locations. For troponin I^{Access} the median content in µg per gram wet weight tissue (and per gram protein) was 41 (0.88), 71 (1.54), 28, (0.66) and 26 (0.65) respectively for the various heart locations. For troponin T in µg per gram wet weight tissue (and per gram protein) we found 75 (1.77), 95 (2.06), 39 (0.99), and 39 (0.88) respectively for the examined heart locations. For myoglobin in mg per gram wet weight tissue (and per gram protein) the values per heart location were 1.90 (45.1), 2.40 (46.1), 1.00 (20.1), and 0.80 (20.9) respectively, and for HBD in U per gram wet weight tissue (and per gram protein) 116 (2.30), 140 (2.87), 50 (1.21), 52 (1.26) respectively. We conclude that there are statistically significant differences between the contents expressed per gram wet weight tissue in the right and in the left ventricle for the biochemical markers troponin I ^{Access}, troponin T, myoglobin and HBD. The biochemical contents expressed per gram wet weight tissue as well as per gram protein in the right and in the left atrium are statistically significant lower for all examined biochemical markers than those in the right and in the left ventricle, whereas there are no statistically significant differences between the biochemical contents in the right and in the left atrium. Furthermore, within the left ventricle there is no statistically significant difference between the locations posterior-, lateral-, anterior wall and interventricular septum.

Introduction
The determination of cardiac markers in blood after myocardial infarction (AMI) is used for a long time as an estimation for the extent of myocardial tissue damage (1). Its relevance is indicated by the fact, that the prognosis of a patient after AMI is related to the amount of myocardial tissue loss (2). A frequently used conventional biochemical parameter for this purpose is hydroxy-butyric acid dehydrogenase (HBD) (3). However, this parameter is non-heart specific, since it is also elevated after hemolysis. An other marker, which can be used, is myoglobin. After AMI the increase in serum concentration of myoglobin is more rapid than the increase of HBD, whereas myoglobin concentration normalises also earlier. But like HBD, myoglobin is non-heart specific, because myoglobin is also a component of skeletal muscle tissue. Moreover, as myoglobin is cleared through the kidneys, the raise in serum concentration after AMI is more complicated than the raise of HBD, since the myoglobin clearence depends on the renal function of the patient.
Recently, more specific biochemical markers for myocardial tissue damage have been reported. These markers include the cardiac troponins (4,5). The troponins are components related to the actin-myosin complexes of the thin filaments of striated muscle. These complexes are involved in the process of muscle contraction and relaxation. Three isoforms of troponin have been described: troponin I, troponin C and troponin T. Each troponin has its own function. Troponin C binds calcium. This binding activates the muscle contraction. Troponin I inhibits troponin C by binding to

this component resulting in muscle relaxation, whereas troponin T binds the troponin I-troponin C complex to tropomyosin.
In contrast to troponin C, the amino acid sequences of troponin I and troponin T from skeletal muscle differ from those of myocardial muscle. In order to separate them immunologically, specific antibodies directed against the cardiac troponins I and T were developed. These antibodies form the basis of the techniques measuring cardiac troponin I and cardiac troponin T concentrations in serum. As the amino acid composition of troponin C from skeletal muscle equals that of myocardial muscle, no cardiac troponin C methodology can be developed to specifically measure myocardial troponin C.
In order to reliably relate the extent of myocardial tissue loss to the increase of cardiac troponin concentration in serum, the troponins should 1. be homogeneously distributed in the myocardium and 2. show inter-individually hardly any difference in myocardial content (6).
So far, a limited number of studies have been reported about the contents of cardiac markers in the heart (7-12). However, these studies concern a limited number of patients, or hearts of non human origin, whether only troponin I or troponin T is considered or only the contents of the ventricles are investigated. Furthermore, two different forms of content expression are used. These include expression per gram wet weight tissue or per gram protein.
The aim of this study is to investigate the distribution and the content (expressed per gram wet weight tissue as well as per gram protein) of cardiac troponin I (measured with two different troponin I methodologies) and cardiac troponin T in the human myocardium in relation to the distribution and the content of the more conventional parameters myoglobin and HBD.

Patients and methods

Postmortem fresh myocardial tissue was obtained from seven different locations in the heart and from the psoas muscle in 35 patients. Care was taken that all tissue samples were free of scarring and necrosis. The heart locations include right ventricle (RV), left ventricle (LV) posterior wall (LV1), LV lateral wall (LV2), LV anterior wall (LV3), LV interventricular septum (LV4), right atrium (RA), and left atrium (LA). Directly after collection, the samples were weighed and frozen in liquid nitrogen. Subsequently, they were destructed in liquified nitrogen using a Micro-dismembrator U (Braun Biotech International, distributed by Salm and Kipp, The Netherlands) at 2000 rpm for one minute. After finishing this procedure, five ml modified PBS buffer (pH=7.0) was added. These solutions were frozen and stored at -20^{o}C until analysis of the biochemical markers troponin I (troponin I^{AxSYM} and troponin $I^{Access)}$, troponin T, HBD, and myoglobin and the protein concentrations.
Troponin I^{AxSYM} was measured on an AxSYM analyser (Abbott Diagnostic Division, Hoofddorp, The Netherlands) according to the instructions of the manufacturer (13). The coefficients of variation (c.v.'s) at three different levels were determined using control sera. These were 6.8 %, 5.2 %, and 5.8% respectively for the levels 2.9, 7.6, and 29.0 µg/l.
An Access Analyser was used to determine the troponin I^{Access} (Beckman, Mijdrecht, The Netherlands) according to the instructions of the manufacturer (14). The c.v.'s of this assay methodology were 8.9%, 8.2% and 7.4% respectively for the levels 0.22, 5.88 and 25.4 µg/l.
Troponin T measurements were performed with an Elecsys 2010 analyser (Roche-Boehringer Mannheim, Almere, The Netherlands). For these measurements the 'third' generation troponin T

reagent was used. This reagent is calibrated with standards of human origin and uses a one amino acid modified signal antibody, which result in a better linearity than the 'second' generation troponin T reagent, which uses standards of bovine origin (15).
Myoglobin was measured using a BNII-nephelometer (Dade-Behring, Leusden, The Netherlands) according to the instructions of the manufacturer.
HBD activities were measured at 37°C using HBD reagent (product nr 1489267, manufacturer Roche-Boehringer Mannheim, Almere, The Netherlands) on a Mega analyser (Merck, Amsterdam, The Netherlands).
For all these methodologies the linearities were validated by using various dilutions of different tissue homogenate-solutions. Furthermore, all assay-methodologies show no blank reaction with the modified PBS-buffer.
The protein measurements were performed using the pyrogallol red methodology on a Mega analyser. The pyrogallol red reagent (product nr. A01217) was manufactured by Biotrol Diagnostic (Chennevières les-Louvres, France) and was distributed by Merck. The instructions of the manufacturer were modified by using 3 µl homogenate-solution and 250 µl reagent. This modification resulted in an enhancement of the linearity up to 4 g/l. The imprecision was determined using two controls. The mean value of control 1 was 0.78 (standard deviation 0.04) g/l and that of control 2 was 2.98 (s.d. 0.11) g/l. The measured concentrations of the tissue homogenate-solutions were between 1 and 3 g/l.
All biochemical results were expressed as µg (for both troponin I methodologies and for troponin T), mg (for myoglobin) and Units (for HBD) per gram wet weight myocardial tissue and per gram protein.
Statistics. Differences of the biochemical markers between the sampled locations were tested by the Friedman test. If significant differences were found with the Friedman test, the Wilcoxon Signed Ranks test was used as post hoc test between the individual

Table 1. Patient characteristics.

Patient Characteristics.

No.	Age(y)	Gender	Cause of death		weight	timelag[a]
1	86	female	Heart failure		380	8 hours
2	56	male	Heart failure	.	620	20 hours
3	91	male	Pneumonia		145	9 hours
4	64	female	Amyloidosis, LVH		400	18 hours
5	75	female	Cardiomyopathy	475	11 hours	
6	60	male	Intracranial bleeding		400	9 hours
7	67	male	Metastatic lung cancer	250	6 hours	
8	62	male	Pulmonary embolism		450	13 hours
9	52	male	Pulmonary embolism		555	12 hours
10	52	female	Metastatic endometrial cancer	270	16 hours	
11	91	female	Resp insuff. in interst lungdisease	185	34 hours	

12	64	female	Metastatic endometr. ca	275	12 hours	
13	73	male	Sepsis and multi organ failure	520	14 hours	
14	47	female	Respiratory insufficiency	350	6 hours	
15	87	male	Bronchopneum, Wegener disease		350	24 hours
16	40	female	Encephalitis		340	24 hours
17	56	female	Meningitis		260	10 hours
18	34	female	Sudden death, right ventricular dyspl		240	48 hours
19	76	female	Pneumonia		600	10 hours
20	54	male	Metastatic lung cancer	450	24 hours	
21	29	female	Metastatic breast cancer	250	10 hours	
22	68	female	Pneumonia		280	48 hours
23	66	male	Intracranial bleeding		600	24 hours
24	70	male	Aortic aneurism	600	12 hours	
25	60	male	Pulmonary embolism		304	24 hours
26	63	male	Septic shock		384	12 hours
27	49	female	Metastatic pancreatic cancer		286	48 hours
28	61	male	Pancreatitis		554	12 hours
29	59	male	Pulmonary embolism		396	24 hours
30	53	female	Haemochromatosis		410	4 hours
31	67	male	AMI		540	6 hours
32	71	female	aneurisma abdom.		700	48 hours
33	74	male	Pancreatitis, ARDS		380	6 hours
34	74	female	Cerebral infection		450	48 hours
35	81	female	Metastatic cancer unknown primary		420	48 hours

[a] time between death and autopsy.

sampled locations using Bonferroni as correction method. Differences in heart weight were considered by testing differences in content of biochemical markers for the sampled locations in 'normal' (heart-weight 450 gram or less) and hypertrophic (heart-weight over 450 gram) hearts using the Mann-Whitney U-test. P values# 0.05 were considered to be statistically significant different. According to this methodology also gender dependency was considered.

Results

The patient characteristics are depicted in table 1. These include age, gender, cause of death, heart-weight and the time between death and autopsy. From table 1 it can be seen that eighteen females were included. The mean age of these patients was 62 (range 29-91) y, the mean heart-weight was 365 (range 185-700) gram and the mean time between death and autopsy was 26 (range 4-48) h. Also seventeen males were included. The mean age of these patients was 66 (range 52-91) y, the mean heart-weight of these patients was 441 (range 145-600) gram, and the mean time between death and autopsy was 15 (range 6-24) h.

Table 2 shows the content of the examined biochemical markers in the different parts of the left ventricle. This table shows that within the left ventricle there is no statistically significant difference

between the sampled areas posterior-, lateral-, and anterior wall, as well as the interventricular septum. Therefore, the mean value of the left ventricle samples will be used in the remaining. In figure 1a the ranges of the contents expressed per gram wet weight tissue of the examined biochemical markers per myocardial location and of the skeletal muscle tissue are shown by the use of a box-plot. In figure 1b this is shown for the contents expressed per gram protein. From figure 1a and figure 1b it can be seen that the contents of troponin I ^{Access}, troponin T, myoglobin, and HBD per gram wet weight myocardial tissue of the right ventricle are significantly lower than those of the left ventricle overall. In contrast, there is no difference, if the contents per gram protein are compared. Furthermore, the contents per gram wet weight tissue as well as per gram protein of all examined markers in the right and in the left ventricle are significantly higher than those in the right and in the left atrium. The contents of biochemical markers in the right atrium do not statistically significant differ from those in the left atrium. For all sampled locations we observe lower results of the troponin I ^{Access} methodology compared with that of the troponin I ^{AxSYM}. The contents of the examined troponins are at least 500-1000 times lower in the skeletal muscle than in the various heart locations. The measured values of the skeletal muscle may be explained by a-specific binding. The content of HBD in skeletal muscle is 1.5 - 5 times lower than in the considered heart locations, whereas the content of myoglobin in skeletal muscle is 1.5 - 4 times higher than in heart muscle tissue. In Table 3 the median contents per gram wet weight myocardial tissue and per gram protein are summarised per sampled location (right ventricle, left ventricle overall, right atrium,

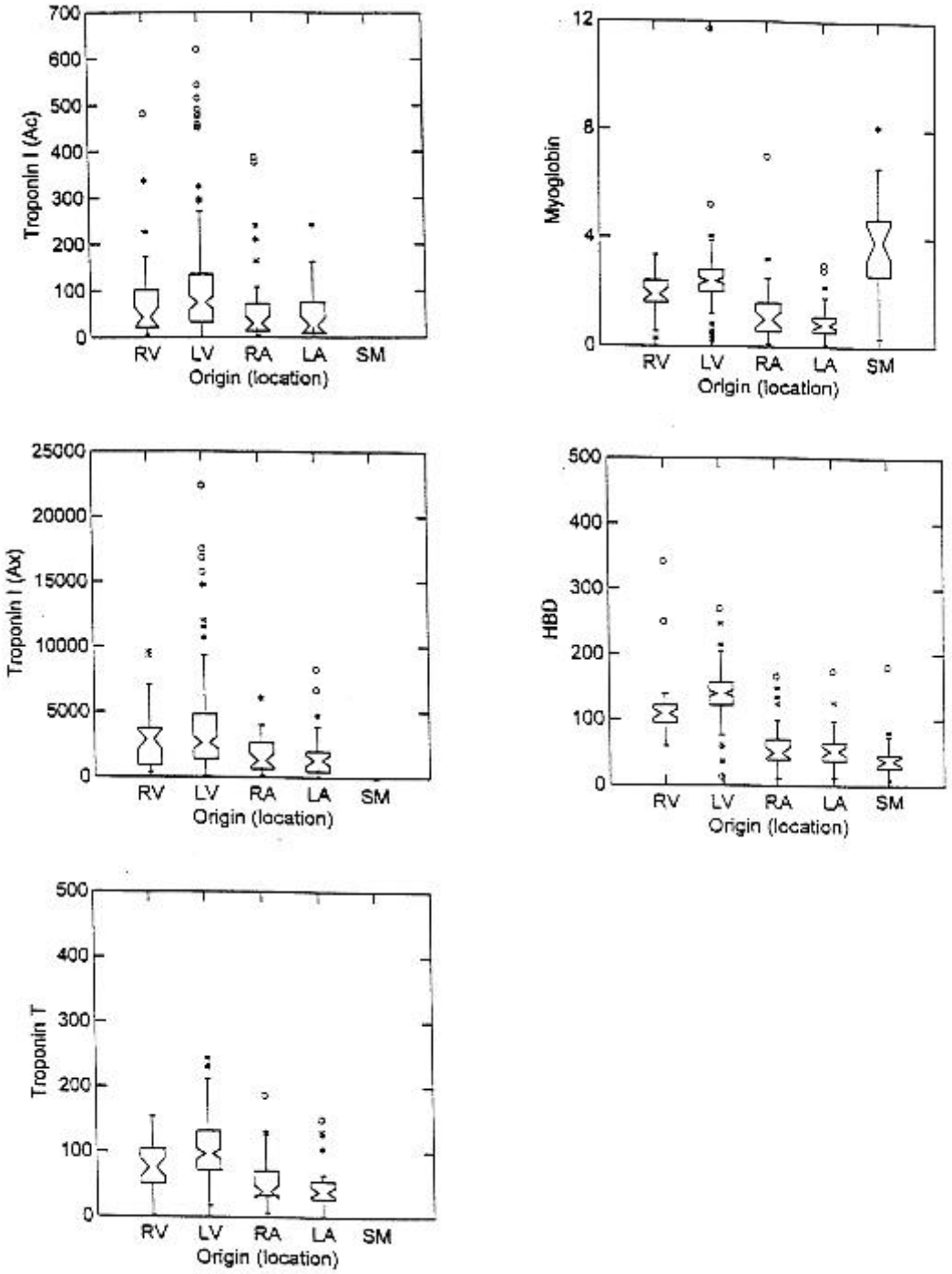

Figure 1. The ranges of contents expressed per gram wet weight myocardial tissue for several cardiac markers sampled at different locations in the heart and the ranges of contents per gram wet weight skeletal tissue are shown. Units: troponin I (Ac) , troponin I (Ax) and troponin T: µg/g wet weight tissue; myoglobin: mg/g wet weight tissue; HBD: U/g wet weight tissue. Abbreviations:RV: right ventricle, LV: left ventricle; RA: right atrium; LA: left atrium; SM: skeletal muscle. Troponin I (Ac): troponin I^{Access} ,Troponin I (Ax): troponin I^{AxSYM}. Statistically significant differences: for all markers the contents of the right ventricle and left ventricle are higher than those of the right atrium as well as the left atrium; for the markers troponin I^{Access}, troponin T, myoglobin and HBD the contents of the left ventricle are higher than those of the right ventricle.

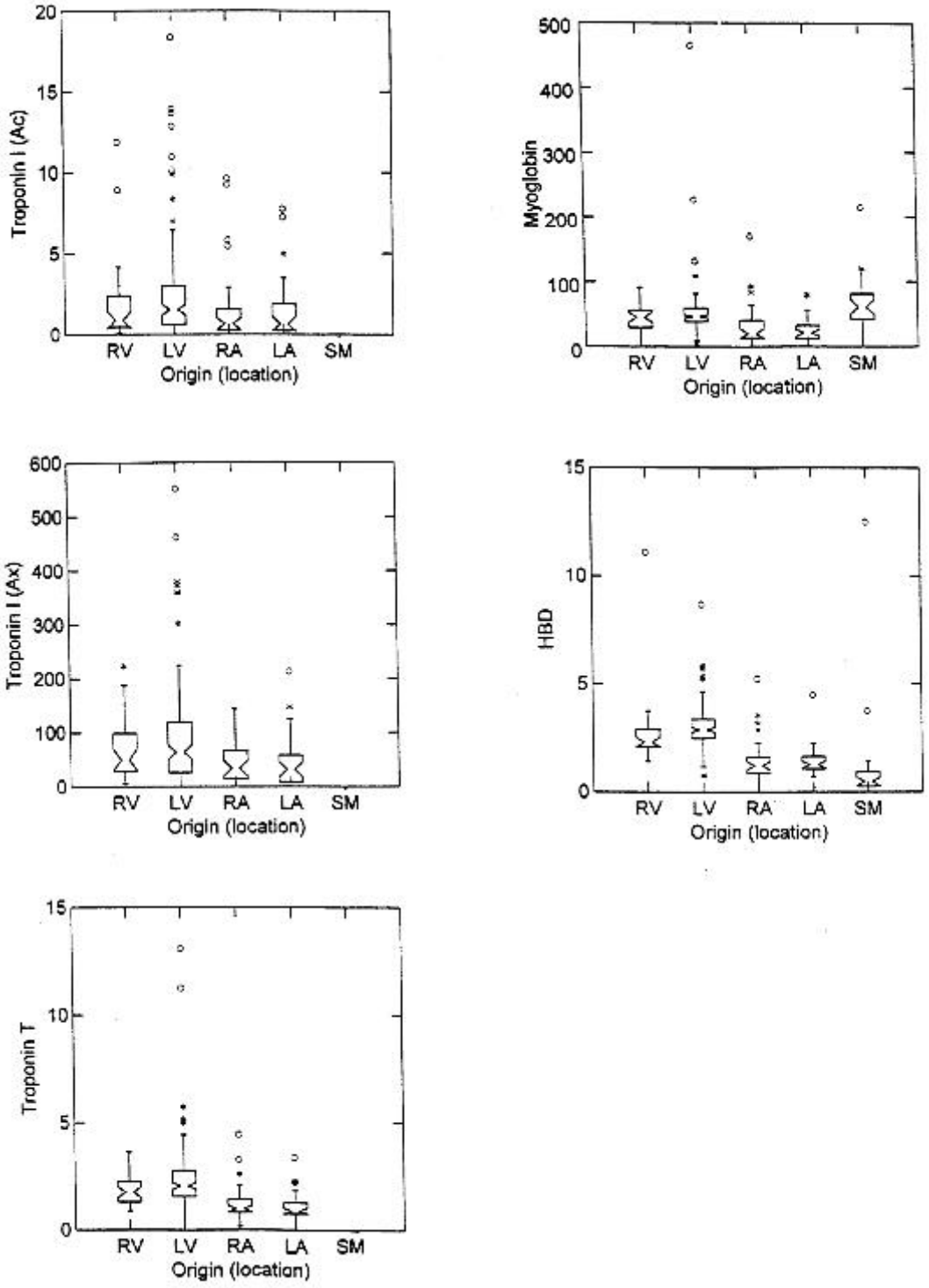

Figure 2. The ranges of contents expressed per gram protein for several cardiac markers sampled at different locations in the heart and the ranges of contents per gram protein in skeletal tissue are shown. Units: troponin I (Ac), troponin I (Ax) and troponin T: µg /g protein; myoglobin: mg/g protein; HBD: U/g protein. For abbreviations see text figure 1. Statistically significant differences: for all markers the contents of the right ventricle and left ventricle are higher than those of the right atrium as well as the left atrium; there is no difference between the right and left ventricle.

Table 2. Median (range) content of various biochemical markers per gram wet weight myocardial tissue sampled at different places in the left ventricle (LV) of the human heart.

Biochemical marker (Unit)	LV posterior wall	LV lateral wall	LV anterior wall	LV septum	LV overall
Troponin I^{Access} (µg)	76.7 (.18 - 543)	69 (.04 - 620)	68.4 (.1 - 459)	66.6 (.1- 491)	71.3 (.04-620)
Troponin I^{AxSYM} (µg)	2072 (15.9 -17520)	2338 (8.4 - 22374)	3182 (8.6 - 15756)	2787 (6.5 - 10678)	2453 (6.5-22374)
Troponin T (µg)	84.5 (17 - 234)	117 (18 - 213)	94 (24 - 244)	107 (13.5 - 230)	95 (13.5-244)
Myoglobin (mg)	2.40 (.78- 4.05)	2.50 (.67 - 5.21)	2.20 (.38 - 11.7)	2.40 (.25 - 3.69)	2.40 (.25-11.7)
HBD (U)	143 (38 - 247)	151 (12.7 - 270)	138 (62 - 196)	135 (5 - 183)	140 (5-270)

No statistically significant differences for each examined cardiac marker could be detected between the various left ventricle locations.

Table 3. Median content per gram wet weight myocardial tissue and per gram protein of various biochemical markers sampled at different places in the human heart and of skeletal muscle tissue.

Biochemical marker (Unit)	Right Ventricle		Left Ventricle		Right Atrium		Left Atrium		Skeletal Muscle	
	w.w[a]	protein[b]	w.w[a]	protein[b]	w.w[a]	protein[b]	w.w[a]	protein[b]	w.w[a]	protein[b]
Troponin I^{Access} (µg)	41.1	0.88	71.3	1.54	28.1	0.66	26	0.65	.08	0.004
Troponin I^{AxSYM} (µg)	65	50.0	2453	64.1	1291	33.9	1264	31.4	2.25	0.04
Troponin T (µg)	75	1.77	95	2.06	39.0	0.99	39	0.88	0.04	0.001
Myoglobin (mg)	1.90	45.1	2.40	46.1	1.00	20.1	0.80	20.9	3.85	60.8
HBD (U)	116	2.30	140	2.87	50	1.21	52	1.26	38	0.49

[a] content expressed per gram wet weight tissue;
[b] content expressed per gram protein.
left atrium) and of the skeletal muscle tissue for both cardiac troponin I methodologies, cardiac troponin T, myoglobin and HBD.

We observe no heart-weight dependency of contents of the examined biochemical markers for all sampled locations between normal (n=25) and hypertrophic (n=10) hearts (all p-values were > 0.05). Evenso, we observe no differences in biochemical contents between the hearts of the male and the female patients (all p-values > 0.05).

Discussion

In this study we have investigated the contents per gram wet weight tissue as well as per gram protein and the distributions of cardiac troponin I, cardiac troponin T, HBD and myoglobin in the human heart. In summary, the person to person variety of the contents is high (the contents differ for some persons more than five times). Furthermore, the distribution of the markers over the heart is not homogeneous. The biochemical contents in the right and in the left atrium are statistically significant lower than those in the right and in the left ventricle, whereas the contents in the right and in the left atrium are not statistically significant different for all examined markers. Furthermore, the contents expressed per gram wet weight tissue in the right ventricle are, in contrast to the contents expressed per gram protein, statistically significant lower than those in the left ventricle for troponin I^{Access}, troponin T, myoglobin, and HBD, whereas the contents within the left ventricle show no differences for all examined biochemical markers regarding the locations posterior-, lateral-, anterior wall and interventricular septum. We have also looked for a gender and a heart-weight dependency. However, we did not observe statistically significant differences between the contents in female and male hearts, although the mean heart-weight of the female patients (365 gram) was lower than that of the male patients (441 gram). Furthermore, we did also not observe statistically significant differences between the contents of 'normal' and hypertrophic hearts.

In clinical practice it is of common use to estimate myocardial tissue damage from levels of cardiac markers in serum. But the afore reported non-homogeneous distribution of cardiac markers in the heart implicates, that it is necessary to know the location of the damage (right or left ventricle?), if the tissue damage is expressed per gram tissue. It is not necessary to differentiate between the locations right and left atrium. However, these locations should be considered different from both locations right and left ventricle. A consequence of the (high) person to person variety in contents is that it is not reliable to state that an individual patient with somewhat higher levels of cardiac markers in serum after AMI experiences more myocardial damage than an other individual patient who has somewhat lower levels of cardiac markers in serum after AMI. In contrast, it is allowed to use the estimations of myocardial damage with levels of cardiac markers in serum to compare groups of patients, if these groups have an equal composition, so that the person to person variety is comparable in both groups, and, if the size of these groups is big enough (to be determined by a power calculation).

The difference between the results of the troponin I^{Access} and the troponin I^{AxSYM} methodologies is caused by the lack of standardization of the troponin I analysis. On the one hand, this is due to the lack of consensus concerning the use of the antibody, which is used to detect troponin I. On the other hand, there is no consensus about the composition of a troponin standard, which may be used by each troponin manufacturer as the 'gold' standard for calibration. The results of the various troponin I methodologies will become more comparable, if such a 'gold' standard will be accepted and introduced by the different troponin manufacturers. However, the composition of a 'gold' troponin standard is complicated, because troponin is in various forms present in circulation. These

forms include free as well as several forms of complex bound troponin such as troponin I-troponin C, troponin I-troponin C-troponin T and troponin I-troponin T (16). Nevertheless an AACC-standardisation subcommittee is working on this subject (17).
Although for a long time increased concentrations in blood of cardiac markers are used to estimate the extent of myocardial necrosis, only a few publications adress to the distribution and content of biochemical parameters in the human heart. Van der Laarse et al. (9) reported no difference between the enzyme content expressed per gram wet weight tissue in the right and in the left ventricle of myocardial tissues obtained by biopsies during open heart surgery. They reported a mean content of 120 U/g wet weight myocadium for HBD. In contrast to our experiments, were these HBD measurements performed at 25 °C. Furthermore, these results were based on left ventricle tissue obtained by biopsies from 6 patients and right ventricle tissue obtained by biopsies from 9 patients. It is unclear whether these tissues were derived from (partly) the same patients. In contrast to their results, we observe statistically significant differences between the contents in the right and in the left ventricle. Besides the already mentioned discrepancy in number of patients for who tissue was derived from right and left ventricles (in our study n=35 versus Van derLaarse n=6 (LV) and n=9 (RV)), also the procedure of collection was different (autopsy versus biopsy). Moreover, we use the Friedman and Wilcoxon Signed Ranks tests to compare the results of the contents in the right and in the left ventricle, whereas Van der Laarse used a *t*-test to test the significance of differences between means of two groups and a Fisher's *F*-test to test the significance of differences between variances of two groups.
Kragten et al. (10) reported the content per gram wet weight tissue of HBD and of troponin T from only the left ventricle in myocardial tissue derived from 17 patients at autopsy. They reported a mean HBD content of 156 U/g wet weight myocardial tissue, which is in agreement with our findings. The mean troponin T content was reported to be 234 µg/g wet weight myocardial tissue, which is more than a factor two higher than we observe. However, in contrast to our measurements, they used the second generation troponin T reagent, whereas we used the third generation. As has recently been reported (7), this third generation troponin T assay (a) is more accurate as well as (b) demonstrates an improved linearity compared with the second generation troponin T and (c) shows lower results above values of 0.2 µg/l than the second generation troponin T assay. Between 0 and 0.2 µg/l there is no difference in results between the second and the third generation assays. The difference in results starts at a level of 0.2 µg/l. At a level of 10 µg/l the results of the third generation are 50% of those determined by the second generation. At a level of 25 µg/l the results of the third generation are 40% of those of the second generation (7). All our measurements in the tissue-homogenates were performed in diluted samples, because the undiluted tissue-homogenate solutions would be out of the analyser range. We diluted the homogenates until a concentration between 10 and 15 µg/l. As already stated, the difference between the second and third generation troponin T assays is at this level a factor between two and two-and-a-half. This might be a reasonable explanation for the difference in troponin T contents between our findings and those reported by Kragten et al.
Katus et al. (11) reported only free troponin T contents in the left ventricles of hearts from three patients undergoing heart transplantation. These patients are different from our population, because most of the patients in our study have no history of heart disease and moreover, none of the patients underwent a heart transplantation.
Bleier et al. (12) reported the content per gram wet weight tissue of myoglobin, troponin I and troponin T in the right atrium from 11 patients undergoing heart surgery. They reported a mean

myoglobin content of 0.97 mg/g wet weight, which is in good agreement with our findings of the right atrium. The troponin I and troponin T contents are different from our results. This might be explained by the already mentioned lack of standardisation of the troponin analysis, as they used other troponin I and troponin T methodologies than we did.
Voss et al. (7) reported the content per gram protein of cardiac troponin T and of myoglobin in the left as well as in the right ventricle in hearts of three healthy and of three diseased humans. The cardiac troponin T measurements were not carried out with the third generation assay, so that these results can not be compared with our results. The mean content of myoglobin of the left ventricle in healthy persons is 18.4 mg/g and in diseased persons 49.8 mg/g (overall mean of these persons is 34.1 mg/g). For the right ventricle these contents were reported to be 28.8 and 66.4 mg/g respectively (overall mean of these persons is 47.6 mg/g). We report median values of 46.1 mg/g for the left ventricle and 45.1 for the right ventricle.
From this study we conclude that there are statistically significant differences between the contents per gram wet weight tissue in the right and in the left ventricle for the biochemical markers troponin I^{Access}, troponin T, myoglobin and HBD. The biochemical contents per gram wet weight tissue as well as per gram protein in the right and in the left atrium are statistically significant lower for all examined biochemical markers than those in the right and in the left ventricle, whereas there are no statistically significant differences between the biochemical contents in the right and in the left atrium. Furthermore, within the left ventricle there is no statistically significant difference between the locations posterior-, lateral-, anterior wall and interventricular septum. For clinical practice these findings implicate that for estimation of myocardial damage with levels of cardiac markers in serum it is useful to know what the location of damage is in the heart. Furthermore, it is not reliable to compare myocardial damage between individual patients with levels of cardiac markers in serum. In contrast, this is allowed for the comparison between groups of patients, if the composition of these groups is equal (e.g. the same person to person variety), and, if the sizes of these groups are big enough.

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CHAPTER 3

Immuno-histochemical detection of cardiac troponin I and cardiac troponin T after myocardial infarction in postmortem obtained myocardial tissue.

J.C.J.M. Swaanenburg and A.T.M.G. Tiebosch

Department of Pathology and Laboratory medicine, University Hospital Groningen.

Introduction

In postmortem examination of patients with sudden, probable cardiac death a diagnosis of a myocardial infarction is usually based on the finding of severe, occlusive atherosclerotic coronary artery disease. Actual detection of the histological sequelae of infarcted myocardium will develop only after significant time-lag between the onset of the myocardial infarction and death. Signs of irreversible abnormalities can be noticed by electronmicroscopy already 120 minutes after infarction (1). Using enzyme-histochemical analysis detectable loss of Lactate Dehydrogenase will be recognizable 5 hours after infarction (2).

Histologically however, the presence of neutrophils, initially in small capillaries and subsequently in the interstitium is the first reliable sign of a myocardial infarction and can not be detected in the first 6 to 12 hours after onset of the infarction (3). Nowadays no reliable histological or immunohistological technique is available which allows detection of a myocardial infarction in the first hours after onset.

Recently the presence of an increase in serum concentrations of the cardiac specific isoform of troponin T as well as troponin I early after myocardial infarction have been described (4,5).

Troponin T is a regulatory protein of the thin filaments of striated muscle tissue. It binds troponin I and troponin C to tropomyosin and transfers calcium-induced conformational changes to the thin filaments of striated muscle. Together with troponin I and troponin C it is a part of the troponin complex of muscle. Cardiac specific isoforms of troponin T and of troponin I have been reported (6,7). In addition to an increase in serum concentrations in patients with myocardial infarction an increase in serum cardiac troponin T and cardiac troponin I concentrations have also been described in patients with unstable angina (8,9) as well as in patients with other causes of myocardial damage such as patients after blunt thoracic trauma (10).

In the present study we evaluated immunohistochemically the expression of cardiac troponin T and cardiac troponin I in the myocardium of patients who died due to the complications of a myocardial infarction. More specifically, we evaluated whether in the first hours after a myocardial infarction loss of cardiac troponin T and/or cardiac troponin I expression could be detected which would assist in making a firm diagnosis of a myocardial infarction in these patients.

Patients and Methods

Post mortem cardiac tissue was obtained from 16 Caucasian patients, 13 males and 3 females, aging from 45 to 79 years of age (median 67). All patients died due to the complications of a myocardial infarction associated with occlusive coronary atherosclerosis. The infarction was located in the left ventricle; anteroseptaal in 7, posterolateral in 7 and lateral in 2 patients. The primary cause of death was related to cardiac failure in 6, rupture of the ventricular wall in 2, and (presumed) untreatable

ventricular arrhythmia's in 8 patients (3 of these presenting with sudden death).
The time between onset of myocardial infarction and death was primarily determined according to patient history and if necessary corrected according to the presence or absence of classical histological parameters. The time between onset of myocardial infarction and death was estimated to be 0-6 hours in 4 patients, 6 – 24 hours in 5 patients; 1 – 3 days in 4 patients, 3 – 7 days in 2 patients whereas 1 patient died 14 days after the onset of symptomatic myocardial infarction.
In all patients a postmortem examination was performed within 36 hours, median 12 hours. Cardiac tissue was obtained from the posterior wall of the right ventricle as well as from the interventricular septum, anterior wall, lateral wall and posterior wall of the left ventricle. In addition biopsies were taken from intercostal muscle as well as psoas muscle.
All tissue samples were snap frozen in isopentane cooled by liquid nitrogen. In addition tissue samples were fixed in 10 % neutral buffered formalin pH: 7.4 and embedded in paraffin. Frozen as well as paraffin sections were cut at 4 micron for both histological (Haematoxylin and eosin stain (H&E)) and immunohistochemical analysis.

Immunohistochemistry
Monoclonal antibodies directed against cardiac troponin T and cardiac troponin I were kindly donated by Roche™ and Abbott™ respectively. A standard immunohistochemical procedure was performed using a commercially available peroxidase labeled rabbit anti-mouse and a peroxidase labelled goat anti-rabbit antibody. (Dako™, Denmark), with a DAB (diaminobenzidine tetrahydrochloride) as a substrate. (11). Either protease pretreatment or heat induced antigen retrieval (HIAR) using a microwave oven in a Tris buffer pH: 8.0 enhanced the immunohistochemical staining in the formalin-fixed paraffin embedded sections. (12). Non-infarcted myocardium acted as an intrinsic positive control whereas striated muscle derived from the psoas and intercostal muscles served as negative controls.

Results
The duration of the myocardial infarction prior to death according to clinical data correlated with the data obtained at classical histopathology on H&E sections.
The expression of both cardiac troponin I and cardiac troponin T expression is still prominent in the first 24 hours after a myocardial infarction. No differences were observed between presumably non-infarcted and infarcted areas of the myocardium. In patients with a myocardial infarction with a duration of several days history,

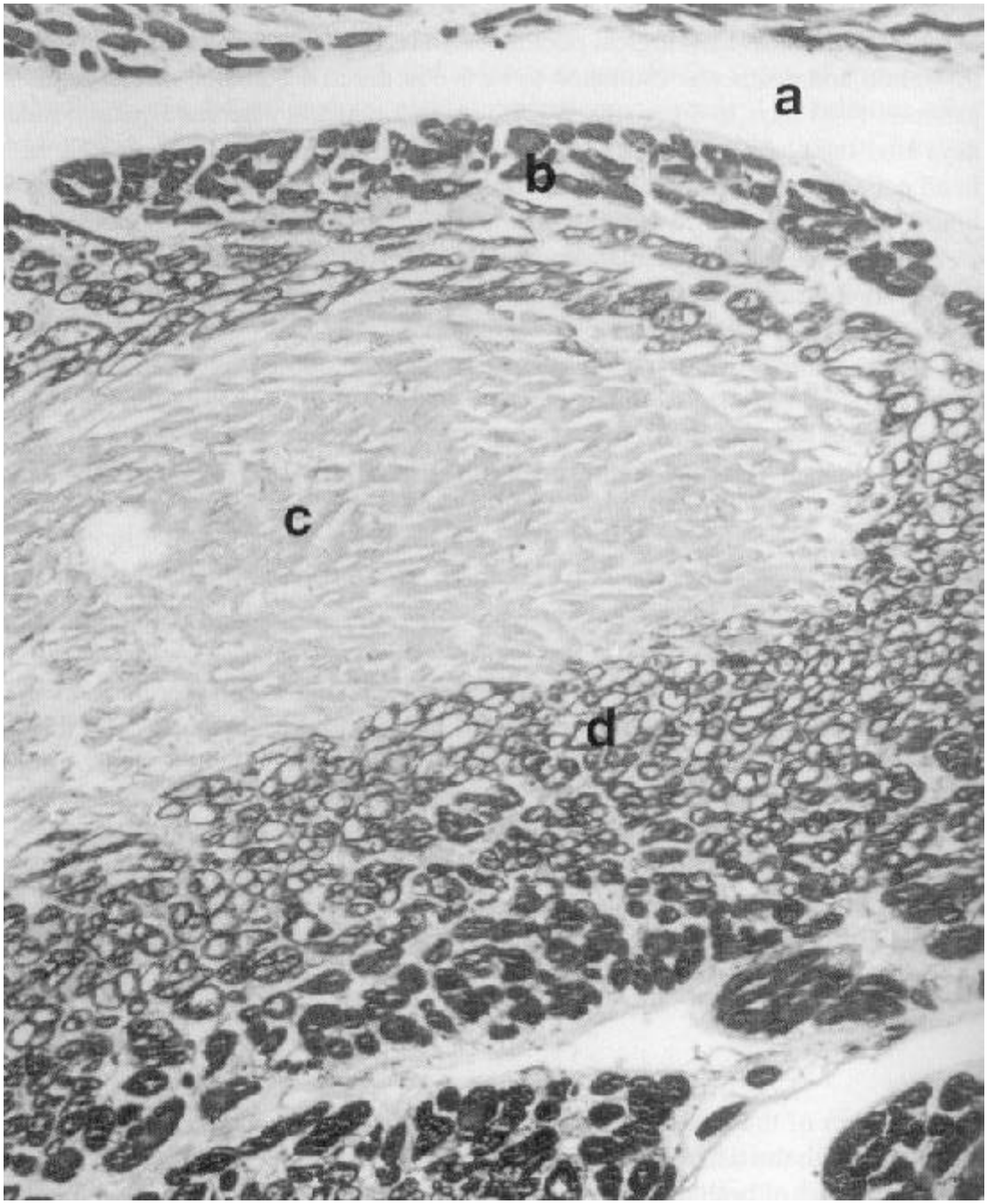

Figure 1. The loss of immunohistochemically detectable cardiac troponin I is shown in infarcted cardiomyocytes 24 hours after a myocardial infarction. Clarification: a is ventricular lumen; b is non-infarcted cardiomyocytes located subendocardially; c is infarcted cardiomyocytes; and d is non-infarcted cardiomyocytes. Original magnification x 100.

expression of both cardiac troponins in the infarcted myocardial cells is gradually diminishing until neither cardiac troponin I nor cardiac troponin T can be detected at 3 days post-myocardial infarction. In all patients both cardiac troponin I and cardiac troponin T can still be detected within the myocardial cells surrounding the infarcted area, as well as in individual surviving myocardial cells within the infarction (see figure 1).
Expression of neither cardiac troponin I nor troponin T was seen in the skeletal muscle biopsies of intercostal and psoas muscle.

Discussion

In the present study we evaluated in patients who died due to a myocardial infarction immunohistochemically the loss of intracellular cardiac troponin I and cardiac troponin T. Our results show that infarcted myocardial cells show a gradual decrease in both cardiac troponin I and cardiac troponin T expression cytoplasmatically, resulting in a total loss of detectable cardiac troponin at day 3 post-infarction. In the first 24 hours after myocardial infarction no decrease in cardiac troponin could be detected immunohistochemically.
Using lightmicroscopy on routine H&E stained sections, histological detection of infarcted cardiomyocytes by examination of cardiac tissue post-mortem is rather difficult. The earliest reliable histological sign at 8 to 12 hours post infarction, is increased eosinophilia of the cardiomyocytes with subsequent loss of cross-striations and degradation of the nucleus , which is established at 8 to 12 hours after the onset of the infarction. This is followed by the inflammatory response characterized by influx of neutrophils at 12 to 16 hours post-infarction. Subsequently, degradation of these neutrophils results in the histological appearance of "nuclear dust" which reaches its maximum at 24 hours post-infarction. In the next days, there is an increasing proliferation of fibroblasts which changes into myofibroblasts immunophenotypically. In addition, there is proliferation of capillaries, influx of lymphocytes and macrophages in the next days post-infarction leading to degradation and phagocytosis of the necrotic cardiomyocytes, finally resulting in scar tissue (3).
Consequently, early detection of ischemic or infarcted cardiomyocytes in the first hours after infarction by routine histological analysis is not reliable.
Using electronmicroscopy, infarcted cardiomyocytes can be detected as early as 2 hours after a myocardial infarction, since only small tissue samples can be examined with this technique, clinical application of electronmicroscopy is not possible.
Enzyme immunohistochemical analysis using Nitro-blue-tetrazolium (NBT) has also been used during the last 20 years. In this procedure the integrity of the cardiomyocyte cell membrane is tested through its ability to retain lactate dehydrogenase intracytoplasmatically. The NBT is not reduced in the absence of the enzyme and consequently, in the infarcted area the (blue) staining will not develop. This technique

can be used both microscopically as well as macroscopically using a cross-section of the heart (13). It has been established that, using the NBT enzyme stain, infarcted myocardial cells can be detected as early as 4 to 8 hours after the onset of the infarction (13).

Recently, studies indicate that the presence of the membrane attack complex of the complementsystem (C5b-C9) and or complement component C9 at the cell membranes of myocardial cells detected immunohistochemically is a parameter indicating myocardial cell necrosis (14,15). Upregulation of genes of the complement components can be detected as early as 1 hour after ischemia (16).

From the present study we conclude that loss of cardiac troponin I and cardiac troponin T can be immunohistochemically observed 3 days after the onset of a myocardial infarction. In the first 24 hours immunohistochemically detectable loss of cardiac troponin I as well as of cardiac troponin T are not present. Their gradual decline correlate with the increased serum levels of detectable cardiac troponin after a myocardial infarction. There is a slow increase in serum levels with a maximum at 24 hours followed by a slow decrease to normal levels in the subsequent days. This indicates that the cardiac troponins are relatively firm bound to other structures in the myocardial cells, e.g. myosins, resulting in a rather slow but longstanding release in the circulation after loss of cellular integrity.

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CHAPTER 4

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.

Joost CJM Swaanenburg[1], Milou Pentinga[2], Mike JL De Jongste[2], Ido P Kema[1], Michiel H Weening[1].

[1]Central Clinical Chemical Laboratory,
[2]Department of Cardiology, University Hospital Groningen.

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Summary
Measurement of creatine kinase (CK) isoforms enables the clinician to detect myocardial tissue damage in an early stage after myocardial infarction. According to the manufacturer's specifications, it should be possible to perform CK isoform analysis automatically using the new Cardio Rep analyser. In order to investigate the suitability of this new analyser we measured the (CK)MM1-3 and (CK)MB1 and 2 isoform patterns firstly in 30 patients with acute myocardial infarction (AMI) for whom CK-total and CKMB levels were ordered and secondly in 23 patients with chest pain suspected as having AMI (n=11) or with unstable angina pectoris (UAP) (n=12) .
The total time for analysis, including 5 min pre- and 10 min post-analyser run time, was found to be 40 minutes. For elevated MB2/MB1 ratios there is a discrepancy between the MB2/MB1 ratios determined from the densitometric scans concerning the surface and the peak height ratios. The MB2/MB1 ratios of the studied AMI patients exceeded the upper reference limits approximately 2 h after the onset of symptoms, whereas the CK-MB and CK total levels increased after about 6 h. The MB2/MB1 ratios from the patients with UAP were either below the detection limit or these patients could be discriminated from patients with AMI when low CK-MB CKtotal levels were considered in conjunction.
From our results we conclude that assessment of CK isoforms can be performed relatively simply with the new analyser within 40 minutes. However, for reliable calculation of the MB2/MB1 ratios, the curve monitoring of the MB2-MB1 densitometric scans should be improved. The CK isoforms are useful as an early marker for AMI as their reference interval is already exceeded approximately 2 h after an AMI. Moreover, CK isoform analysis might prove to be useful in discriminating at an early stage between AMI and other causes of chest pain. This could decrease the number of - patients with a false-positive diagnosis admitted to Coronary Care Units, resulting in a reduction of costs.

Introduction

Acute Myocardial Infarction (AMI) is an important cause of death in the Western World. Nevertheless, the diagnosis is not always easy to confirm. The recommendation of the World Health Organisation (1) for the diagnosis AMI states that two of the following three criteria should be fulfilled:
(1) typical anamnestic signs (chest pain >20 min, resistance to nitroglycerine);
(2) typical ECG findings (serial ECG >24 h: ST elevation 1.5 mV in precordial leads or/and ST elevation >1.0 mV in standard leads followed by T-wave inversion);
(3) increase in the serum cardiac enzymes lactate dehydrogenase (LD, EC 1.1.1.27), aspartate aminotransferase (ASAT, EC 2.6.1.1) and creatine kinase (CK, EC 2.7.3.1).
Several criteria have been proposed for an 'ideal' marker for detection of AMI (2). It should only be present in high concentration in myocardial tissue. After an AMI the marker should be released rapidly and its level should be proportional to the damage of myocardial tissue. The period following an AMI during which the marker can be demonstrated in the blood should be long enough for detection. On the other hand, the period should not be too long, so that reinfarctions might be overlooked. To date, no commercially available fulfils all these criteria.
For more than 20 years the enzyme activities of CK total and CK-MB have been used for the early detection of AMI. Early detection is important, since the prognosis of patients with AMI is

considerably improved, if therapy to restore the coronary blood flow is started within 6 h of the onset of symptoms (3-5). Early detection of myocardial damage depends on the appearance in the blood of increased levels of markers of myocardial tissue damage. The latter increase depends partly on the molecular mass of the marker. Therefore, the determination of smaller proteins in serum or in plasma is a reliable tool for the early diagnosis of AMI. Myoglobin (6,7) and heart fatty acid binding protein (8,9) are examples of such small muscle proteins. Troponin T (10-13) and Troponin I (14-17) are alternative markers. Recently glycogen isophosphorylase BB (18) and CK isoforms (19-22) have been reported as ideal markers for the early detection of AMI.

Creatine kinase consists of the isoenzymes CK-MM, CK-MB and CK-BB (23). CK-MM has three subforms (24,25): (CK-)MM1 (plasma), (CK-)MM2 (intermediate plasma) and (CK-)MM3 (tissue). The conversion of MM3 into MM2 and of MM2 into MM1 by cleaving the C-terminal amino acid lysine of each CK-M-subunit is catalyzed by plasma carboxypeptidase (26). For CK-MB only 2 subforms (27) are detectable in serum: (CK-)MB1 (plasma) and (CK-)MB2 (tissue). The conversion of MB2 into MB1 is also catalysed by plasma carboxypeptidase. MB1 is inactivated by proteolysis in the lymph (28).

Puleo et al. have shown that at 6 h after an AMI plasma CK isoforms have a diagnostic sensitivity and specificity of 92% and 96% (29), respectively. In the latter study the Rep analyser (Helena Laboratories, Beaumont, USA) was used to determine the levels of the CK isoforms. Analysis with this instrument is labour-intensive and time-consuming because of the numerous manual actions involved in loading of the sample, electrophoresis, detection and scanning of the isoform bands. Thus this procedure is not suitable for CK isoform analysis at any time, as needed. The new Cardio Rep has overcome this drawback.

We investigated the performance of the new analyser and the changes in CK-MM and CK-MB isoform patterns as a marker for the detection of myocardial tissue damage in patients in whom there was a high suspicion of AMI. In addition, we discuss the possible implications, for the patient and the hospital organisation, if the automated CK-isoform determination is introduced into everyday clinical practice.

Materials and Methods

Patients

To investigate the performance of the new analyser and to find out whether it is user-friendly, CK isoforms were measured from 30 patients with AMI for whom determinations of CK total and CKMB-activities were ordered.

Also 23 patients were studied who were admitted to our hospital with chest complaints, where there was a suspicion of unstable angina pectoris (UAP) (n=12) or AMI (n=11). All patients were treated with intravenous nitroglycerin, heparin, and oral aspirin. Out of these 23 patients, 11 patients (seven male, mean age 60 years, median 61; four female, mean age 64 years, median 62) had had an AMI and 12 patients (six male, mean age 63 years, median 62; six female, mean age 74 years, median 72) had experienced UAP.

The patient materials used for electrophoresis were serum samples. Serum was separated from the blood cells after the blood was centrifuged at 1000x g during 10 minutes. The analyses were performed within 24 h after blood sampling. Although the manufacturer recommends EGTA plasma samples, we found no significant difference between results obtained from EGTA plasma samples and those obtained from serum samples.

Methods

The MM1-MM3 and MB1 and MB2 isoform patterns were determined using the Cardio Rep. Before the analyser run can be started, it takes 5 min to apply the thin-layer 12 g./l agarose gel, to put the sera samples into the wells and to enter the sample identification.

After the analyser run has been started "sample applications" appears on the screen. During this procedure, lasting 3 min, excess buffer is removed, the applicators are washed and the samples are applied and absorbed in the gel.

The next procedure is electrophoresis at 25 °C, 900 V, 40 mA for 6 min. The gel is cooled by a thermostatically controlled peltier support to prevent melting of the agarose and denaturation of the CK enzyme.

Electrophoresis is followed by reagent application procedure. During this procedure, which takes 3 min, the standard reagent for CK analysis (30) is poured, spread and absorbed, and finally the excess of reagent is removed. The next step is incubation for 5 min at 50 °C. After incubation the gel is dried at 55 °C for 4 min.

Finally, the scan procedure begins with cooling of the gel to 20 °C, followed by determination of the optimum PMT voltage (approximately 400 V), scanning (wavelength 340 nm) of the MB1 and MB2 bands of the samples, editing and saving the scans. The scan procedure takes 4 min. The entire standard analyser run, which measures only the MB2/MB1 ratio, takes 25 min.

After the standard analyser run has finished, there is another 10-min procedure for additional scanning, editing and saving of the MM1-MM3 bands, the calculation of MM3/MM1 ratios, and inspection and printing of the Mb and the MM scans.

Thus, the total analysis time takes 40 min, and consists of 25 min for the actual analyser run time, plus 5 min before and 10 min after. Five patient samples can be analysed simultaneously with one standard sample on one gel in the same run.

The levels of CK total (reference values men < 70 U/l and women <50 U/l) and CK-MB (immune-inhibition) (reference values < 10 U/l) were determined using an Ektachem analyser (Johnson and Johnson, Beerse, Belgium).

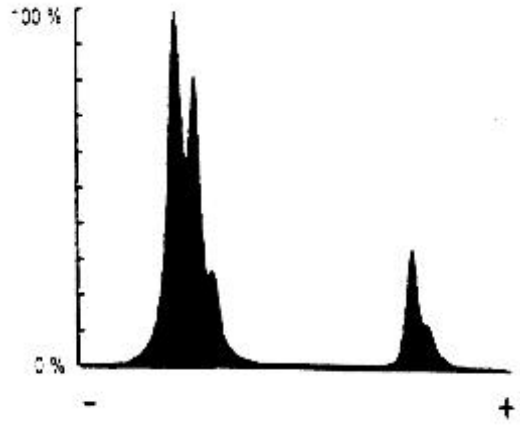

Figure 1. A typical isoform pattern after electrophoresis and scanning of the creatine kinase MM1, MM2, and MM3, and MB1and MB2 isoforms in a patient several hours after myocardial infarction. The cathode is represented by - and the anode by +. The most intense isoform band is defined as 100%.

Results

A typical pattern of the densitometric scan after CK isoforms analysis from a patient with AMI is shown in figure 1: between the cathode and the anode the MM3, MM2, MM1 and the MB2, MB1 peaks are shown. As already described in the Patients and methods section, the total running time of the determination is about 40 min.

In Figure 2A the MB2/MB1 ratios obtained from 30 AMI patients are plotted calculated from the MB2/MB1 surface ratios and from the MB2/MB1 peak height ratios. The surface ratio was calculated by the analyser from the densitometric scan.

A **B**

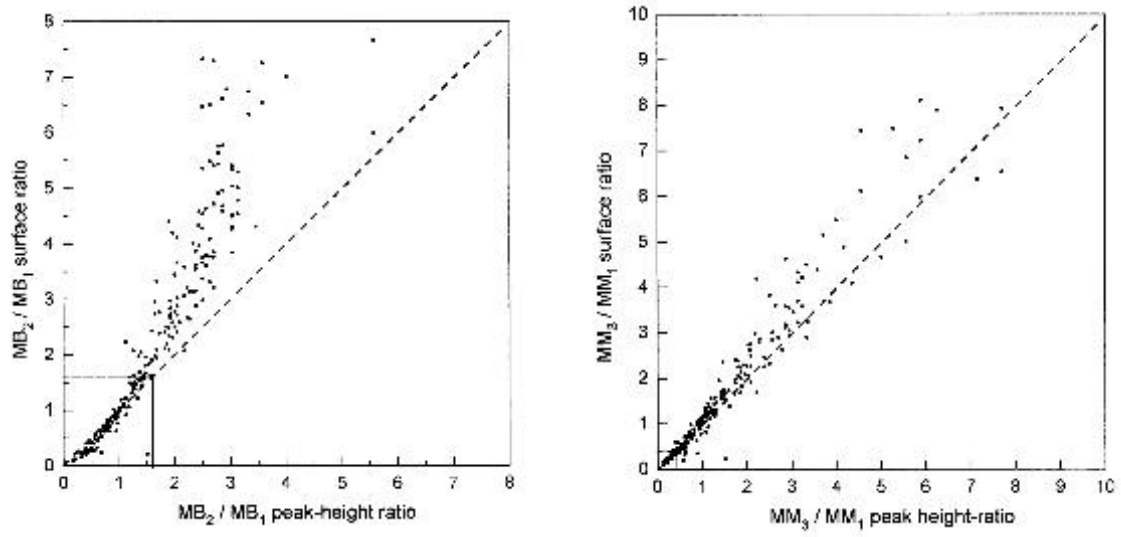

*Figure 2.*The relations between (A) the MB2/MB1surface ratios and the MB2/MB1 peak height ratios, and (B) between the MM3/MM1 surface ratios and the MM3/MM1 peak height ratios. The surface ratios are generated from the scans after electrophoresis by the standard software of the analyser. The peak height ratios are calculated manually from the scans visualised on the monitor of the analyser. For the MB2/MB1 ratio the cut off value line is drawn at 1.6 and for the MM3/MM1 ratio at 0.4. The results are derived from 30 patients with acute myocardial infarction (AMI).

The peak height ratio was manually determined from the densitometric scan visualised on the screen of the monitor. It can be seen from Figure 2A that up to the cut-off value of 1.6 there is no discrepancy between these differently calculated MB2/MB1 ratios. Above the 1.6 cut-off value the surface ratios are higher than the peak height ratios. The MM3/MM1 surface and peak height ratios are plotted in Figure 2B, showing that there is a good correlation between the MM3/MM1 surface and peak height ratios.

The MB2/MB1 surface ratios have also been measured in 23 patients who were hospitalised for typical chest pain and ECG changes. In Figure 3 the MB2/MB1 surface ratios and the CK-MB levels (still routinely determined in our hospital) plotted against the time after infarction for the first 24 hours after the infarction, are represented for 11 patients with AMI. The MB2/MB1 ratios are above the cut-off value of 1.6 (12) at approximately 2 h after the infarction. At that time the CK total and the CK-MB levels are still within reference ranges for AMI as well as UAP patients. At admission to the hospital the CK total and the CK-MB levels of the UAP patients (mean activities 45, 3 U/l respectively) are comparable with those of the AMI patients (60, 4 U/l, respectively). The CK-MB activities are 'positive' (i.e. above the upper limit of the reference interval) approximately 6 h after the onset of the anginal complaints. At that time the MB2/MB1 ratios have maximal values,

whereas the CK-MB activities reach these maximal values at about 15 h after the onset of the anginal complaints.
Out of eleven patients with AMI, eight had maximal MB2/MB1 surface ratios between 4 and 6, one had a ratio lower than 4 (i.e. 2.6) and two had ratios higher than 8 (i.e. 8.1, 9.3). The MB2/MB1 ratios for UAP patients are lower. For seven out of 12 patients with UAP it proved impossible to determine the MB2/MB1 ratios, because the MB2 and MB1 levels were below the detection level. For five patients the maximal MB2/MB1 ratios were 1.1, 1.7, 1.8, 1.9, 2.9 respectively. None of these patients had maximal CK activities above 100 U/l or maximal CKMB activities above the upper limit of reference interval.

Discussion

CK isoform analysis with the new Cardio Rep analyser is relatively simple as a result of the automated electrophoresis procedure. The total running time of the analysis is approximately 40 min consisting of 5, 25 and 10 min pre-run, actual run and post-run time. This is comparable to the assay time of most of the other cardiac markers. In these experiments the analyser proved to be user-friendly and easy to operate.
However, the analyser would become more user-friendly, and the post-run time can be shortened by some minutes, if the MM1-MM3 bands were scanned, edited and saved together with the MB1 and MB2 bands in the same standard program.
Furthermore, the CK isoform assessment would become more reliable, if the curve monitoring of

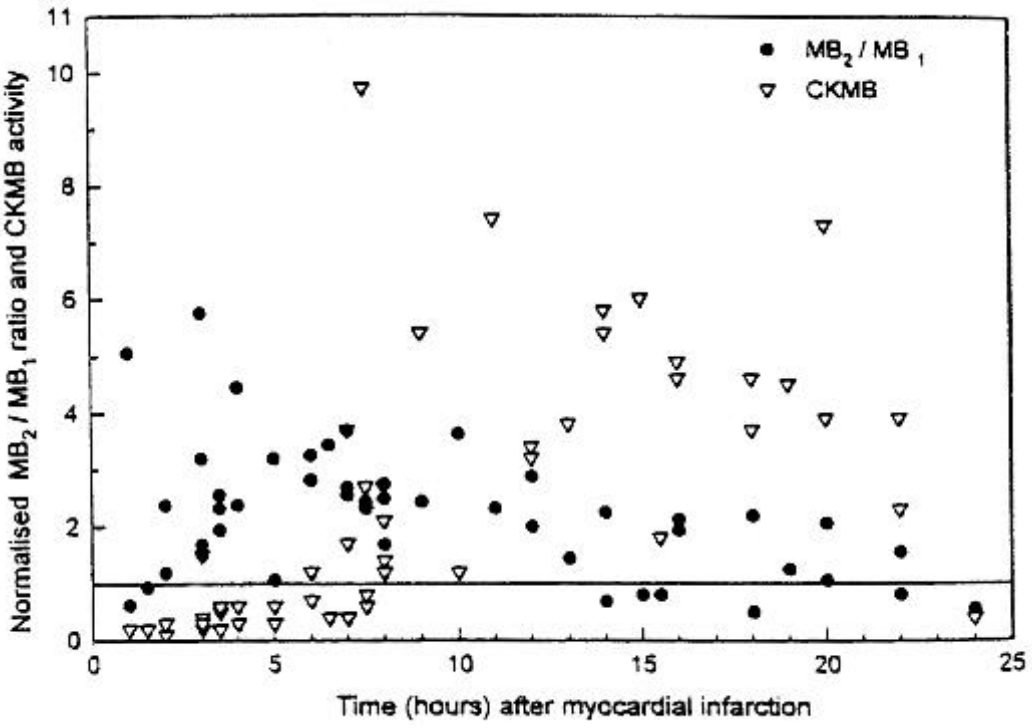

Figure 3. Normalised MB2/MB1 ratios (!) and normalised CKMB levels (L) versus the time after infarction, for 11 patients with AMI for the first 24 h after infarction. The normalised MB2/MB1 ratios and the normalised CKMB levels are multiples related to the cut off values of the MB2/MB1 ratio (1.6) and CKMB level (10 U/L).

the MB1 and MB2 peaks was improved. From the data presented in Figure 2 it is concluded that the curve monitoring of the CK isoforms must be improved in order to measure the MB2/MB1 isoforms ratios more accurately. It is difficult to determine the MB2/MB1 ratio accurately especially when the CK levels are elevated and the densitometric curves partly overlap each other,.

The discrepancy between the MB2/MB1 surface ratios and the MB2/MB1 peak height ratios is caused by the insufficient separation of the isoform bands by the densitometer. The separation of the MB1 and MB2 bands can be enhanced either by improving the electrophoretic separation or by improvement of the curve monitoring software of the densitometer. As increasing the time of electrophoresis requires more gel, this is not a possible solution for the problem of the overlapping MB1 and MB2 bands because of the volume available for the gel in the analyser. Thus, the only solution is to improve the curve monitoring software of the densitometer. With the present analyser software the MB2/MB1 ratios can only be used for the qualitative detection of myocardial tissue damage.

If the MB2/MB1 ratio is above 1.6, we recommend the additional use of the MM3/MM1 ratio. - The MM3/MM1 ratios are more reliable, because the MM3 and MM1 bands cannot overlap each other as they are separated by the MM2 fraction. Moreover, we have found that this ratio is a more reliable indicator of the time after infarction in a single blood sample. The MM3/MM1 ratio cannot be used as a primary criterion for myocardial tissue damage, because skeletal muscle accounts for 97% of MM. For this reason MM has poorer clinical specificity for myocardial tissue damage compared with the MB isoforms.

From our comparison of CK isoforms with CK-MB levels, we conclude that the MB2/MB1 isoform ratios show an earlier increase after AMI than the CK-MB levels still routinely used in our hospital. To provide a reliable diagnosis after an AMI the CK-MB level should show a manifold increase to exceed the reference interval. However, if the diagnosis is based on a change in the ratio of the MB isoforms, a release of small amounts of the MB2 isoform into the plasma leads to a significant change in the MB2/MB1 ratio.

Recently, equivalent early sensitivities of myoglobin, creatine kinase MB mass, creatine kinase isoform ratios and cardiac troponins T and I have been reported for AMI (31). In this study Mair et al. Used the Rep for the measurement of CK isoforms. They reported an increase in the MB2/MB1 ratio 3 h after infarction. In our study we found this time to be approximately 2 h. This might possibly be explained by modifications to the new analyser. As a result of these modifications, the gel is no longer exposed to daylight during anaslysis so that weaker MB1 and MB2 bands can be detected.

There are two essential differences between the information from the CK isoform analysis and that from the other commercially available cardiac markers. First, the CK isoform analysis reports ratios, giving relative rather than absolute results. Second, only the CK isoform measurement offers the possibility of indicating the time between infarction and blood sampling in a single blood sample with a single measurement.

From our experience, we suggest the following use of the Cardio Rep. Usually, patients are diagnosed on the basis of symptoms and ECG changes; laboratory test results are more frequently used for confirmation and quantification of the AMI. The CK isoform analysis can be used in those patients where the diagnosis is doubtful, since the MB2/MB1 ratio is already raised 2 h after infarction. As this technique indicates the time since infarction using a single analysis in a single blood sample, another reason for CK isoform analysis arises when it is not clear how much time has passed since the start of the episode.

The introduction of routine automated CK isoform analysis will increase laboratory costs. On the other hand, a 1-day patient stay in the CCU is much more expensive than the measurement of CK-isoforms. The savings as a consequence of more efficient patient treatment are impressive, considering that in the USA the annual costs of caring for 'non-myocardial infarction' in CCU's are $6 -$13 billion (32,33).
Further studies should be carried out to determine the clinical relevance, for differentiation between AMI and UAP patients of the changes over time in the CK-MB and CK-MM isoform ratios, and to determine the cost-benefit consequences. in time for differentiation between AMI- and UAP-patients and to determine the consequences for the cost- benefit relation. The kinetics of CK isoform alterations (i.e. maximal value of the MB2/MB1 ratios, and the time at which these values are reached) may indicate the time required for patient recovery and patient stay in CCU's. To date, it is not clear whether there are associations between CK isoform kinetics, infarct size and the prognosis for the patient.
In conclusion, with regard to the automated CK isoform analysis using the Cardio Rep, the following is noted: it takes about 40 min in total, including 5 min pre- and 10 min post-analyser run time to automatically measure the CK MM1-MM3 and MB1 and MB2 isoforms, and the analysis is relatively simple; however, the curve monitoring of the MB2-MB1 scan should be improved for reliable isoform ratio calculations; the CK isoforms have the potential to discriminate in an early stage between AMI and other causes of chest pain.
As a consequence of the introduction of CK isoform analysis in daily practice, patients with an AMI can be diagnosed earlier. Moreover, the number of patients admitted to CCU's with a false-positive diagnosis may decrease, resulting in a reduction of costs.

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CHAPTER 5

Analytical aspects of the automated CKMB1,2 and CKMM1,2,3 isoform determination and its relation to other biochemical markers.

Joost CJM Swaanenburg[1], Mike JL De Jongste[2], Marcel Volmer[1], Ido P Kema[1].

[1]Central Clinical Chemical Laboratory,
[2]Department of Cardiology, University Hospital Groningen.

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Summary

The automated (CK)MB1,2/MM1,2,3-isoform measurement, based on electrophoresis, has been simplified to the point that it has become possible to perform this analysis on a 24 h routine basis. We studied analytical aspects of this analysis and its clinical relevance in relation to other biochemical markers (CK total, CKMB activity, CKMB mass, myoglobin, troponin I and troponin T) in patients with acute myocardial infarction (AMI), patients with unstable angina pectoris (UAP), and healthy donors. Furthermore, the additional significance of the analysis was evaluated in patients with clinically unexpected, raised CKMB/CK total activities. The storage of serum at 4EC does not influence the MB2/MB1-ratios, whereas storage at 20EC changes them significantly. MM3/MM1 and normal MB2/MB1 ratios show lower coefficients of variation (3%) than increased MB2/MB1 ratios (9%). Between 2 and 30 h after myocardial tissue damage, AMI patients showed a characteristic change in CK isoform patterns. At a mean time of 3.6 h after the onset of symptoms we found raised MB2/MB1 ratios in 94% of these patients. With the information of the CK-isoform analysis, unexpected abnormal CK activities could be explained by CK-macro enzymes (Ig-bound and mitochondrial), insufficient CK-clearance capacity, enzyme activities 4 h after (re-)infarction, and raised CK-activity 15 h after skeletal muscle damage. We conclude that the CK-isoforms are relatively simply to assess; they are adequate tools with which to indicate the CK kinetics over a period lasting between 2 and 30 h after tissue damage with a single blood sample and a single analysis; the CK-isoform analysis has additional value in explaining inappropriate CKMB/CK total activities, and the MB2/MB1-ratios show to be one of the best early parameters for discriminating patients with AMI on admission to the hospital.

Introduction

The enzyme creatine kinase (CK) is composed of two subunits (M and B), and consists of 3 iso-enzymes (CK)-MM, (CK)-MB and (CK)-BB (1). The existence of CK-isoforms was initially reported in 1977 (2). The difference in structure between CK-isoforms is determined by the presence of the amino acid lysine at the C-terminal position of the CK-M-subunit. The iso-enzyme MM (3) consists of three isoforms (or 'subforms') MM3 (tissue form), MM2 (intermediate form) and MM1 (plasma form). Two (CK)-MB-isoforms (4) are recognisable in the blood circulation: MB2 (tissue form) and MB1 (plasma form). Loss of the amino acid lysine from the CK-M subunits (in the blood circulation catalysed by the enzyme carboxypeptidase) is associated with a minor change in molecular weight and no change in catalytic activity. However, this loss does induce a unique isoelectric point characteristic of each CK-isoform. The CK-isoform assay methodologies based on chromatofocusing (3) isoelectric focusing (5), and immunoblot techniques (6) are not suitable for clinical use, because of the tedious and time- consuming procedure. With the introduction of the Cardio Rep™ analyser, the CK isoform analysis has been automated in such a way that this analysis has become more accessible as a stat analysis for 24 h per day, 7 days a week, and could potentially be the diagnostic application of MM and MB isoforms on a routine basis (7).

The established enzymatic criteria for acute myocardial infarction (AMI) by measurements of plasma CK-MB activities every 4-6 h, has long been regarded as the most specific, sensitive, and cost-effective means (8,9) by which to diagnose myocardial tissue damage. Because the prognosis of the patient with AMI is related to the time elapsed between onset of symptoms and start of

therapy, much effort has been made to detect (new) early markers of myocardial tissue damage. Small protein molecules such as myoglobin (mol mass 17 kDa) (10,11), Troponin T (mol mass 33 kDa) (12,13), troponin I (mol mass 23.5 kDa) (14,15) and heart Fatty Acid Binding Protein (mol mass 15 kDa) (15) are such new markers. Glycogen isophosphorylase BB (17) and the CK isoforms (18,19) were also introduced as early markers for myocardial tissue damage.
We investigated analytical and clinical aspects of the automated CK isoform analysis on a routine basis by measuring CK isoform patterns from patients with AMI, patients with unstable angina pectoris (UAP), healthy donors, and patients with inappropriate CKMB/CK total activities (CKMB/CK total ratios >20% or clinically unexplainable raised CK total activities). The parameters CK total, CK-MB activity, CKMB mass, myoglobin, troponin I and troponin T were also analysed from the patients with AMI, those with UAP, and the healthy donors in order to determine the relevance of the CK isoforms in relation to these other markers.

Patients and methods

Patients

During a 3-month period, blood samples were collected at regular intervals from 39 consecutive patients (27 male [mean age 61 , range 42-78 years], 12 female [mean age 63, range 48-83 years]) who suffered from an AMI in accordance with the WHO criteria (abnormal ECG, clinical signs, increased cardiac markers) [20]. The levels at which the cardiac markers were considered to be increased are given in Table 1. A total of 192 CK isoform analyses were performed from these patients in order to measure the MB2/MB1 and MM3/MM1 ratios in time after AMI. During a successive period of 2 months, CK isoforms were analysed as well as CK total, CKMB activity, CKMB mass, myoglobin, troponin I and troponin T from another 25 AMI-patients on admission to the hospital (19 male [age 57, range 35-85 years], 6 female [age 59, range 23-80 years]) from 54 UAP patients at admission to the hospital (39 male [age 62, range 35-85 years], 15 female [age 66, range 53-82 years]), and from 69 healthy donors (45 male [age 38, range 30-45 years], 24 female [age 37 , range 31-44 years]). The interval between the onset of symptoms and admission to hospital was calculated from information given by the patient. This information was retrospectively validated by the time- related enzyme activity changes in blood after AMI.
Furthermore, the CK isoforms were measured in sera from patients (age 55, range 36-71 years) with clinically unexplainable, raised CK-MB/CK total activities.

Methods

Blood was centrifuged for 10 minutes at 1000 g and 20EC in order to separate the blood cells from the serum. The stability of CK isoforms was tested by analysing 15 patient samples immediately after blood sampling and 24 h after storage of the sera at 4EC and at 20EC.
The CK-MM1,2,3/CK-MB1,2 isoform patterns were measured by electrophoresis on agarose gel using the Cardio Rep™ (Helena Laboratories, Beaumont, USA). After electrophoresis at 25E C, 900 V, and 40 mA, the "reagent application" procedure, and determination of the optimum PMT voltage for scanning, the MB1 and MB2 bands are scanned. Modifying the standard procedure the operator must additionally edit, inspect and save the MM1,2,3 bands.
The MM3/MM1 and MB2/MB1 ratios are calculated by the analyser from the area under the curves of the densitometric scans. Five patient samples can be analysed simultaneously with one

standard sample on one gel in the same run. The whole procedure takes about 40 min [21].
The activities of CK total and CK-MB (based on immune inhibition) were measured on a Vitros 750C analyser (Johnson and Johnson, Beerse, Belgium).
Myoglobin and troponin I were measured on an Access analyser (Sanofi Diagnostics Pasteur BV, Vlaardingen, The Netherlands). After incubation of patient serum with assay specific reagents, and separation of the bound and unbound fractions, a dioxetane chemiluminescent substrate provides a long-lasting luminescent signal which is proportional to the analyte concentration [22].
CKMB mass was measured on a MAGIA 7000 analyser (Merck, Amsterdam, The Netherlands). The principle of separation after the one step competition reaction of the enzyme-immunoassay is based on magnetisation of "paramagnetic micro particles". After this procedure substrate is added for the chemical reaction catalysed by alkaline phosphatase [23].
Troponin T was measured with an Elecsys 2010 analyser (Boehringer Mannheim, Almere, The Netherlands). After incubation of serum with a biotinylated monoclonal troponin T- antibody and the addition of streptavidin coated micro particles, the complex becomes magnetically bound to the surface of the electrode. A photomultiplier measures a chemiluminescent emission after the application of a voltage to this electrode [24].

Statistics
A paired t-test was used to compare the results of the stability study.
The within run reproducibility was tested with sera from 2 patients. The samples with various levels for the MB2/MB1- and MM3/MM1-ratios were analysed five times each. One level was within the reference limits (mean MB2/MB1 0.85; mean MM3/MM1 0.33); the other level was beyond the reference limits (mean MB2/MB1 3.63; mean MM3/MM1 2.82). The run to run reproducibility was tested with a commercial control sample (Helena product nr 3320) at one level (MB2/MB1 ratio 1.13) for 41 runs in 23 days. This control serum contains no detectable MM3 fractions. Therefore, it was not possible to determine the run to run reproducibility of the MM3/MM1 ratio.
The area under the curve, the sensitivity, the specificity with the corresponding 95% confidence intervals, and the cut-off values based on the most optimal point of the ROC-curves were calculated from the ROC curves (ROC 2.0, University Hospital Groningen).

Results

Analytical aspects. The storage of sample material during 24 hours at 4 EC did not influence the MB2/MB1 ratio (p=0.768). Whereas, storage at 20 EC showed a significant change of the MB2/MB1 ratio (p=0.003).
The reproducibility of the normal MB2/MB1 ratio showed a coefficient of variation (c.v.) of 3.5%, the c.v. of the MB2/MB1 level beyond the reference limits was 9.9%. The c.v. of the normal MM3/MM1 ratio was 3.0%, and the c.v. of the raised MM3/MM1 ratio was 2.8%. The run to run reproducibility of the MB2/MB1-ratios showed a c.v. of 8.8%.
Clinical aspects. Figure 1 shows the time related changes in CK-isoform patterns after myocardial infarction. The basic tissue turnover characteristic of the CK-isoform pattern is depicted in fig 1a. The top of the MB2-peak equals the MB1 peak; the MM3 peak is lower than the MM2- and MM1-peak. The first changes are observed approximately 2 hours after the myocardial tissue damage (fig 1b). The MB2 peak exceeds the MB1 peak and the MM3 peak exceeds the MM2-

and MM1-peak. By conversion of MB2 into MB1 as well as of MM3 into MM2 and of MM2 into MM1, the isoform-peaks are shifting in time from MB2 to MB1 and from MM3 through MM2 to MM1 (fig 1b - fig 1h). Until 18 hours after tissue damage, the MB2 peak exceeds the MB1 peak. Between 18 and 30 hours after myocardial damage MB2 is lower than MB1 and after 30 hours MB2 is comparable to MB1. From the MM-isoforms, MM3 is the most intense up to 9 hours after tissue damage. Between 9 and 20 hours MM2 is the most intense and after 20 hours MM1. After 30 hours the CK-isoform pattern has returned to that of basic tissue turnover (compare fig 1a with fig 1h).

In figures 2a-2b the patterns of the median MB2/MB1- and MM3/MM1-ratios with the 95% confidence intervals are shown up to 75 hours after AMI. About 2 hours after AMI the MB2/MB1 ratio starts to increase, the maximum is reached after approximately 8 hours and normalises after about 30 hours. The MM3/MM1 ratio also starts to raise about 2 hours after tissue damage, the maximum is reached after approximately 14 hours and normalises after about 30 hours.

The mean and range of time of admission to the hospital of the AMI patients are 3.6 hours and 2 - 6 hours, respectively. The medians and ranges of the CK-total, CKMB-activity, CKMB-mass, the MB2/MB1- and the MM3/MM1-ratios, myoglobin, Troponin I, and Troponin T from the AMI- and the UAP-patients at admission to the hospital and the donors are shown in table 1. Table 1 shows that the median results of

Table 1. The medians and ranges of the test results of the indicated parameters from healthy donors, and at time of admission to the hospital from patients with AMI and patients with UAP.

parameter	unit	upper reference limit	donors median	donors range	AMI patients median	AMI patients range	UAP patients median	UAP patients range
CK-total	U/L	70	37	12-159	150	37-1256	37	10-116
CKMB-act	U/L	10	2	1-4	13	2-58	3	1-9
CKMB-mass	µg/L	5.00	1.15	0.26-5.10	29.9	2.68-176.5	2.23	0.80-15.0
MB2/MB1		1.50	n.d.	n.d.-1.38	2.78	1.35-4.76	1.16	n.d.-2.63
MM3/MM1		0.60	0.18	0.04-1.80	2.35	0.72-5.03	0.70	0.38-2.44
myoglobin	µg/L	70	25	6-115	614	71-9840	32	20-323
Troponin I	µg/L	0.1	0.00	0.00-0.21	0.11	0.00-8.66	0.00	0.00-0.15
Troponin T	µg/L	0.1	0.00	0.00-0.03	0.14	0.00-5.95	0.01	0.00-0.42

n.d.: not detectable
AMI: acute myocardial infarction
UAP: unstable angina pectoris.

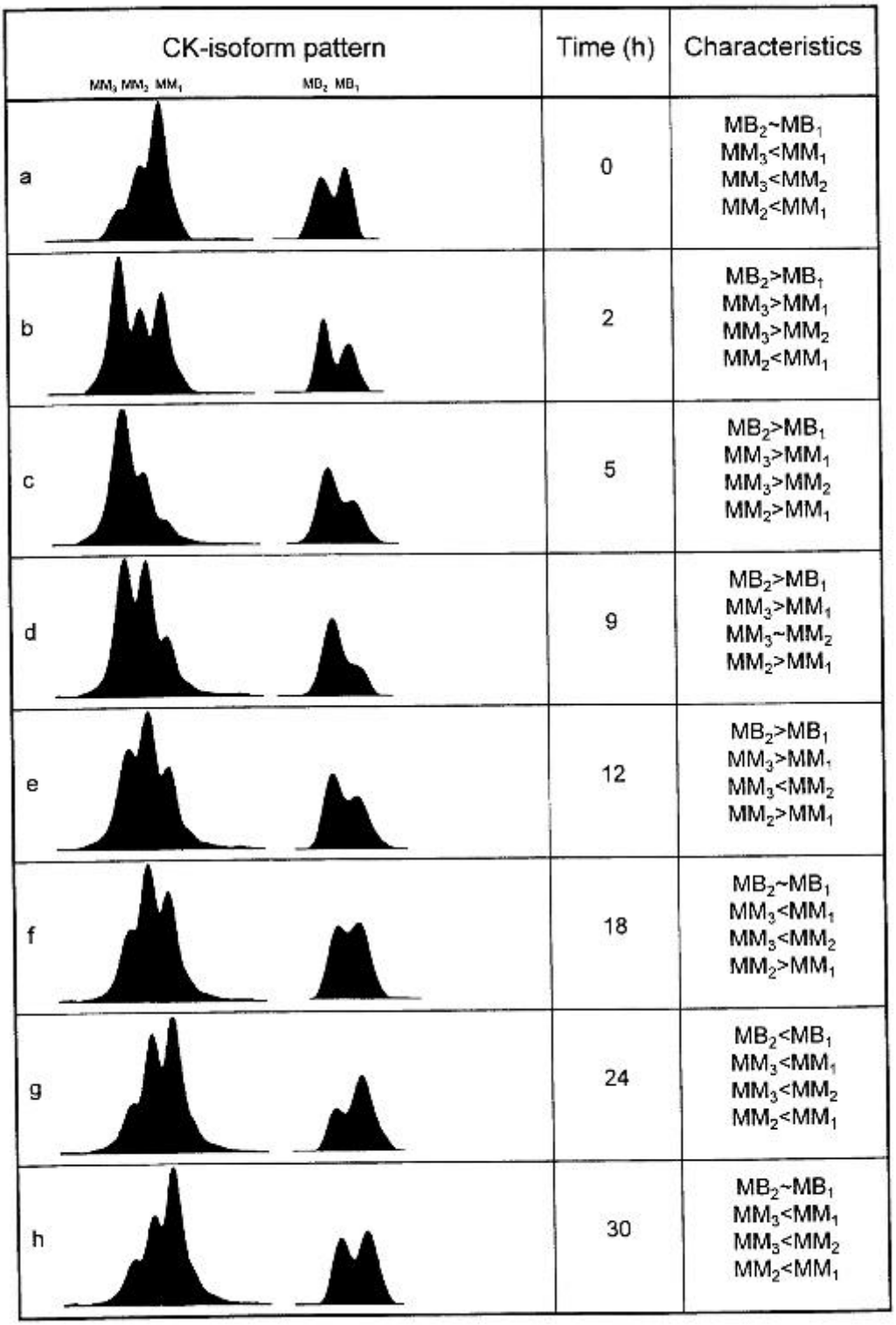
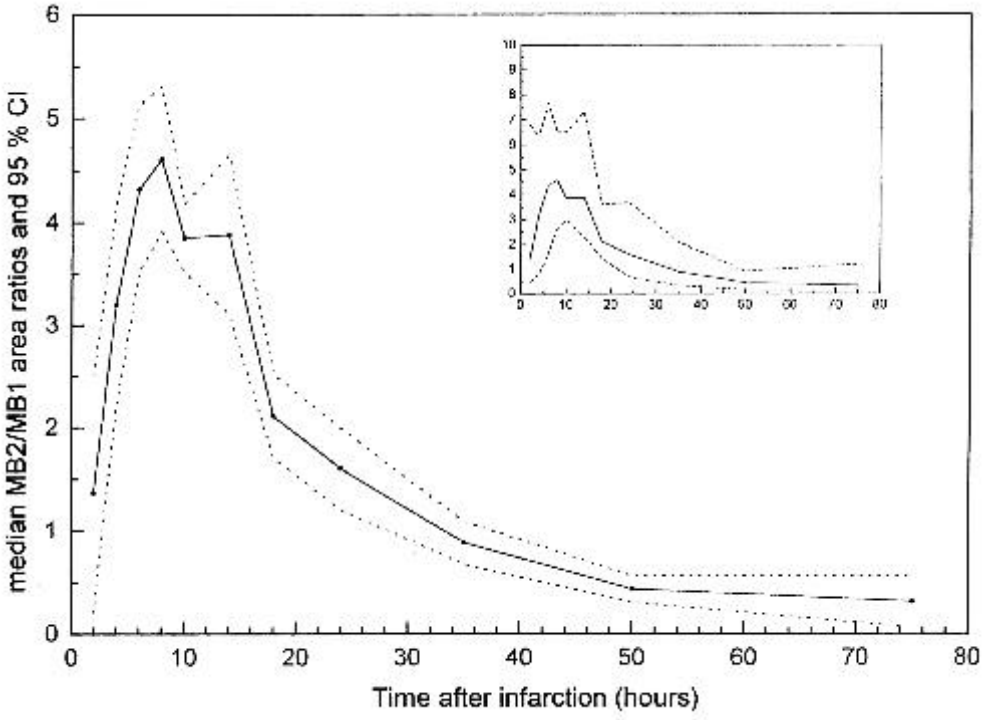


Figure 1. Characteristic patterns of time-related changes in CKMM1,2,3/CKMB1,2 isoforms after myocardial tissue damage. From left to right the densitometric scans after electrophoretic separation of the (CK)MM3-, MM2-, MM1-, and the (CK)MB2-, and MB1 bands are shown. Time indicates the interval in hours after tissue damage. Time 0 is characteristic for basic tissue turnover.

A



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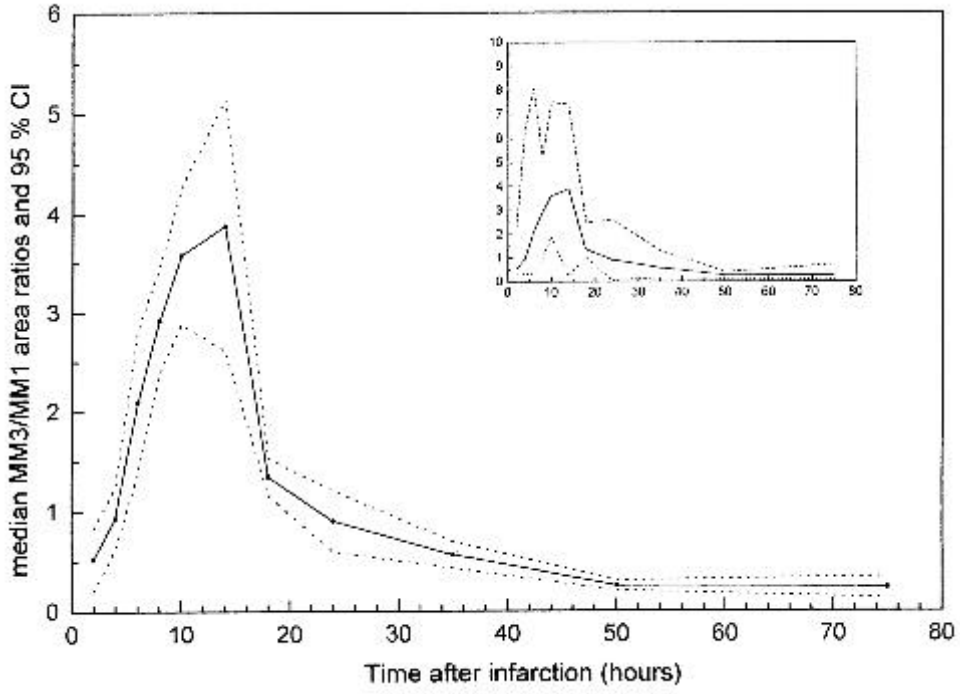

Figure 2A, B. The patterns of the median MB2/MB1 and MM3/MM1 ratios with the 95% confidence intervals (CI) up to 75 h after myocardial tissue damage. In the right upper corner the same median MB2/MB1 and MM3/MM1 ratios are shown with the 95% CI of all observed values.

all parameters from the donors are within the reference limits. The MB2/MB1-ratios could not be calculated from 64 out of 69 donors because the CK-MB activities were so low that the isoform bands on the electrophoresis gel could not be detected by the densitometer. The MB2/MB1-ratios were detectable, if the CKMB activities were more than 3 U/l. If the MB2 and MB1 bands could not be detected, the result was interpreted as negative. Therefore, for mathematical purposes (36), these ratios were assumed to be beyond the lowest measured patient ratio (0.50).
The medians of all parameters from the AMI-patients at time of admission to the hospital exceed the upper reference limits. From the UAP-patients the MM3/MM1 ratio is the only parameter, of which the median exceeds the upper limit of reference range.
In table 2 the characteristics for all 8 biochemical parameters of the ROC curves related to the three groups of individuals are presented. It concerns the areas under the curves, the sensitivities and the specificities (all 3 with the 95% confidence intervals), and the corresponding cut off values. Table 2 shows, that the areas under the curves of the various parameters except for Troponin I and Troponin T are hardly different. The MB2/MB1 ratios has the highest value for the donors vs. the AMI-patients and the second highest for the UAP vs. AMI-patients.
Figure 3 shows 5 CK-isoform patterns (patient 1-5) from patients with inappropriate CKMB/CK-total activities.
The CK-isoform pattern from patient 1 shows an extra peak, located between the MM1- and MB2- peaks; this extra peak approaches more the MM1- than the MB2-peak. For patient 2 the CK-isoform pattern shows an extra peak close to the MM3-peak in the direction of the cathode. The CK-isoform pattern of patient 3 with a permanently abnormal CK-total activity shows also consistently a very intense MM1-peak. The CK-isoform pattern from patient 4 shows that MM3>MM2>MM1 and MB2>MB1. The CK-isoform patterns from patient 5 show firstly a basic tissue turnover pattern, secondly a pattern approximately 15 hours after tissue damage and thirdly a basic tissue turnover pattern with an intense MM1 peak.

Discussion

Analytical aspects. The in vitro stability of CK-isoforms at 4EC storage conditions during 24 hours show reliable results, which is in contrast to the results for storage at 20EC. These findings are confirmed by other investigators (37).
The within run reproducibility show acceptable results for the MB2/MB1-ratios within the reference limits and for the MM3/MM1-ratios within and outside the reference limits (c.v.'s less than 3.5%). Raised MB2/MB1-ratios show less reproducible results (c.v. up to 10%). This is caused, as has been reported earlier (32), by the inappropriate separation of the MB2 and MB1 bands by the densitometer, especially with high CKMB-activities.
Clinical aspects. From fig 2a-2b it may be concluded that the MB2/MB1-ratios maximises earlier in time than the MM3/MM1-ratios. This may be explained by the **Table 2**. ROC characteristics from the parameters CK-total, CKMB-activity, (CK)MB mass, (CK)MB2/MB1-ratio, (CK)MM3/MM1-ratio, myoglobin, Troponin I and Troponin T.

UAP-patients vs. AMI-patients 2-6 hours after the start of the complaints

Parameter	area under curve	c.o.v.	sensitivity	specificity

CK-total	0.95	(0.89-1.00)	53	0.91	(0.76-0.99)	0.85	(0.74-0.94)
CKMB-act	0.92	(0.80-1.00)	6	0.86	(0.64-0.99)	0.87	(0.76-0.94)
CKMB-mass	0.95	(0.89-1.00)	5.77	0.93	(0.68-1.00)	0.84	(0.71-0.92)
MB2/MB1	0.96	(0.91-1.00)	1.96	0.95	(0.74-1.00)	0.88	(0.71-0.97)
MM3/MM1	0.92	(0.85-1.00)	1.09	0.89	(0.65-0.99)	0.91	(0.78-0.97)
myoglobin	0.98	(0.95-1.00)	255	0.93	(0.68-1.00)	0.94	(0.84-0.99)
Troponin I	0.90	(0.80-1.00)	0.11	0.53	(0.28-0.77)	0.98	(0.91-1.00)
Troponin T	0.78	(0.60-0.96)	0.02	0.80	(0.52-0.96)	0.69	(0.54-0.81)

donors vs. AMI-patients 2-6 hours after the start of the complaints

Parameter	area under curve		c.o.v.	sensitivity		specificity	
CK-total	0.93	(0.87-0.99)	49	0.95	(0.76-0.99)	0.77	(0.65-0.86)
CKMB-act	0.95	(0.87-1.00)	5	0.91	(0.70-0.99)	1.00	(0.96-1.00)
CKMB-mass	0.97	(0.95-1.00)	5.77	0.93	(0.68-1.00)	0.99	(0.94-1.00)
MB2/MB1	1.00	(1.00-1.00)	1.35	1.00	(0.85-1.00)	1.00	(0.96-1.00)
MM3/MM1	0.98	(0.96-1.00)	0.72	1.00	(0.85-1.00)	0.94	(0.85-0.98)
myoglobin	0.98	(0.96-1.00)	71	1.00	(0.82-1.00)	0.95	(0.90-0.99)
Troponin I	0.76	(0.64-0.87)	0.02	0.71	(0.44-0.90)	0.93	(0.84-0.98)
Troponin T	0.89	(0.76-1.00)	0.01	0.87	(0.60-0.98)	0.99	(0.92-1.00)

UAP: Unstable angina pectoris; AMI: acute myocardial infarction; c.o.v.: cut-off value; units: CK-total and CKMB-activity U/l; CKMB-mass, myoglobin, Troponin I and Troponin T µg/l; values between parentheses are 95% confidence intervals.

kinetics of the MB and MM-isoforms. MB2 is directly converted into MB1, whereas MM3 is converted into MM1 via MM2. Therefore, there is more time for the MM3/MM1-ratio to raise. Approximately 30 h after myocardial tissue damage the MM- and MB-isoform patterns have returned to basic tissue turnover.

Regarding the test results of CK-total, CKMB-activity, CKMB-mass, MB2/MB1- and MM3/-MM1-ratios, myoglobin, Troponin I and Troponin T, the MB2/MB1-ratio is at

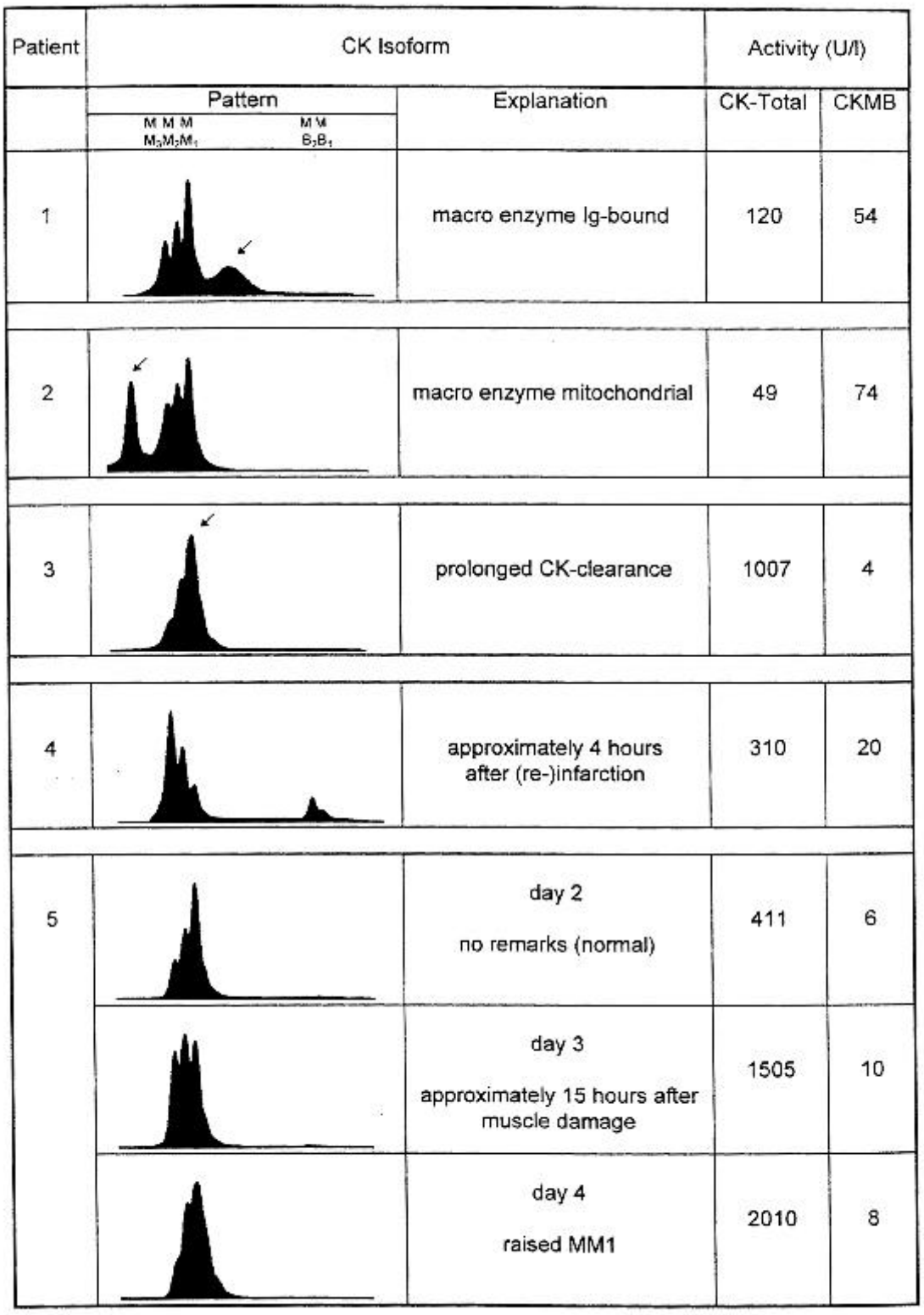

Figure 3. Examples of CK isoform patterns from patients with different causes for unreliable CK-MB/CK total activities. The (CK)MM3, MM2, and MM1 peaks are shown from left to right. The (CK)MB2 and MB1 peaks are only detectable for patient 4. The arrows in the CK isoform patterns for patients 1-3 indicate the disturbances and relate to the explanation column.

time of admission to the hospital one of the best of the examined parameters to discriminate AMI-patients from UAP-patients and healthy donors. This is supported by recently reported findings (38). The ROC-curves for the UAP-patients vs. the donors are not presented, since the areas under the ROC-curves are not significant. This implicates, that there are no reliable cut-off values to discriminate between these
populations. The parameter with the highest area under the curve (0.89) is the MM3/MM1-ratio, a parameter more representative for skeletal muscle than for myocardial tissue damage. Although Troponin I and Troponin T are more specific for myocardial tissue damage, they turn out to be less sensitive at time of admission to the hospital than the CK-isoform ratios, because these ratios are earlier raised in circulation after myocardial tissue damage.
Laurino et al. (37) reported comparable sensitivities and specificities of the mass measurements of CK-MB2, CKMB and myoglobin. In concordance with Roberts et al. (39), we prefer the measurement of MB2/MB1-ratios above CK-MB2 mass, because a release of minute amounts of MB2 into the plasma after myocyte necrosis will lead to a significant change in the MB2/MB1-ratio.
As the analyser reports only MM3/MM1- and MB2/-MB1-ratios, the CK-isoform analysis cannot be used for the quantification of tissue damage. For this purpose other cardiac markers such as Troponin I or Troponin T should be used. However, only the CK-isoform analysis offers the possibility of indicating the interval between infarction and blood sampling in a single blood sample with a single analysis. So, we suggest to use the CK-isoform analysis as marker for early damage and as marker for indication of the interval between AMI and time of blood sampling.
Concerning the 5 CK-isoform patterns from patients with different causes for inappropriate CKMB/CK-total activities, the CK-MB/CK-total ratios >20% for patient 1 and for patient 2 were caused by CK macro-enzymes. The occurrence of these two types of CK macro-enzymes is a common phenomenon in a small part of the population (40,41). Type 1 is CK associated with immunoglobulin and type 2 appears to be oligomeric CK-mitochondrial. Most of the time an extraordinary raised CKMB/CK-total ratio (>20%) is caused by this macro-enzyme artifact (42). However, an accompanied raised CKMB-activity can never be excluded. With the investigated CK-isoforms technique it is possible to discriminate on a routine basis 24 hours a day between an increased MB-activity, a CK-macroenzyme, or a combination of both phenomena. Patients 3 - 5 are examples of the additional information of the CK-isoform analysis in comparison with the CK-isoenzyme analysis. With the information of the CK-isoform pattern the permanently abnormal CK-total activity (1004 U/l) from patient 3 can be explained by the also permanently raised MM1-isoform, indicating that the maximum CK-inactivation capacity of the lymphoid system is exceeded (43). Patient 4 and patient 5 are examples of the possibility to indicate the time elapsed after tissue damage with a single CK isoform analysis. With the result of the CK isoform analysis the correct time after (re-)infarction of 4 hours could be estimated for patient 4, from whom the clinician could not get reliable information about the time since onset of symptoms. Patient 5 had raised CK-total, CKMB activities (1505, 10 U/l, resp.) three days after Coronary Artery Bypass Grafting. On basis of the MM1,2,3/MB1,2 isoform pattern, it was concluded that there was no myocardial tissue damage, but only skeletal muscle damage caused by a phlebography on day 2, approximately 15 h before blood sampling.
From this study we conclude that the CK-isoform pattern is relatively simply to assess on a routine basis with the new analyser. However, the analyser should be improved for better reproducibility of raised MB2/MB1 ratios. The CK-isoform analysis has additional value in monitoring the CK-

kinetics over a period from 2 h untill approximately 30 h after tissue damage. When the investigated parameters are compared, the MB2/MB1-ratio has shown to be one of the most reliable tests of the examined parameters to detect patients with AMI at time of admission to the hospital. Moreover, the CK-isoforms are additional tools in explaining inappropriate CKMB/CK-total activities.

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CHAPTER 6

Evaluation of the Stratus Cardiac Status and the Triage Cardiac Panel point- of- care testing devices for performing troponin I, CKMB-mass and myoglobin measurements.

Joost CJM Swaanenburg[1], Stan AJ van den Broek[2], Harry de Wit[1].

[1] Department of Pathology and Laboratory Medicine,
[2] Department of Cardiology, University Hospital Groningen.

Introduction

For many years the cardiac enzyme CKMB has been the gold standard for the detection of myocardial injury. However, CKMB is not heart-specific. As cardiac troponin I and cardiac troponin T are reported to be fully heart-specific (1,2), these parameters are more suited to diagnose myocardial necrosis. Moreover, these parameters are recently recommended by the National Academy of Clinical Biochemistry (NACB) (3) to be the new (biochemical) standard for the diagnosis of acute myocardial infarction (AMI) and thus replacing CKMB as one of the three WHO-criteria (4). Furthermore, the NACB recommended that two decision limits are needed for the optimum use of sensitive and specific cardiac markers such as cardiac troponin I and cardiac troponin T: a low abnormal value establishes the first presence of true myocardial injury, and a higher value is suggestive of injury to the extent that it qualifies as AMI. An other issue of which the NACB did some recommendations is the turn around times (TAT's) of laboratory testing of cardiac markers. It was recommended that results have to be reported within one hour after the collection of blood. If this requirement cannot be fulfilled consistently, point of care (POC)-testing should be considered.

Recently, it became possible to measure troponin I, CKMB-mass and myoglobin quantitatively as POC-test. The Stratus Cardiac Status analyser and the Triage Cardiac Panel are examples of these quantitative POC-testing devices.

The aim of this study was to investigate the performances of the Stratus CS™ analyser and the Triage CP™ in relation to the established central laboratory AxSYM™ analyser (5). We evaluated the correlations between the point of care testing devices and the central laboratory equipment for the cardiac markers cardiac troponin I, CKMB mass, and myoglobin. Furthermore, the analytical imprecisions of the POC-analysers were determined.

Patients and methods

Patients, who were admitted to the emergency department with complaints of acute chest pain, were included during a six weeks period for the evaluation of the POC testing devices.

After collection, blood was centrifuged at 1000x g and serum was separated from the cells in order to measure the cardiac troponin I, CKMB mass and myoglobin on the AxSYM analyser (Abbott Diagnostic Division, Hoofddorp, The Netherlands). The established cut off value of cardiac troponin I for acute myocardial infarction (AMI) has been reported to be 2.0 µg/l (5). At the same time Li-heparin anticoagulated blood was collected to measure all three biochemical parameters on the Stratus CS analyser (Dade-Behring, Leusden, The Netherlands) and the Triage CP (Biosite Diagnostics, manufactured by Merck, Amsterdam, The Netherlands).

The Stratus Cardiac Status is a fluorometric enzyme immunoassay based analyser for quantitative determination of cardiac troponin I, CKMB mass and myoglobin. Although preprocessed plasma specimens can also be measured, the system has been designed to analyse closed sample tubes containing Li-heparin anticoagulated whole blood. In order to separate plasma from the cells, a centrifugation step has been incorporated into the test system. Furthermore, a bar-coded test pack must be placed into the analyser for each particular test to be performed. Up to four test packs can be placed into the analyser at the same time. All required reagents have been enclosed within the test

pack. The test system utilises radial partition immunoassay technology which has been improved by the use of monoclonal capture antibody coupled to Starburst^{R} dendrimers (6). The dendrimer technology provides for better presentation and functionality of the capture antibody on the glass fiber solid phase surface used in the assay. This in turn leads to more efficient capture of the target antigen. The assay process is initiated by applying the dendrimer-antibody reagent onto the glass fiber matrix to form a reaction zone, which serves to capture the analyte of interest. Subsequently, centrifuged plasma is added, followed by the first incubation period. Thereafter, the alkaline phosphatase-labeled second antibody is applied to the matrix, followed by a second incubation period. The unbound, labeled antibody fraction is removed from the reaction zone by radial elution using substrate-wash reagent. Captured phosphatase-labeled antibodies convert the included enzyme-substrate into a fluorescent product; this permits quantification of the cardiac marker by front surface fluorescence measurement. The whole analytical procedure takes 14 minutes for one parameter analysis and 16 minutes for determining all three parameters of the same sample. The manufacturer recommended via a packet insert a cut off value for AMI of 1.5 µg/l (7).
The Triage Cardiac Panel is a self calibrating fluorescence immunoassay system for the quantitative determination of cardiac troponin I, CKMB mass, and myoglobin in heparin-treated whole blood and plasma specimens. After addition of the sample to the sample port, the cells are separated from the plasma via a filter which has been incorporated in the device. A predetermined quantity of plasma is allowed to react with fluorescent antibody conjugates within the reaction chamber. After incubation, the reaction mixture flows down the device detection lane. Complexes of the analytes and fluorescent antibody conjugates are captured on discrete zones, producing binding assays that are specific for each analyte. The concentration of each measured analyte is directly proportional to the detected fluorescence. Each testing device measures simultaneously cardiac troponin I, CKMB mass, and myoglobin. All results are available in 15 minutes. The cut off value for AMI is recommended by the manufacturer to be 1.0 µg/l (8).
The correlations between the cardiac marker test results of the Stratus CS, the Triage CP and the AxSYM were performed using Passing and Bablok regression analysis. The between day imprecision was determined using samples with different concentrations for all three analytes.

Results

Evaluation Stratus CS. One-hundred-thirty-seven patients (91 men, mean age 56.5 (standard deviation (sd) 11.6) y; 46 women mean age 66.2 (sd 11.8) y) were included for the analytical evaluation. From these patients 197 measurements were performed. The correlations between cardiac troponin I, CKMB mass, and myoglobin measurements performed on the Stratus CS and the AxSYM analysers are shown in figure 1. From this figure it can be concluded that for both CKMB mass and myoglobin there is a reliable correlation (slope is 0.73 (confidence interval (CI) 0.67-0.77), intercept is -0.25 (CI -0.34- -0.10) and coefficient of correlation (r) is 0.993 for CKMB mass, and for myoglobin slope is 0.68 (CI 0.63-0.74), intercept is 7.3 (CI 4.6-10.2) and r is 0.988 respectively), whereas there is a moderate correlation for cardiac troponin I (slope is 0.25 (0.23-0.28), intercept is 0.01 (CI 0.01-0.02), and r is 0.943). Furthermore, figure 2 shows that the concentration 2.0 µg/l for the AxSYM (the established cut off value for AMI) corresponds with a concentration 0.5 µg/l for the Stratus CS. In table 1A the frequency distribution is shown of the combinations whether or not the corresponding patient results of the Stratus CS and AxSYM are below or beyond various cut off values. In this table also the efficiencies are shown which have been

calculated from these data. The efficiency for the combination troponin I cut off value 1.5 µg/l (Stratus CS, recommended by the manufacturer) and 2.0 µg/l (AxSYM) is 87.3%, whereas the efficiency is 95.9%, if for the Stratus CS 0.5 µg/l is used as cut off value. In our hospital a concentration 0.5 µg/l for the AxSYM is used as low cut off value for the detection of minimal myocardial injury. This corresponds to a concentration 0.15 µg/l for the Stratus CS (resulting in an efficiency of 92.9%, see table 1A).

The between day imprecision was tested by measuring three samples with different concentrations during forteen consecutive days. The troponin I concentrations of these samples were 0.61 µg/l, 8.3 µg/l, and 16.2 µg/l respectively and the corresponding coefficients of variation 4.5%, 3.5% and 2.7% respectively. For CKMB mass the between day imprecisions were 3.2% for the low concentration (3.6 µg/l), 1.7% for the elevated concentration (18.1 µg/l) and 4.4% for the high concentration (53.0 µg/l). Myoglobin showed imprecisions of 2.6% for the low concentration (46 µg/l), 1.2% for the medium concentration (228 µg/l), and 3.0% for the high concentration (477 µg/l).

Evaluation Triage CP. For the correlation between the AxSYM and the Triage CP seventy-eight patients were included. From these patients 112 measurements were performed. The correlations between cardiac troponin I, CKMB mass, and myoglobin measurements performed on the Triage CP and AxSYM analysers are also shown in figure 1. From this figure it can be concluded that there is a reliable correlation for CKMB mass (slope is 1.01 (CI 0.84-1.05), intercept is -0.21 (CI -0.43- -0.08), and r is 0.979), whereas there are moderate correlations for cardiac troponin I (slope is 0.15 (CI 0.12-0.21), intercept is 0.0, r is 0.922) and myoglobin (slope is 1.31 (CI 1.13-1.59), intercept is 16.8 (CI 5.3-24.6), and r is 0.877 respectively). From figure 2 it can be deducted that the troponin I concentration of 2.0 µg/l for the AxSYM analyser corresponds with a concentration of 0.4 µg/l for the Triage. In table 1B the frequency

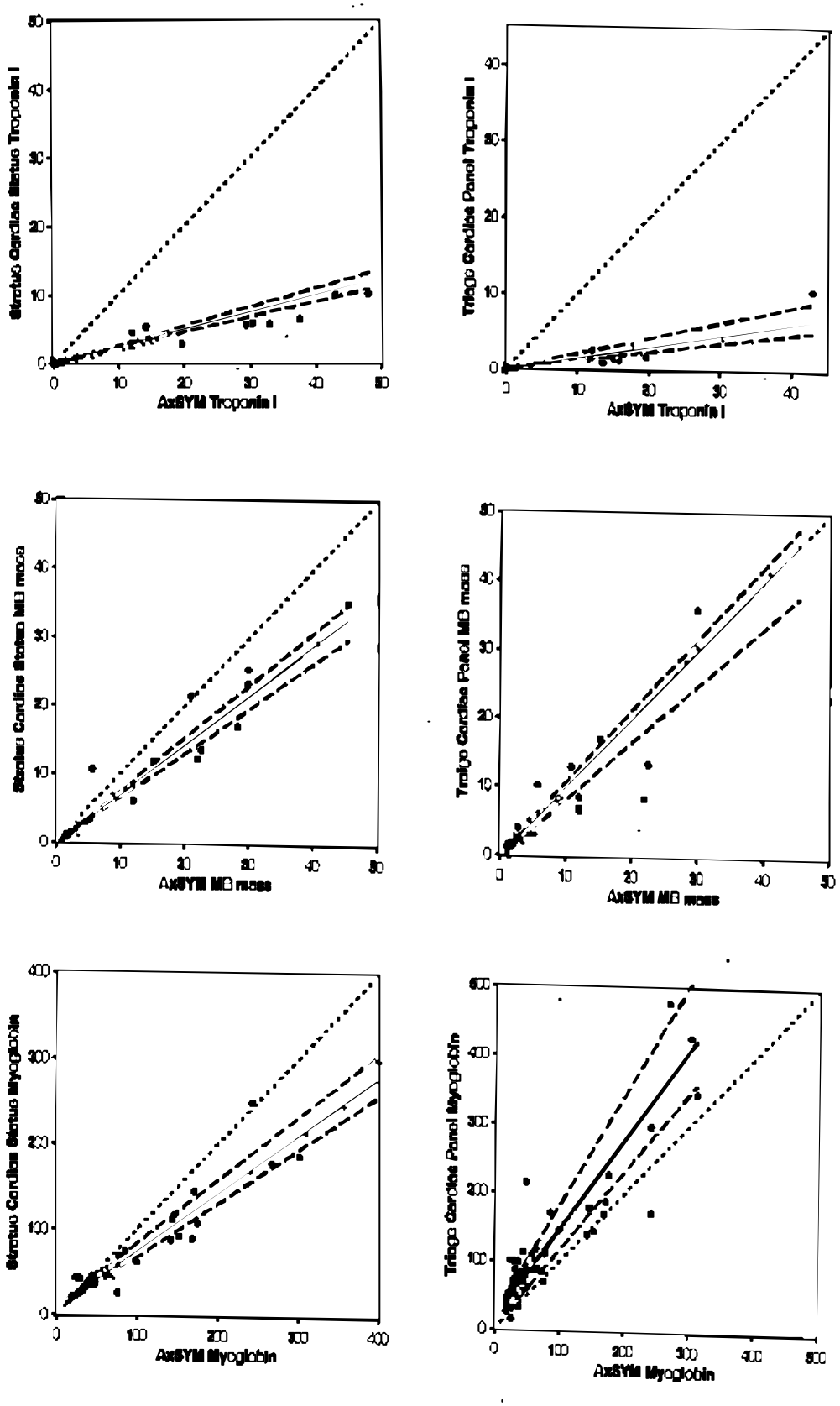

Figure 1. Regression analysis performed according to Passing and Bablok is shown between the AxSYM and Stratus CS and between the AxSYM and the Triage CP for the cardiac markers troponin I, CKMB mass and myoglobin (all units µg/L).

Table 1. Two by two tables for various cut off values of cardiac troponin I depicting frequencies of non-elevated and elevated values of the AxSYM versus the Stratus CS (A) and versus the Triage CP (B) analysers.

A

		AxSYM	
		# 2.0	> 2.0
Stratus CS	# 1.5	149	24
	> 1.5	1	23

Efficiency 87.31%

		AxSYM	
		# 2.0	> 2.0
	# 0.5	146	4
	> 0.5	4	43

Efficiency 95.94%

		AxSYM	
		# 0.5	> 0.5
	# 0.15	129	7
	>0.15	7	54

Efficiency 92.89%

B

		AxSYM	
		# 2.0	> 2.0
Triage	# 1.0	88	12
	> 1.0	0	8

Efficiency 88.89%

		AxSYM	
		# 2.0	> 2.0
	# 0.4	85	2
	> 0.4	3	18

Efficiency 95.37%

		AxSYM	
		# 0.5	> 0.5
	# 0.2	76	5
	> 0.2	3	24

Efficiency 92.59%

Efficiency: (frequency of both non-elevated + both elevated values) / total frequency.

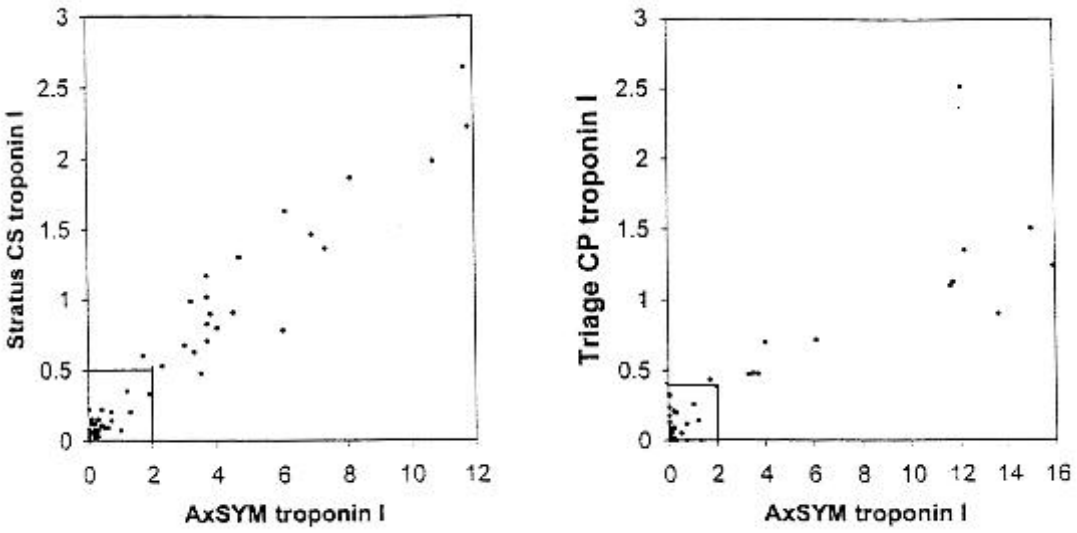

*Figure 2.*The correlation between the AxSYM and the Stratus CS, and between the AxSYM and Triage CP in the low concentration area. The cut off values for AMI of the Stratus CS and the Triage CP are related to the established AxSYM cut off value 2.0 µg/L.

distribution is shown of the combinations whether or not the corresponding patient results of the Triage CP and AxSYM are below or beyond various cut off values. From this table it can be seen that the efficiency for the cut off values 2.0 µg/l (AxSYM) and 0.4 µg/l (Triage) is 95.4%, whereas this is 88.9%, if for the Triage the manufacturer's recommended cut off value of 1.0 µg/l is used. To a low cut off value 0.5 µg/l for the AxSYM analyser corresponds a value 0.2 µg/l for the Triage CP. This combination of Triage CP and AxSYM low cut off values results in an efficiency of 92.6% (see table 1B). The imprecision of the Triage CP was between 3 - 6% for the three analytes at low, moderate and high concentration levels, respectively.

Discussion

In this study we investigated the performance of two recently introduced POC testing devices. We found that for CKMB mass and myoglobin measured with a Stratus CS analyser there is an acceptable correlation with the established AxSYM analyser. However, the results of the Stratus CS are 25% lower than those of the AxSYM. Furthermore, cardiac troponin I showed a moderate correlation because of the lack of a world wide accepted cardiac troponin I standardisation. The comparison between the Triage CP and the AxSYM analysers showed less acceptable correlations. Especially, the myoglobin correlation data showed more discrepancy between the Triage CP and AxSYM than between Stratus CS and AxSYM. In contrast to the comparison between the Stratus CS and the AxSYM analysers, we found no systematic differences between the Triage CP and the AxSYM.

The recommended cut off value for AMI of the Stratus CS manufacturer (1.5 µg/l) is too high, as we found a lower value (0.5 µg/l), which correlates better to the AxSYM (fig 2) resulting also in a better efficiency (87.3% versus 95.9%). Moreover, for those patients for who the troponin I AxSYM

values were elevated in contrast to the troponin I Stratus CS values, the clinical history was more in agreement with the elevated troponin I AxSYM values. Moreover, subsequent cardiac troponin I measurements of these patients resulted in elevated concentrations for both methodologies. The same applies for the Triage CP: the cut off value for AMI recommended by the Triage CP manufacturer (1.0 µg/l) appeared to be too high compared with the AxSYM. A cut off value 0.4 µg/l is more in agreement with the established AxSYM value of 2.0 µg/l. This is also illustrated by the better efficiency (88.9% compared with 95.4% respectively). Evenso, the clinical histories of the patients with elevated troponin I AxSYM values and non-elevated troponin I Triage CP values were more according to the elevated troponin I AxSYM values.

For the low cut off values for the detection of minor myocardial injury we found for the Stratus CS analyser 0.15 µg/l, whereas this was 0.2 µg/l for the Triage CP. The lower cut off values which we report for both POC testing devices are in agreement with reported findings from other investigators (7,8).

Since it has been reported that elevated levels of cardiac troponin I and cardiac troponin T are prognostic factors for patients with acute chest pain complaints, the turn around time for these determinations are recommended to be within one hour. If the central laboratory cannot fulfill this requirement, POC testing must be considered (3). For several years it has been possible to measure the cardiac troponin I or cardiac troponin T contents in blood as POC-test using 'strip'-technology (9,10). These 'strip'- testing devices require to put 150-200 µl whole blood on a strip containing a capture-antibody to bind the troponin and a label-antibody to measure the intensity of the capture bound troponin. The results of these methodologies are qualitative, because test results are only reported as 'negative'or 'positive' (10). However, for reliable trend analysis quantitative results are required. Recently, a measuring device has been developed to quantitate the troponin T results of the strip-test technology (11). To measure quantitatively troponin I as POC test both evaluated testing devices seem to be useful alternatives, as the way of performing of these POC testing devices is such simple that non analytically educated personnel can reliably perform these tests.

After the analytical evaluation period the Stratus CS analyser was located at the Coronary Care Unit (CCU). During this period it turned out that this testing device is suitable for decentralised testing in daily clinical practice. Indeed, we found an excellent performance regarding the convenience and reliability when the analyses were carried out by non analytically educated personnel. These people slightly prefer the Stratus CS, because of the closed tube system. Because of this aspect, there is no risk for contamination with blood of the patient. Furthermore, in contrast to the Triage CP, where always all three parameters are simultaneously measured, it is possible to choose with the Stratus CS which parameter has to be determined. In favour of the Triage CP are the low costs of investment and the compactness of the device allowing easy reallocation near by the individual patient.

From this study we conclude that the Stratus CS analyser shows good correlations with the AxSYM analyser for CKMB mass and myoglobin, whereas the correlation for cardiac troponin I is moderate. The cardiac troponin I low cut off value for the detection of minor myocardial injury appears to be 0.15 µg/l, whereas the cut off value for AMI is 0.5 µg/l. The Triage Cardiac Panel shows a good correlation for CKMB mass, whereas the correlation data for cardiac troponin I and myoglobin are moderate. The cardiac troponin I low cut off value appears to be 0.2 µg/l, and the cut off value for AMI 0.4 µg/l. Furthermore, both POC testing devices are suited for point of care testing performed by non-analytically educated personnel.

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CHAPTER 7

Troponin I, Troponin T, CKMB-activity and CKMB-mass as markers for the detection of myocardial contusion in patients experiencing blunt trauma.

Joost CJM Swaanenburg[1], Joost M Klaase[2], Mike JL DeJongste[3], Kees W Zimmerman[2], HenkJan ten Duis[2].

[1]Central Clinical Chemical Laboratory,
[2]Department of Surgery,
[3]Department of Cardiology, University Hospital Groningen.

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Summary
Myocardial contusion is an infrequent, but sometimes serious complication in patients experiencing deceleration (blunt) trauma. We investigated the assessment of the new cardiac markers Troponin I (cTnI) and Troponin T (cTnT) in relation to the conventional CKMB-activity, the CKMB-activity/CK-total ratio, CKMB-mass and the CKMB-mass/CK-total ratio for the detection of myocardial contusion in 89 patients with blunt trauma (38 patients with thoracic injuries and 51 patients without thoracic injuries). All parameters were analysed at admission (t_1) and 24 hours after admission (t_2). For the patients with thoracic injuries, at t_1 cTnI was elevated in three and cTnT in four patients; at t_2 both cTnI and cTnT were elevated in nine patients. At t_1, eighteen to thirty patients had increased levels of the conventional parameters; at t_2 this was true for six to thirty-five patients. For the patients without thoracic injuries all cTnI and cTnT levels were within the reference ranges at t_1. At t_2 one patient, who experienced an acute myocardial infarction, had elevated cTnI and cTnT levels. At t_1, five to thirty-five patients had increased levels of the conventional parameters; at t_2 this was true for four to forty-two patients. From this study we conclude, that the conventional parameters are not useful for the detection of myocardial contusion in patients experiencing blunt trauma. The parameters cTnI and cTnT are equally accurate and more reliable for the selection of patients, who require intensive cardiac monitoring. If at admission the cTnI or the cTnT levels are within the reference ranges, a second analysis after admission is necessary to reach a reliable conclusion concerning myocardial contusion as a result of trauma on basis of the troponin levels.

Introduction

Myocardial contusion can result from blunt thoracic trauma and is commonly suspected in deceleration injuries after a traffic accident or a fall from great height. Myocardial contusion has a patho-anatomical substrate that comprises subepicardial or subendocardial sharply-defined small areas of bleeding. Microscopically, disruption of myo(cardial)fibrils with leucocyte infiltration and oedema are found. Complications of myocardial contusion, such as arrhythmias, can potentially be life-threatening. However, not every patient with a blunt chest trauma has to be admitted to a hospital ward with cardiac monitoring facilities.

The reported incidence of myocardial contusion in patients with blunt chest injury varies among 0% (1-3) and 76% (4) and depends on the diagnostic criteria used and the severity of the blunt chest trauma (5,6). Biffl et al. (7) reported recently a 30% incidence in 359 patients with high-risk blunt chest trauma; complications (dysrhythmia and cardiogenic shock) requiring treatment occurred in 5% (8). Postmortem evidence of myocardial contusion was found in 14% of the immediate fatalities from blunt injuries (9).

Diagnosing myocardial damage as a result of trauma may be a problem. There is a lack of a 'gold standard' for establishing the diagnosis. It is generally accepted that patients with blunt thoracic injury are suspected of having myocardial contusion on basis of their complaints, ECG findings at admission, and an elevated creatine kinase (CK) MB-activity, whether or not expressed as a fraction of the CK-total activity (10,11). The measurement of CKMB-activity is complicated by artifacts as CK-macro-enzymes and CKBB in the blood. These artifacts do not influence the CKMB-mass measurements. However, CKMB-mass as well as CKMB-activity measurements are also elevated after severe skeletal muscle injuries.

The 12-lead ECG is another often performed diagnostic procedure. However, in the first few hours following a major injury ECG abnormality may simply reflect metabolic changes (12). In a review of twelve prospective studies, abnormal ECGs were reported in 33% of trauma patients (range 11-

81%), but in all these studies there was no uniform definition of what constituted an abnormal ECG (13). Another procedure is transthoracic echocardiography. This is a widely available bedside procedure that is used to assess the performance of the myocardium. Recent transthoracic studies have shown wall-motion abnormalities to occur in about 25% of blunt chest injury victims (14-16). However, sub-optimal examinations influence the reliability of this tool (17-19). So, there is much confusion as to how to diagnose myocardial contusion.
Recently, the cardiac markers troponin I (cTnI) and troponin T (cTnT) have become available. Troponin I, C and T form a complex that regulates the calcium-modulated interaction of actin and myosin in striated muscle. Troponin I from cardiac muscle and slow- and fast-twitch skeletal muscle are products of different genes with unique amino acid sequences (20-22). Skeletal muscle in animals and in humans does not express cTnI at any developmental stage or in response to any pathological stimuli (23,24). In contrast, cTnT is expressed in fetal and neonatal skeletal muscle in humans and experimental animals, but is suppressed in healthy adult skeletal muscle (25-27).
We investigated the assessment of the new biochemical parameters cTnI and cTnT, in relation to the more conventional CKMB-activity, the CKMB-activity/CK-total ratio, CKMB-mass and the CKMB-mass/CK-total ratio for the detection of myocardial contusion in patients who have experienced blunt trauma.

Patients and methods

Patients and Controls

Between July and December 1996 we investigated the value of CKMB-activity, CKMB-activity/CK-total ratio, CKMB-mass, CKMB-mass/CK-total ratio, cTnI and cTnT in 89 consecutive patients experiencing blunt trauma. At admission an ECG and chest X-ray were performed on these patients according to the hospital trauma protocol. When myocardial damage was suspected, a cardiologist was consulted. The patients were divided into two groups: patients with and without thoracic injuries. The group with thoracic injuries consisted of 38 patients. If thoracic injury was excluded (normal ECG, normal X-ray, no obvious external chest injury and no complaints of chest pain) patients were categorised in the group patients without thoracic injuries (n=51). Some of this group (17) were patients with injuries of the extremities only.
The reference ranges of the biochemical parameters were confirmed with sera from 69 healthy control persons (45 male, mean age 38 years; 24 female, mean age 37 years).

Methods

Blood was taken at admission (t_1) and 24 hours after admission (t_2) for analysis of the biochemical parameters. After centrifugation at 1000 g at 20 EC the serum was separated. CK-total and CKMB-activities were analysed immediately. If the CKMB-activity/CK-total ratio was more than 3%, a CK-isoenzyme electrophoresis was carried out to exclude CK-macro-enzymes and CKBB. For retrospective analysis of cTnI, cTnT and CKMB-mass, serum was stored at -20 EC. These measurements were performed in one batch on the same day after the inclusion of patients was finished.
CK-total and CKMB-activity (immune-inhibition) were measured with a Vitros 750C analyser (Ortho Clinical Diagnostics, Beerse, Belgium).
For cTnI measurements an Access analyser (Sanofi Diagnostics Pasteur, Vlaardingen, The Netherlands) was used. After separation of the bound and unbound fractions, a dioxetane chemiluminescent substrate provides a long-lasting signal which is proportional to the cTnI concentration (28).

cTnT measurements were performed on an Elecsys 2010 analyser (Boehringer Mannheim, Almere, The Netherlands) using the more cardiac specific 'second generation' cTnT antibodies (29). After the forming of a 'sandwich'-complex, in which ruthenium is incorporated, the application of a voltage induces chemiluminescent emission which is measured by a photomultiplier (30).
CKMB-mass was measured with a MAGIA 7000 analyser (Merck, Amsterdam, The Netherlands). The principle of separation after the one step competition reaction is based on magnetising of 'para-magnetic microparticles'. Substrate is added after this procedure for the chemical reaction catalysed by alkaline phosphatase (31).
CK-isoenzyme analyses were performed electrophoretically with a Cardio Rep analyser (Helena Laboratories, Beaumont, TX, USA) (32).
To compare the various parameters the test results were normalised by dividing the result of the analysis by the cut off value (cov) of the parameter. The covs are the upper limits of reference ranges of the biochemical parameters. The parameters CKMB-mass, cTnI and cTnT are not in common use in our hospital. For these parameters the reference ranges recommended by the manu-facturers are used.

Results

The patient and trauma characteristics are summarized in Table 1. The levels of the biochemical parameters in the sera of the control persons were all below the upper limits of the reference ranges.
On basis of the trauma protocol used in this study, three patients with thoracic injuries were suspected for myocardial contusion.

In Figure 1, the normalised results (for reasons of clarity only the range 0 - 10) are depicted of the

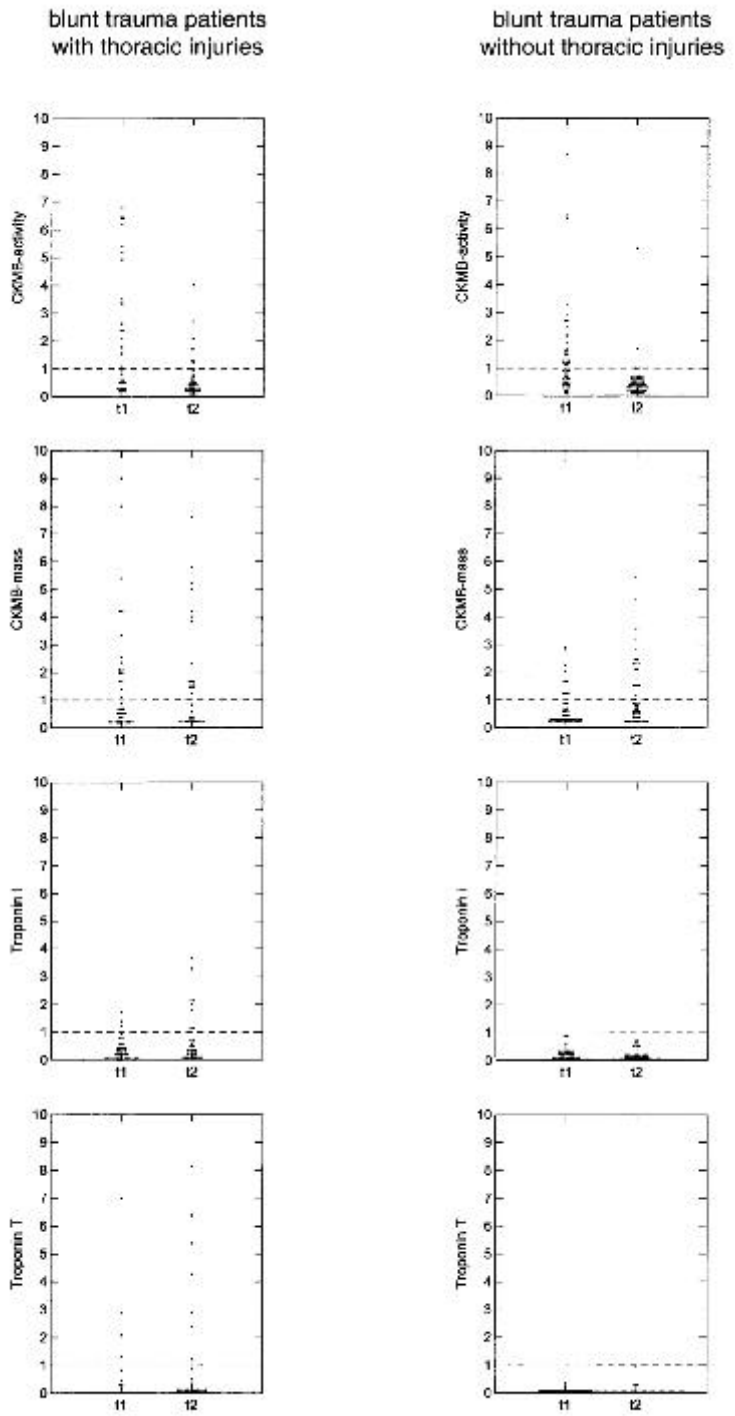

Figure 1. Normalised results of biochemical markers are shown for 38 blunt trauma patients with thoracic injuries and 51 blunt trauma patients without thoracic injuries at admission *(t_1)* and 24 h after admission *(t_2)*. For reasons of clarity, only the results out of the range 0-10 are shown. The dashed lines indicate the upper limits of the reference ranges. Patients with thoracic injuries, who have increased troponin results at t_1,have also increased results at t_2.

biochemical markers CKMB-activity, CKMB-mass, cTnI and cTnT at admission (t_1) and 24 hours

after admission (t_2) for both groups of patients. As the patterns for the CKMB-activity/CK-total ratios and the CKMB-mass/CK-total ratios are similar to those of CKMB-activity and CKMB-mass, these pictures are not shown for reasons of clarity.

Table 1. Patient and trauma characteristics.

		total patients	patients with thoracic injury	patients without thoracic injury
Number		89	38	51
Mean age in years (range)		36 (5-84)	37 (8-82)	33 (5-84)
Cause of the accident (freq.):				
Traffic	62	31	31	
Fall from height		22	5	17
Other		5	2	3
Accompanying injuries*				
Trauma capitis	48	23	25	
Abdomen		23	15	8
Pelvis		4	4	0
Extremity		51	19	32
Back		6	2	4
Other		1	0	1

* Patients could have more than one injury.

In Table 2 the percentages of elevated results of all the examined biochemical parameters at t_1 and at t_2 for both groups of patients are shown.
In the thoracic injury group, three patients had elevated cTnI and four patients had elevated cTnT results at t_1. For nine patients out of this group both cTnI and cTnT results were above the cov at t_2. None of the conventional markers was elevated in these nine patients at t_1 and at t_2. On the other hand, there were elevated levels of CKMB-activity (five patients), CKMB-activity/CK-total ratio (four patients), CKMB-mass (fifteen patients) and CKMB-mass/CK-total ratio (seven patients) in the twenty-nine patients with thoracic injuries for whom the cTnI and cTnT results were not elevated and for whom myocardial damage was also not suspected clinically.
Although in the group consisting of patients without thoracic injuries none could have myocardial damage resulting from trauma, a number of them had elevated results at t_1 and t_2 for the markers CKMB-activity (twenty-nine and four patients, respectively), the CKMB-activity/CK-total ratio (forty-six and ten patients, resp.), CKMB-mass (ten and twenty patients, resp.) and the CKMB-mass/CK-total ratio (nineteen and eleven patients, resp.). Even in the subpopulation of seventeen patients with only trauma of the extremities, 50% of the results of these biochemical parameters were elevated. All patients without thoracic injuries had cTnI and cTnT results within the reference ranges at t_1. At t_2 one patient had elevated cTnI and cTnT results after experiencing an acute myocardial infarction (AMI) 7 hours after admission, not as a direct result of trauma.

The CKMB-activity/CK-total ratios above 15% (normalised ratio 5) from 37 out of the total 89 patients were all shown to be influenced by an increased concentration of CKBB by performing a CK-isoenzyme analysis. The phenomenon of elevated CKBB was seen for all patients only at t_1.

Table 2. Cut off values and the percentages of elevated results of all examined biochemical parameters at admission and 24 h later for blunt trauma patients with thoracic injuries and for blunt trauma patients without thoracic injuries.

Parameter	% Elevated results of 38 blunt trauma patients with thoracic injuries		%Elevated results of 51 blunt trauma patients without thoracic injuries		Cov[a]
	t_1	t_2	t_1	t_2	
CKMB-activity	63	24	57	8	10 U/L
Ratio[b]	79	13	90	20	3.0 %
CKMB-mass	47	61	20	39	5.0 µg/L
Ratio[c]	55	32	38	22	3.0 %
Troponin I	8	24	0	2	0.1 µg/L
Troponin T	10	24	0	2	0.1 µg/L

t_1: admission; t_2: 24 hours after admission;
[a]Cov: cut off value (upper limit of reference range);
[b]Ratio: CKMB-activity/CK-total;
[c]Ratio: CKMB-mass/CK-total.

Discussion

It is often very difficult to establish the diagnosis of myocardial contusion. The seriously injured trauma patient usually cannot provide adequate information about injury mechanism and often cannot complain about chest pain. Clinicians use a variety of tools for this diagnosis. To date, blood analyses (conventional and recent markers) are the most-often used tools to determine myocardial contusion.

Conventional markers. Although CKMB-isoenzyme measurement is currently a recommended biochemical test for the detection of myocardial damage (11), it is less accurate in patients with chest trauma. As CKMB is present in myocardial muscle and in skeletal muscle, CKMB from both types of tissue is released and causes blood values to be elevated in response to the injury (33,34). Also in our study population many patients without thoracic injuries had elevated levels of CKMB-activity, CKMB-activity/CK-total ratio, CKMB-mass and CKMB-mass/CK-total ratio. Even in the 17 patients who only had trauma of the extremities, 50% of the results for these biochemical parameters were elevated. If these variables had been used as criterium for myocardial damage, these patients could have been incorrectly classified. Subsequently, since patients with myocardial damage should

be monitored for 48 h (12), they could have been unnecessarily exposed to an expensive intensive care unit.
The false positive finding of CKMB-activity/CK-total-ratios over 15% (for 37 out of the total 89 patients) is related to the CKBB-isoenzyme. The CKBB-isoenzyme is normally not detectable in the blood. CKBB is present not only in brain tissue, but also in other tissues such as stomach, intestine, kidney, bladder, prostate and uterus (35). Thus, after an accident with involvement of these tissues the CKMB-activity/CK-total ratio may be elevated.
Because the CKMB-mass measurement is not influenced by the CKBB-isoenzyme, the CKMB-mass/CK-total ratio is also used as index for myocardial damage (36), despite the fact that CKMB-mass and CK-total are expressed in different units. If we compare the results of these ratios with those of the CKMB-activity/CK-total ratios for patients without thoracic injury, the CKMB-mass/CK-total ratios are more reliable. However, there are still eight patients for whom at t_1 both the CKMB-mass and the CKMB-mass/CK-total ratio are elevated and at t_2 this is true for five patients. Thus, the CKMB-mass/CK-total ratio is also not specific for the detection of myocardial damage in patients after blunt trauma.

Recent markers. Adams et al. (36) reported that measurement of troponin is more sensitive than the conventional diagnostic tools for the detection of myocardial damage in patients after trauma. Moreover, it is also easier and less costly than using echocardiography as a routine screening test to detect cardiac injury after blunt chest trauma (36). The determination of troponin is more specific, as has been reported by Bodor et al. (37). They showed by histological experiments on biopsies of skeletal and myocardial muscle tissues, that cTnI was only detectable in myocardial tissue. For cTnT they show cross reactions with skeletal muscle biopsy specimens. However, they used their own produced antibodies, and these are different from those used by the manufacturer of the commercially available reagent. This may explain why we did not find elevated cTnT results in the patients without thoracic injuries. Moreover, we used the 'second generation' reagent for the cTnT measurements. As has recently been reported (29), the antibody used in this reagent is more specific for myocardial tissue and shows less cross-reactivity with skeletal muscle tissue.
As we did not find elevated troponin results in the group of patients without thoracic injuries and for reasons of reported sensitivity and specificity (36-39), we prefer the troponin results as criteria for the detection of traumatic myocardial damage and for the selection of patients, who require intensive cardiac monitoring. However, as can be concluded from Table 2, measurement of cTnI and cTnT at admission can be too early for a definite conclusion: the cTnI and cTnT levels were still within the reference ranges at t_1 for most patients with thoracic injuries who have elevated cTnI and cTnT results at t_2. This phenomenon is similar to that of patients experiencing an AMI. For these patients it takes several hours for the troponins to be elevated after AMI. Further studies should be carried out to determine the clearance characteristic curves of cTnI and cTnT in circulation after myocardial damage caused by trauma. From such studies the earliest reliable time after trauma can be determined, at which traumatic myocardial damage can be detected.
We conclude from this study, that CKMB activity, the CKMB-activity/CK-total ratio, CKMB-mass and the CKMB-mass/CK-total ratio are not useful for the detection of myocardial damage in patients experiencing blunt chest trauma. The parameters cTnI and cTnT are equally accurate and more reliable than the other biochemical markers for the detection of myocardial damage and for the selection of patients, who require intensive cardiac monitoring. If at admission the cTnI or the cTnT results are still within the reference ranges, a second analysis after admission is necessary to reach a reliable conclusion concerning myocardial contusion as a result of trauma on basis of the troponin results.

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CHAPTER 8

The performance of troponin I and troponin T in relation to conventional criteria for the detection of myocardial injury in patients undergoing major noncardiac surgery.

JCJM Swaanenburg[1], JPC Sonneveld[2], H Delwig[3], JJM Ligtenberg[4], JMKH Wierda[2], ARJ Girbes[5].

[1] Department of Laboratory Medicine,
[2] Department of Anesthesiology,
[3] Surgical Intensive Care,
[4] Intensive and Respiratory Care, Department of Internal Medicine, University Hospital Groningen;
[5] Department of Surgical Intensive Care, University Hospital Vrije Universiteit, Amsterdam.

Summary
Biochemical markers together with the ECG are commonly used parameters for the detection of peri-operative acute myocardial infarction (AMI) in patients undergoing major non-cardiac surgery. We investigated the performance of troponin I and troponin T in relation to the conventional criteria CKMB-activity, CKMB-mass and ECG changes for the detection of AMI in sixty patients undergoing major noncardiac surgery. AMI was diagnosed according to WHO-criteria. All biochemical parameters were analysed at the start and at the end of surgery, and six hours, one day and three days thereafter. Two patients experienced AMI, and both patients had elevated troponin I and troponin T concentrations, whereas one of these patients had elevated CKMB-act and both patients had elevated CKMB-mass concentrations. Another four patients had ischemia characterised by ECG changes, but no infarction, from who only one patient had slightly elevated troponin I concentrations. One patient had seriously elevated troponin I and troponin T concentrations without notification of ECG changes. Troponin I concentrations were elevated in five patients in at least one of the five timepoints, whereas this applies for troponin T concentrations in three patients. In contrast, twenty patients had elevated CKMB-act at at least one of the five timepoints and moreover, this was true for thirty-two patients concerning CKMB-mass concentrations. We conclude that troponin I and troponin T are more specific and reliable for the detection of myocardial injury after major noncardiac surgery than the conventional markers CKMB-act and CKMB-mass. Furthermore, the measurement of troponin I or troponin T looks like to be more accurate than (discontinuous) ECG monitoring for the detection of myocardial injury after major noncardiac surgery.

Introduction

Peri-operative myocardial infarction (AMI) has been known to be a serious complication in patients undergoing major noncardiac surgery (1). Chest pain complaints, ECG changes and elevation of biochemical markers in the blood are the three WHO-criteria to diagnose AMI in patients after ischemic episodes (2). For this reason, biochemical markers are, besides the ECG, of common use for the detection of AMI. Among the conventional biochemical markers, the creatine kinase (CK)MB isoenzyme has already for a long time been accepted as the gold standard for the detection of myocardial tissue injury (3). However, CKMB is not a specific parameter for myocardial tissue damage. In addition, the CKMB/CK total ratio is also used as a marker for myocardial tissue damage. But this ratio may be falsely negative, if myocardial tissue damage is accompanied by massive skeletal muscle damage (4). Therefore, this ratio is only reliable in patients with solitary ischemic myocardial damage. Thus, a correct interpretation of elevated CKMB-levels and CKMB/CK-total ratios in patients after (noncardiac) surgery may be a problem for reasons of the non-heart specificity of the CKMB isoenzym.
Recently, new biochemical parameters have been introduced for the detection of myocardial tissue damage. Troponin I (5) and troponin T (6) belong to this new category of biochemical parameters. Both troponins are related to the actin-myosin complexes of the thin filaments of striated muscle. Troponin I as well as troponin T are part of skeletal and myocardial muscle tissue and both have an unique protein structure (7,8). The sequence of amino acids of both troponin I and troponin T from skeletal muscle differs from those of myocardial muscle. For this reason, they can be separated immunochemically by the use of specific antibodies directed against these parts of the proteins for

which the sequences of the amino acids differ in skeletal from myocardial muscle tissue.
Several studies concerning myocardial damage in patients at the Intensive Care Unit (ICU) or after major non-cardiac surgery have been reported (9-13). Mostly, it concerns the investigation of only the troponin I or troponin T marker. Moreover, in most of these studies the first generation troponin T measurement has been used. This first generation troponin T reagent has been characterised by cross reactivity of the label-antibody with skeletal muscle troponin T (14).
The aim of this study is to investigate the performance of the new biochemical markers troponin I and the more heart specific second generation troponin T in relation to the conventional criteria CKMB-activity, CKMB-mass and ECG changes for the detection of myocardial injury in patients undergoing major noncardiac surgery. This study is part of a randomised and controlled trial studying the effects on clinical outcome of standard preoperative treatment at the ward compared to a preoperative haemodynamic optimisation at the ICU.

Patients and methods

Sixty consecutive patients who underwent extensive non-cardiac surgery were included during a ten months period. The patients were characterised as high surgical risk according to criteria earlier described (15). These include: previous severe cardiorespiratory illness; extensive ablative surgery planned for carcinoma; age over 70 years and evidence of limited physiologic reserve of one or more vital organs; septicaemia, positive blood culture or septic focus, white blood cell count >13.000 /ml, spiking fever to 38.3 ^{o}C for 48 h; acute renal failure; and late stage vascular disease involving aortic abnormality. All patients were preoperatively screened by extensive interview, physical examination and resting ECG. When indicated, additionally non-invasive and invasive diagnostics were performed to evaluate the existence and severity of myocardial ischemia. The patients of the protocol group (n=31) were admitted to the ICU at the preoperative day for haemodynamic optimisation (15), whereas the control group patients (n=29) received standard treatment at the ward. Anaesthesia was standardised in both groups.
Peri-operative AMI was diagnosed according to WHO-criteria (2) on basis of 12-lead ECG's in combination with elevations of CK-total and CKMB-activity measurements at day 1 directly after surgery, at day 2, 3 and 4 and at the final day of the hospital stay before the patient was discharged from the hospital. New ST-T depression of more

Table 1. Patient and surgical characteristics.

Number of male (female) patients	37 (23)
Mean age in years (standard deviation, range)	65 (12, 32-81)
Type of surgery:	
Aorta surgery	14
Laparotomy for septic focus	2
Laparotomy for malignancy:	33
- upper digestive tract	6
- modified Whipple procedure	7
- liversurgery (hemihepatectomy / cryosurgery)	11

- cystectomy	3
- colonsurgery	2
- other	4
Restricted surgical procedure (inoperability):	11
- hepatico-jejunostomy / gastro-jejunostomy	5
- 'open-close'	4
- other	2
Per-operative period:	
- mean time of operation in minutes (standard deviation)	309 (164)
- mean peroperative bloodloss in ml (stand.dev., range)	1850 (2050, 0-10000)
epidural catheter	52

than 1 mm in 2 or more adjacent leads, in the absence of electrolyte disturbances and use of digoxin, was defined as ischemia. ST-T elevations of more than 1 mm in 2 or more adjacent leads together with T-top changes were defined as AMI. The ECG's made at the day of hospital discharge were screened for new Q's or T-top inversions;
AMI was defined as a Q wider than 1.5 mm and deeper than 3 mm.
Blood was collected at day 1 at the start (t_0) and at the end (t_1) of the surgical procedure; six hours after the end of the surgery (t_2); at day 2 (t_3) and at day 4 (t_4). The blood was centrifuged at 1000x g and at 20 ^{o}C. After this procedure the serum was separated from the cells. The CK-total and CKMB-activity measurements were performed immediately. Until analysis of CKMB-mass, troponin I and troponin T, the sera were stored at -20 ^{o}C.
The CK-total (upper reference limit (url) 70 U/l) and CKMB-activity (immune-inhibition, url 10 U/l) were measured on a Vitros 750C analyser (Ortho Clinical Diagnostics, Beerse, Belgium).
Troponin I (AMI cut off value 2.0 µg/l) measurements were performed on an AxSYM analyser (16) (Abbott Diagnostic Division, Hoofddorp, The Netherlands).
For troponin T (AMI cut off value 0.1 µg/l) and CKMB-mass (url 5.0 µg/l) measurements an Elecsys 2010 analyser was used (Roche-Boehringer Mannheim, Almere, The Netherlands). The more cardiac specific 'second generation' troponin T-antibodies were used for the troponin T measurements (14).
All test results were normalised by dividing the result of the assay by the corresponding url in order to be able to compare the various biochemical parameters. The parameters CKMB-mass, troponin I and troponin T were not of common use in our hospital. For these parameters the url's were used recommended by the particular manufacturers. The specificities and the positive predictive values (PPV's) with the corresponding exact 95% confidence intervals were calculated for the parameters CKMB-act, CKMB-mass, troponin I and troponin T by the use of StatXact-4 (Cytel Software Corporation, Cambridge, MA, USA).

Results

Patient and surgical characteristics are depicted in table 1. Eleven patients have undergone a less extensive surgical procedure than planned, because of (abdominal) metastasis (ten patients), and because of preoperative development of cardiac ischaemia during haemodynamic optimisation (one patient).

Two patients (patients A and B) have been classified as having experienced an AMI during hospital stay. Both patients have had elevated concentrations of CKMB-mass, troponin I and troponin T, whereas only patient B has had elevated CKMB-act. One patient (patient C) has initially been diagnosed at the ICU as experiencing a non Q-wave AMI because of ECG changes (ST-T depression), history of coronary artery disease and elevation of CK-total and CKMB-act (CK-total 176 U/L, CKMB-act 18 U/L). All troponin I and troponin T concentrations of this patient have been below the url's.

Another three patients (patients D, E and F) have had a period of ischemia without AMI. All troponin I and troponin T concentrations from the patients D and E have not been elevated at any of the five time points. From patient F the maximum troponin I concentration (2.4 µg/l) has been slightly elevated, whereas the maximum troponin T concentration (0.08 µg/l) has remained below the url.

Another patient (patient G) has had elevated maximum troponin I (30.4 µg/l) and maximum troponin T (0.78 µg/l) concentrations without notification of ECG changes.

In figure 1 the normalised results are shown of the biochemical parameters CKMB-act, CKMB-mass, troponin I and troponin T at the five different time points. CKMB-act has been elevated in twenty patients at at least one of the five time points. Moreover, this is true for thirty-two patients concerning the CKMB-mass concentration. In contrast, the troponin I concentration has been elevated in five patients at any of the five time points, whereas this applies for the troponin T concentration in three patients.

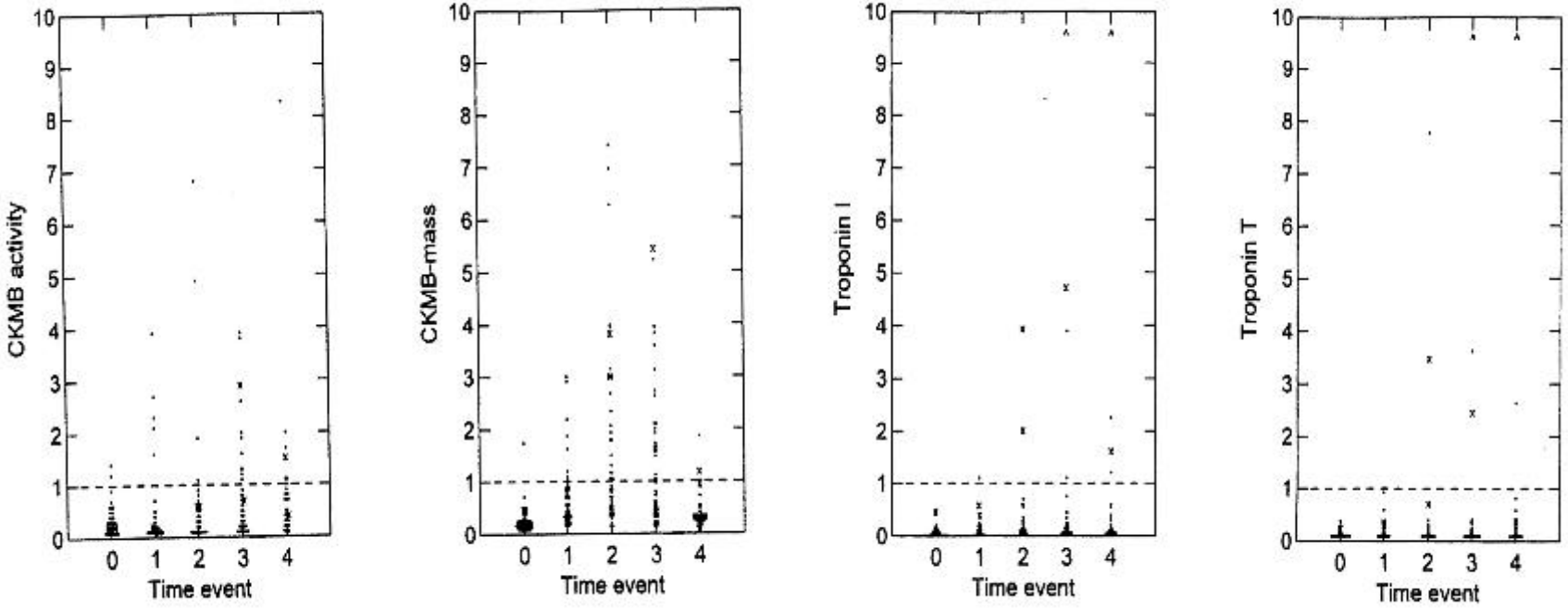

Figure 1.The normalised values between 0 and 10 at five timepoints for CKMB-activity, CKMB-mass, troponin I and troponin T from sixty patients undergoing major noncardiac surgery. Two patients experienced an acute myocardial infarction. The values of these 2 patients are indicated with (x). ^ indicates a value beyond 10.

Table 2. The specificities and positive predictive values (PPV's) with their corresponding 95% confidence intervals of the parameters CKMB-act, CKMB-mass, troponin I and troponin T in sixty patients undergoing major non-cardiac surgery, from who two experienced an AMI.

Parameter	Url	Specificity (95% CI)	PPV (95% CI)
CKMB-act	10 U/l	0.67 (.52-.79)	0.05 (.01-.25)
CKMB-mass	5.0 µg/l	0.47 (.34-.61)	0.06 (.01-.21)
troponin I	2.0 µg/l	0.95 (.85-.99)[a]	0.40 (.05-.85)
troponin T	0.1 µg/l	0.98 (.91-1.00)[a]	0.67 (.09-.99)

Url: upper limit of the reference range;
PPV: positive predictive value;
CI: confidence interval;
[a] $p<0.01$ compared to CKMB-act and CKMB-mass.

In table 2 the specificities and the PPV's with their corresponding 95% confidence intervals are depicted for the parameters CKMB-act, CKMB-mass, troponin I and troponin T. From this table it can be seen, that the new biochemical markers troponin I and troponin T show statistically significant higher specificities and higher PPV's than the conventional markers CKMB-act. and CKMB-mass.
The two groups of patients do not differ regarding the prevalence of myocardial infarction (either group contains one AMI patient).

Discussion

Patients planned for extensive surgery are at risk for the development of myocardial ischemia and infarction (1). The risk for the development of cardiac ischemia or infarction is related to age, preexisting cardiovascular morbidity, the type and length of operation and anaesthesia (1,17). The establishment of myocardial infarction after surgery may be complicated. The patient experiences tissue and muscle damage from other than myocardial origin as a result of the surgical procedure. Furthermore, the patient may not always be able to complain about chest pain. The mainly used parameters for the diagnosis of cardiac ischemia and myocardial infarction in these patients are ECG changes and elevation of biochemical markers. Conventionally, the biochemical marker CKMB is still the gold standard to evaluate the prevalence and extent of myocardial damage (3). Recently, it has been reported, that troponin I and troponin T have been a more sensitive and more specific biochemical marker for the detection of myocardial damage in ischemic patients (18,19) and in patients with combined myocardial and skeletal muscle damage (20). The troponin I measurement has been complicated, because troponin I is released in circulation after tissue necrosis in several forms. These forms include free as well as various forms of complex-bound troponin I (21). Consequently, several different troponin I measurements have been possible and commercially available (22). On the other hand, only one troponin T measurement has been

commercially available, because the use of the troponin T antibody has been patented. But the troponin T measurement has also been complicated. This applies, especially, for the first generation of the troponin T reagent, which has been characterised by cross-reactivity of the label-antibody with skeletal muscle troponin T (14). This cross-reactivity may happen, if there has been a massive skeletal muscle damage.

Therefore, in our study, we have investigated the performance of both the troponin I and the troponin T parameters in relation to the conventional CKMB-markers and ECG changes for the detection of myocardial injury in patients undergoing major non-cardiac surgery. The conventional marker CKMB-activity has been elevated in twenty different patients, although for only two patients the diagnosis AMI could be established by the WHO-criteria. CKMB-mass, which has been known to be more sensitive for minor myocardial tissue damage than CKMB-act., has even been elevated in thirty-two different patients. The use of the non heart specific CKMB-activity as parameter for the detection of AMI has initially been resulted for one patient (patient C) in the unjustified diagnosis non Q-wave AMI, and, subsequently, in an unnecessarily prolonged stay of this patient at the ICU. The diagnosis AMI has been established because of 1. ECG changes, 2. history of coronary artery disease of this patient and 3. elevation of CK-total and CKMB-act. However, the CKMB-act measurement in the serum of this patient has been resulted in falsely elevated activities by the presence of the CKBB isoenzyme. This CKBB isoenzyme was caused by the bowel surgery. In contrast, all troponin I and troponin T concentrations of this patient C were below the url's at any time point.

The new markers troponin I and troponin T have been elevated in three and five different patients, respectively. This is more in agreement with the number of patients for who an AMI was diagnosed. As can be seen from table 2, this results also in significantly higher specificities and higher PPV's of the troponin I and troponin T parameters than those of the CKMB-act and CKMB-mass. As the number of AMI patients is (too) small compared to the number of non-AMI patients, it has no sense to calculate sensitivities and negative predictive values.

Two patients (D and E) have had ECG changes without elevations of the troponin I and troponin T concentrations. For these patients it should be considered, that changes of the 12-lead ECG do not necessarily have to be a result of AMI. Especially, in the first few hours following major injury, there is metabolic chaos and any ECG abnormality may reflect this (23). On the other hand, one patient (G) has had elevated troponin I and troponin T concentrations at three succeeding timepoints (t_2-t_4) without notification of ECG changes. The findings of the biochemical parameters strongly suggest that this patient experienced myocardial injury, although all routine ECG's were normal. The findings of these three patients are examples, from which it appears that ECG changes are not 100% specific for the detection of AMI.

The influence of the haemodynamic optimisation on the incidence of AMI after major non-cardiac surgery can not be statistically considered in this study. For this purpose much larger populations are needed. A sample size of 769 patients in the control group and 769 patients in the protocol group will be needed to detect a significant difference of 2% on the incidence of AMI. These sample sizes are based on a normal incidence of 3% AMI after major noncardiac surgery achieving a power of 80% given an alpha of 0.05. Unfortunately, it will take too much time to get such large populations.

From this study we conclude that troponin I and troponin T are more specific and reliable for the detection of myocardial injury after major noncardiac surgery than the conventional biochemical

markers CKMB-act. and CKMB-mass; furthermore, the measurement of troponin I or troponin T looks like to be more accurate than (discontinuous) ECG monitoring for the detection of myocardial injury after major noncardiac surgery.

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CHAPTER 9

The release of CKMB, troponin I and troponin T after coronary artery bypass surgery with or without cardiopulmonary bypass, and after aortic- and mitral valve surgery.

J.C.J.M. Swaanenburg[1], B.G. Loef[2], M. Volmer[1], P.W. Boonstra[2], J.G. Grandjean[2], M.A. Mariani[2], A.H. Epema[3].

[1] Department of Pathology and Laboratory Medicine,
[2] Department of Cardio Thoracic Surgery,
[3] Department of Anesthesiology, University Hospital Groningen.

Summary
We prospectively investigated the release patterns of the biochemical markers CK-total, CKMB-activity, CKMB-mass, troponin I (two different methodologies troponin I^{AxSYM} and troponin I^{Access}) and troponin T after various forms of cardiac surgery in patients without complications during the first 48 h after surgery. We studied patients undergoing coronary artery bypass grafting (CABG) with the use of cardio-pulmonary bypass (CPB) (group A, n= 36) or without CPB (group B, n=23). In addition, we analyzed cardiac markers in a group of patients undergoing aortic valve replacement (group C, n=14) or mitral valve replacement (group D, n=9). Preoperatively, all patients had normal renal, hepatic and cerebral function. Exclusion criteria included recent myocardial infarction, unstable angina and emergency procedures. Blood samples were collected before induction of anaesthesia (baseline), at the start of surgery, after release of aortic cross-clamping (CPB procedures), respectively opening of the graft(s) (procedures without CPB), admission to the intensive care unit, and at fixed daily moments (2am, 7am, 2pm, 9pm). The median areas under the curves of the different release patterns indicate that the values of all studied markers except CK-total, are the lowest in the CABG without CPB group. In the CABG with CPB sub-group of one or two anastomoses (n=10) CKMB-activity, CKMB-mass, and troponin T are lower compared to the three or more anastomoses sub-group (n=26). All markers except CK-total are lower in both CABG groups than in the aortic and mitral valve replacement groups. Comparison between the aortic and mitral valve replacement groups showed no difference in the examined markers. For most parameters, the highest 97.5 percentile values were observed at 6-8 hours after release of aortic cross clamp respectively opening of the graft(s). These values are for troponin I^{AxSYM} 23, 3.5, 44, 50 µg/l respectively for the A, B, C, D patient groups, for troponin I^{Access} 0.8, 0.15, 2.2, 2.2 µg/l respectively, for troponin T 0.6, 0.15, 1.0, 1.8 µg/l respectively, and for CKMB mass 34, 8, 80, and 80 µg/l respectively.
We conclude that the release patterns of cardiac markers after cardiac surgery depend on the type of and circumstances during surgery. Patients undergoing CABG without CPB show the lowest levels, whereas patients undergoing CABG with CPB show higher levels, and patients undergoing valve replacements show the highest levels.

Introduction

Perioperative myocardial infarction is a serious complication of open heart surgery resulting in increased morbidity and mortality. Myocardial infarction after coronary artery bypass graft (CABG) surgery occurs in at least 5% of the patients (1). It is important to detect the acute myocardial infarction (AMI) early, to initiate immediately an appropriate therapy. Currently, the diagnosis AMI is assessed by changes of the ECG and increase in the release of biochemical markers. However, changes of the ECG are not very sensitive and specific in the peri-operative period for detection of AMI. Also, the conventional biochemical marker, CKMB, which is still used as the gold standard for the detection of myocardial tissue damage, is not cardiac specific (2)
With the introduction of the new markers troponin I and troponin T, it has become possible to discriminate between myocardial and skeletal muscle damage. Troponin I and troponin

T are peptides and they are part of the actin-myosin complex of the thin filament of striated muscle. The amino acid composition of myocardial troponin I and troponin T differ from those of the skeletal isoforms. Antibodies directed against these specific sequences of cardiac amino acids have been developed to measure the cardiac troponins (3,4)
Cardiac surgery may be indicated for patients with coronary or valvular heart disease. Different surgical strategies have been developed to treat these patients including bypass surgery or valve replacement. Coronary artery bypass grafting (CABG) surgery can be performed with or without (minimally invasive CABG (MICAB)) the use of cardiopulmonary bypass (CPB or heart lung machine). In patients undergoing the CPB procedure (e.g. CABG and valve surgery) the heart is arrested and protected by cardioplegia. During this period the heart is ischemic. At the end of the CPB the heart is reperfused and the cardiac action is resumed. This reperfusion after the ischemic period results in myocardial damage and eventually in necrosis (5). In contrast, during MICABG the heart keeps beating, and thus reperfusion injury is avoided (6). Consequently, these types of cardiac surgical procedures may result in different release patterns of the biochemical markers for myocardial damage. Moreover, release of the markers after surgery may not only be caused by the surgery itself, but also by myocardial infarction. The cutoff values of the cardiac markers for AMI patients presenting with acute chest pain complaints have already been reported (7,8,9). In contrast, these values are not well established for patients after cardiac surgery.
The aim of this study is to investigate the release patterns of the biochemical markers CK-total, CKMB-activity, CKMB-mass, troponin I and troponin T after various forms of cardiac surgery in patients without complications during the first 48 h after surgery.

Patients and methods
This study was approved by the medical ethical committee of the University Hospital Groningen. After informed consent, we prospectively studied patients scheduled for different types of elective open heart surgery e.g. coronary artery bypass surgery (CABG) with (group A, n= 42) or without the use of cardio-pulmonary bypass (CPB) (group B, n=25), aortic valve replacement (group C, n=14) and mitral valve replacement (group D, n=9). Preoperatively all patients had normal renal, hepatic and cerebral function. Exclusion criteria included recent myocardial infarction, unstable angina and emergency procedures.
Anesthesia and CPB management
Anesthesia was performed according to a fixed protocol (10). Preoperative data include a clinical examination, routine blood screening and ECG. Premedication consisted of diazepam (10-15 mg orally) 2 hours preoperatively. All routine medication was continued except diuretics and digoxin preoperatively. After insertion of a peripheral venous line and radial arterial cannulation under local analgesia (lidocaine 1%), anesthesia was induced with sufentanil (1-3 µg/kg) and midazolam (0.05-0.1 mg/kg). Tracheal intubation was facilitated with pancuronium (0.1 mg/kg). A flow directed pulmonary artery catheter was inserted via the right internal jugular vein. Anesthesia was maintained with a sufentanil (0.1 µg/kg/hour) and a midazolam (0.1 mg/kg/hour) continuous infusion and pancuronium. Volatile anesthetics were not used. After induction hydroxyethyl starch 6% solution and ringer lactate solution were used to maintain mean arterial blood pressure (MAP) $60 mmHg) and

cardiac index $2.2 l/m^2. All patients operated with CPB received dexamethason (1 mg/kg) after the induction of anesthesia.
Standard CPB technique with hypothermia was used in all patients of group A, C and D. Nonpulsatile flow was performed with a roller pump (Stockert, Munchen, Germany) and membrane oxygenator (Cobe Laboratories, Lakewood, CO) The circuit was primed with hydroxylethyl starch 6% solution (500 ml) and ringer solution (1000 ml). Heparin was injected into the central venous port of the pulmonary artery catheter (3 mg/kg) to obtain an activated clotting time greater than 400 sec before cannulation of the aortic root and the right atrium or the superior and inferior caval vein. Flow during CPB was maintained at 2.4 L.min/m^2 with mild hypothermia (32 ^{o}C) as assessed by monitoring the nasopharyngeal temperature. Blood pH was regulated using "-stat management. For myocardial protection cardioplegic arrest during aortic cross clamping was obtained with cold (4 ^{o}C) St Thomas solution infused into the aortic root or retrogradely into the coronary sinus. Administration of cardioplegia was repeated every 30 min. MAP was maintained between 60-90 mmHg during CPB and, if necessary, corrected with nitroglycerin (to decrease MAP) or phenylephrine (to increase MAP). Heparin was neutralized with protamine (3 mg/kg for CPB, and 0.5 mg/kg for non CPB procedures) within 10 min after weaning from CPB. Mannitol and ultrafiltration were not used during the entire procedure. Patients were weaned from bypass using dopamine.
Perioperative ECG-monitoring consisted of three-lead (I, II, V5) continuous automated ST-T segment analysis (Marquette, Milwaukee,WI. Postoperatively, full 12-lead ECG-registration was obtained at admission to the ICU, and at day 1, and day 2. The diagnosis AMI was established according to the WHO-criteria; ECG-changes (new Q-wave > 0.4 seconds, ST-elevation in two or more leads > 0.1 mV, and a typical rise and fall of CKMB. After completion of the study patients were excluded from further analysis if they experienced re-operation, AMI, or periods of sustained supra- or ventricular arrhythmia.
Surgical procedure
Group A: *conventional CABG*. Cardiopulmonary bypass was instituted by means of cannulation of the ascending aorta and right atrium (two-stage cannula). The mammary arteries and the right gastroepiploic artery were used as pedicled grafts. Occasionally, the saphenous vein graft and the radial artery were used as free-grafts. Cardiac arrest was obtained with infusion of cristalloid cardioplegic solution in the aortic root, and in the free-grafts whenever present.
Group B: *"off-pump" CABG (11).* "Off-pump" CABG was performed through a midline sternotomy. The mammary arteries and the right gastroepiploic artery were used as pedicled grafts. Coronary anastomoses were performed by means of mechanical stabilization of the anastomotic site and temporary segmental occlusion of the target coronary artery.
Group C: *aortic valve surgery*. Cardiopulmonary bypass was instituted by means of cannulation of the ascending aorta and right atrium (two-stage cannula). Cardiac arrest was obtained with infusion of cristalloid cardioplegic solution both antegrade in the coronary ostia selectively and retrograde through the coronary sinus.
Group D: *mitral valve surgery*. Cardiopulmonary bypass was instituted by means of cannulation of the ascending aorta and of both vene cavae selectively. Cardiac arrest was obtained with infusion of cristalloid cardioplegic solution in the aortic root. A standard

longitudinal left atriotomy was used to reach the mitral valve.

Cardiac markers

Blood samples were obtained before induction of anesthesia (base line), at the start of the surgery, during the surgical procedure after release of aortic cross clamping for CPB procedures and opening of the graft(s) for operations without CPB. Postoperatively, blood was collected directly at admission to the ICU, at fixed daily moments (2am, 7am, 2pm, 9pm) and at the second day postoperatively, together with routine blood sampling for patient care. All sampling times were recalculated to the time after the start of the procedure (baseline sample). Blood samples were immediately centrifuged at 1000 g and subsequently serum was separated from the cells. CK total and CKMB activity were measured immediately. For the determination of CKMB mass, cardiac troponin I and cardiac troponin T, the serum samples were stored at -20 °C until analysis.

CK-total and CKMB-activity measurements were performed with a Vitros analyzer (Ortho, Beerse, Belgium). The upper limits of reference range are for men 70 U/L, for women 50 U/L and for CKMB-activity 10 U/L.

Troponin I was measured using an Access (7) analyzer (Beckman, Mijdrecht, The Netherlands) and an AxSYM (8) analyzer (Abbott Diagnostics Division, Hoofddorp, The Netherlands). The upper reference limit of the Access analyzer is 0.1 µg/l, whereas the upper limit of reference range for the AxSYM analyzer is 2.0 µg/l.

CKMB-mass and troponin T were measured on an Elecsys 2010 (9) analyzer (Roche, Almere, The Netherlands). The upper limit of the reference range for CKMB-mass is 5.0 µg/l and for troponin T 0.1 µg/l.

Statistical analysis

Patient characteristics are expressed as mean ± SD. Results of the release patterns of the examined biochemical markers are smoothed and expressed as the 2.5-, 50- and 97.5-percentile of the concentrations from the individual patients at the various time points according to NACB recommendations (12). The area under the curve (AUC) of the release patterns of the various biochemical markers from the individual patients were calculated with the program AUCv1.0 (University Hospital Groningen, Dep. Path. and Lab.Med., Groningen, The Netherlands) using the trapezium method (13).

In order to be able to compare the results of the AUC?s of the various markers, all test results were normalized by dividing the test result by the upper limit of the reference range of that particular marker (2). Differences between the median AUC of the four different heart surgery methodologies were analyzed using the Mann-Whitney U test and the Kruskal-Wallis test. $P < 0.05$ was considered to be statistically significant different.

Table 1. Patient characteristics.

Type of surgery	men n	age years mean (SD)	women n	age years mean (SD)
CABG with CPB	25	66 (9.8)	11	68 (11.2)
CABG without CPB 19	61 (14.4)		4	63 (5.7)
AVR	8	67 (9.2)	6	65 (15.0)
MVR	6	64 (13.9)	3	73 (7.6)

CABG: coronary artery bypass grafting; CPB: cardio-pulmonary bypass; AVR: aortic valve replacement; MVR: mitral valve replacement.

Results

Six patients from the CABG with CPB group were excluded because of AMI (n=2), atrial fibrillation (n=3) and re-operation (n=1). Data analysis is based on the remaining group consisting of patients without complications (total n=36). Two patients were excluded from the CABG group without CPB because of AMI (total n=23). The peri-operative period in the group of patients undergoing aortic valve (total n=14) and mitral valve replacement (total n=9) was uneventful. The patient characteristics are depicted in table 1.

In figure 1a and figure 1b the smoothed 2.5-, 50- and 97.5-percentile values of both troponin I methodologies, of troponin T, and of CKMB-mass are presented for the four groups of patients. For most parameters the highest measured values were at 6-8 hours after baseline. These values are for troponin I^{AxSYM} 23, 3.5, 44, 50 ìg/l respectively for the A, B, C, D patient groups, for troponin I^{Access} 0.8, 0.15, 2.2, 2.2 ìg/l respectively, for troponin T 0.6, 0.15, 1.0, 1.8 ìg/l respectively, and for CKMB mass 34, 8, 80, and 80 ìg/l respectively. For further analyses we subdivided the CABG with CPB population into two groups. The first group consisted of patients receiving one or two anastomoses (n=10), and the second group of patients received three or more anastomoses (n=26). The median areas under the curves with the corresponding ranges of the normalized release patterns from the examined biochemical markers after the various forms of heart surgery are summarised in table 2. From this table 2 it can be seen that the values for all measured markers, except CK-total, are statistically significantly lower ($p<0.05$) in the CABG without CPB group compared to the other types of surgery. In addition, in the CABG with CPB sub-group of one or two anastomoses CKMB-activity, CKMB-mass, and troponin T are statistically significantly lower compared to the three or more anastomoses sub-group. All markers except CK-total are lower in the two CABG groups than in the aortic and mitral valve replacement groups. Comparison between the aortic and mitral valve replacement groups showed no difference in the examined markers.

Table 2. Median area under the curve (range) of the normalised release patterns from the examined biochemical markers after various forms of heart surgery.

Parameter	CABG + CPB[a] 1-2 anas[e] n=10	CABG + CPB[a] $ 3 anas[e] n=26	CABG + CPB[a] all patients n=36	CABG - CPB[b] 1-2 anas[e] n=23	Aorta VR[c] n=14	Mitralis VR[d] n=9
CK-total	61 (29 , 153)	97 (30 , 1126)	90 (29 , 1126)	96 (21 , 267)	100 (17 , 354)	132 (26 , 257)
CKMB-act	12 (7 , 19)	17 (10 , 68)	16 (7 , 68)[$]	8 (3 , 14)[#]	25 (11 , 138)	45 (16 , 126)
CKMB-mass	83 (40 , 160)	108 (72 , 339)	104 (40 , 339)[$]	24 (8 , 44)[#]	196 (97 , 1280)	268 (181 , 530)
Troponin T	84 (18 , 166)[*]	124 (54 , 241)	106 (18 , 241)[$]	5 (2 , 41)[#]	198 (49 , 561)	285 (193 , 662)
troponin I (AxSYM)	111 (78 , 242)	164 (67 , 234)	151 (67 , 242)[$]	6 (1 , 55)[#]	246 (34 , 1022)	428 (322 , 732)
troponin I (Access)	116 (55 , 269)	134 (19 , 532)	122 (19 , 532)[$]	14 (1 , 48)[#]	288 (85 , 1621)	252 (146 , 736)

[a] Coronary Artery Bypass Grafting (CABG) with Cardio-Pulmonary Bypass (CPB);
[b] CABG without CPB;
[c] Aorta Valve Replacement;
[d] Mitralis Valve Replacement;
[e] number of anastomoses.

[*] statistically significant different from CABG + CPB $ 3 anastomoses;
[#] statistically significant different from all other methodologies;
[$] statistically significant different from Aorta VR and Mitralis VR.

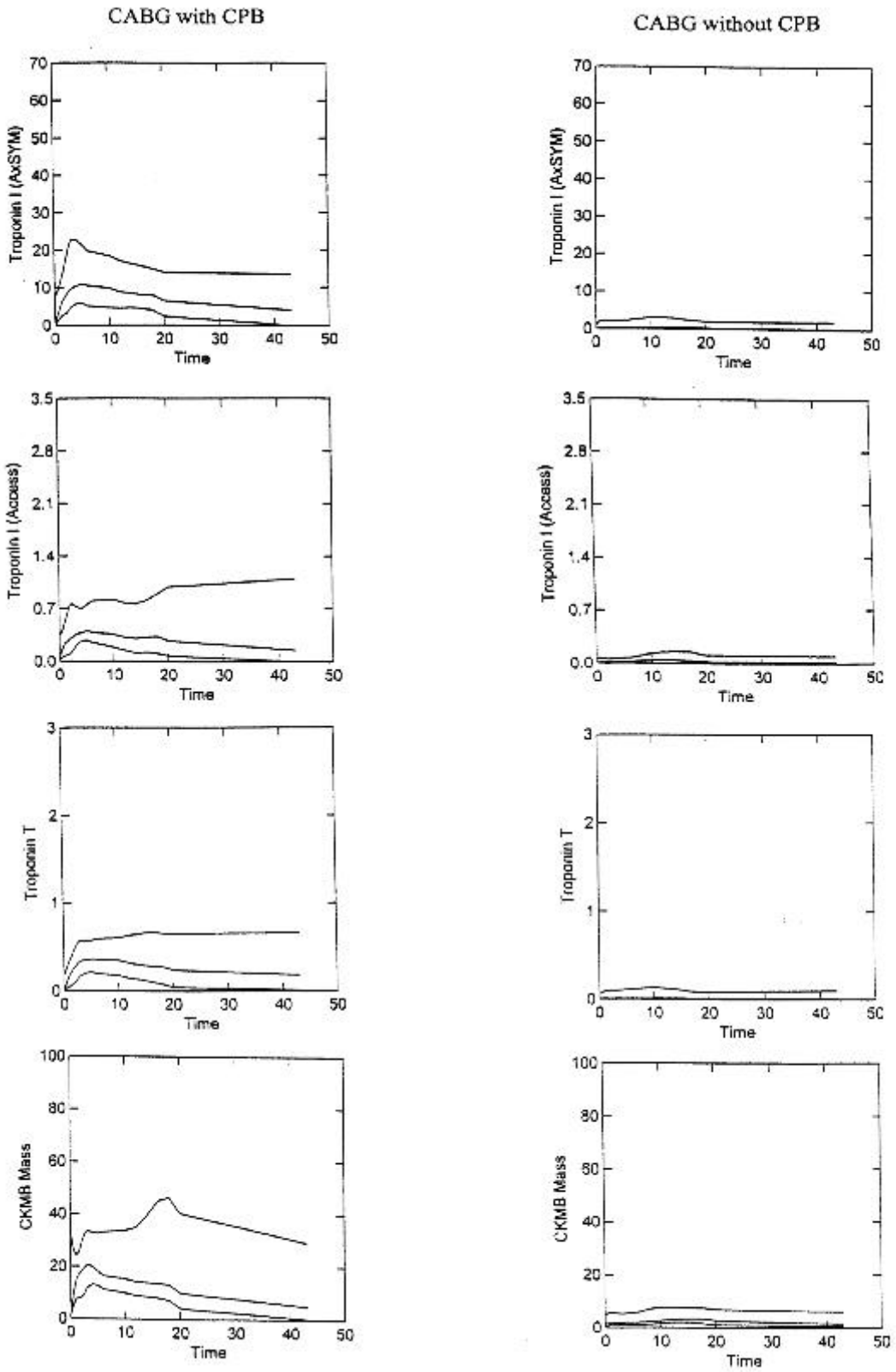

Figure 1a. The smoothed 2.5-, 50-, and 97.5-percentile values of the release patterns of two different troponin I methodologies, troponin T and CKMB mass (all concentrations in µg/l) are shown after coronary artery bypass grafting (CABG) with the use of cardiopulmonary bypass (CPB) and CABG without CPB. Time indicates hours after the start of the surgery.

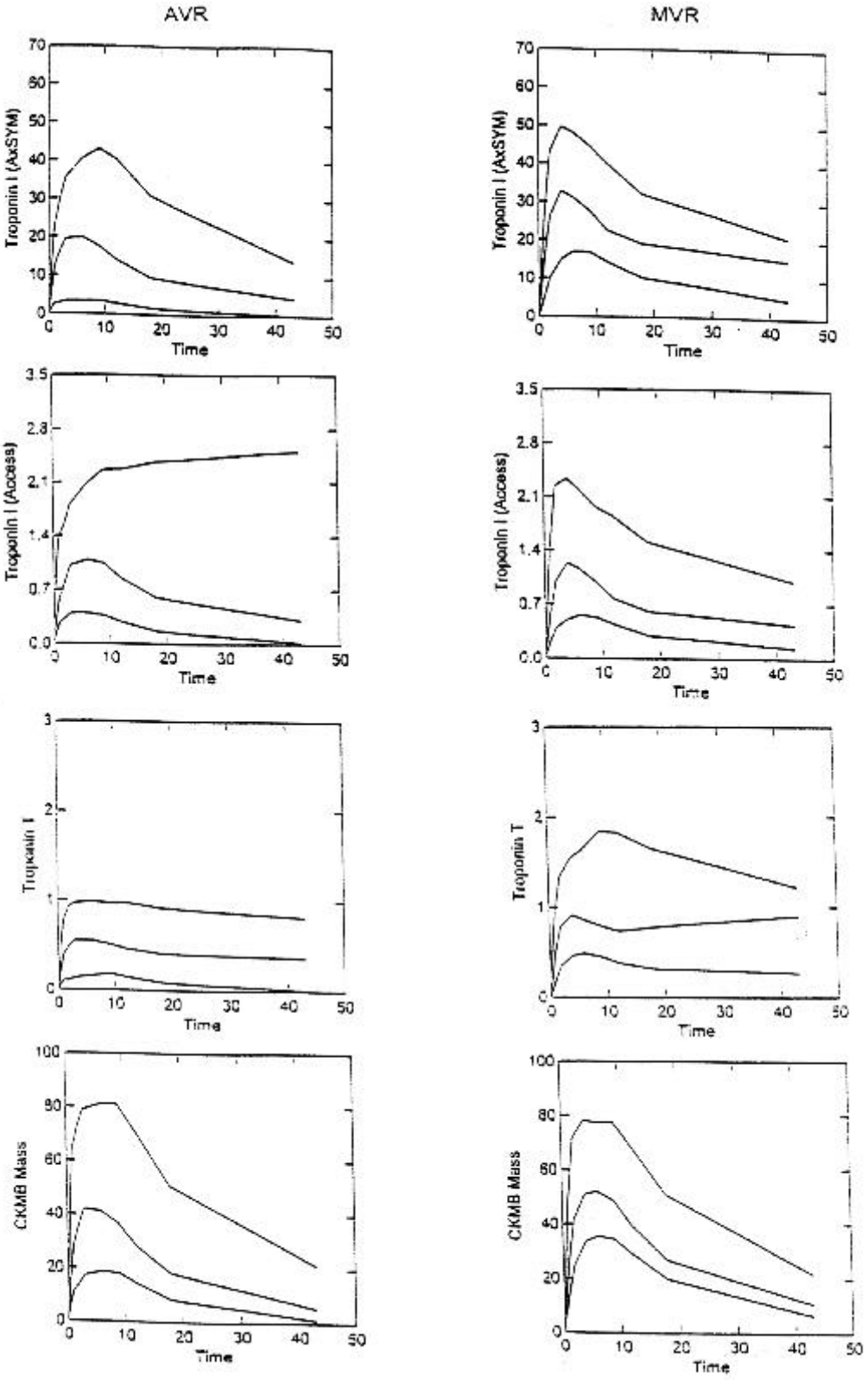

Figure 1b. The smoothed 2.5-, 50-, 97.5-percentile values of the release patterns of two different troponin I methodologies, troponin T, and CKMB mass (all concentrations in µg/l) are shown after Aortic Valve Replacement (AVR) and Mitral Valve Replacement. (MVR). Time indicates hours after the start of the surgery.

Discussion

This study demonstrates, that the release patterns of cardiac markers in patients after uncomplicated heart surgery are dependent on the type of surgery. Cardiac troponin I and cardiac troponin T are slightly increased from baseline in the group of patients after CABG without CPB. In contrast, all procedures using CPB resulted in significantly higher values of the release patterns of the examined cardiac markers. Release of troponins in CABG patients is lower than in valve surgery patients. Consequently, the measured 97.5 percentile values of the release patterns are different for the studied cardiac operations. These values increase after the start and reach the highest level after 6-8 hours. The lowest levels were obtained for the beating heart surgery group, the highest values for patients undergoing valve surgery whereas the values of the CABG with CPB patient group were in between.

For the detection of AMI in patients with chest pain, cut-off values are reported for all commercially available biochemical markers (7-9). In contrast, patients after heart surgery may experience a certain amount of myocardial injury. This injury is multifactorially determined including use of CPB, surgical technique, aortic occlusion and pre-existing coronary artery disease. This study clearly demonstrates that the release pattern of cardiac markers for each type of operation is different, Therefore, to detect AMI in the postoperative period dedicated cut off values related to the release time frame are necessary.

An indication of the amount of cardiac damage is reflected in the area under the curve of the release pattern of a cardiac marker (14). In order to compare the quantity of myocardial tissue damage for the various forms of surgery, the areas under the curves from the numerous release patterns were calculated using the trapesium method. As not all examined biochemical markers are expressed in the same units, all results were normalised by dividing the testresults by the upper limit of the reference range. The use of areas under the curves in order to compare different types of heart surgery is more reliable than comparing 'peak' (e.g. highest measured) concentrations, since the peak concentration does not have to be the real maximal concentration. Moreover, working with one sample collected at the estimated peak moment demands fixed time intervals from intraoperative events, i.e. opening cross clamp respectively opening grafts. Fully individualized blood sampling is impractical in an intensive care unit. Most often routine blood collection in the ICU takes place at fixed protocollized time points. Therefore working with area under the curves is not only less complicated but also more accurate. However, for a reliable estimation of the AUC at least 3 to 4 sample points are necessary (for instance every 6 hrs during the first 24 hrs postoperatively). Table 2 shows that patients undergoing CABG without the use of CPB have the lowest areas under the curves values. Cardiac markers did hardly change from baseline and thus these patients did hardly experience myocardial damage. The other groups experienced a certain amount of damage. The areas under the curves from the patient category CABG with CPB are statistically significant lower than those for the mitral valve and aorta-valve replacement. A number of commercial methodologies is available for the determination of cardiac troponin I whereas there is only one manufacturer for the patented troponin T. This study shows that all types of surgery except the beating heart surgery result in elevation beyond the upper limit of the reference range of each cardiac marker concentration, and thus, in measurable cardiac damage as a result of the surgical procedure

itself. Comparison between the AUC's of the different surgery groups shows, that all examined cardiac markers except CK-total can differentiate between the types of surgery. These data show evidently, that CK-total is not applicable for discrimination between the various groups. In contrast to both troponin I methodologies, can troponin T discriminate between the CABG patients with 1 or 2, and the CABG patients with 3 or more distal grafts. This finding suggests that troponin T may be clinically more sensitive to monitor myocardial damage in the peri-operative period.

So far, most studies reported results from heterogeneous groups of patients undergoing bypass and/or valve replacement surgery. Furthermore, no discrimination has been made between patients with and without complications or only one new cardiac marker has been investigated.

Banning et al. (15) reported the release patterns of cardiac troponin T, CK and CKMB-isoenzyme after coronary bypass graft surgery. For cardiac troponin T peak values were reported of 3.5 µg/l. These values are higher than we found, however, they used the first generation cardiac troponin T reagent, which was known to be susceptible to interference by skeletal muscle tissue. Etievent et al. (16) investigated cardiac troponin I values after aortic valve replacement and after CABG. In contrast to our findings, they reported higher values for the CABG group than for the aortic valve replacement group at 6 and at 12 hrs. Harff et al. (17) reported the results of several biochemical assays after CABG. Most of these markers concern the conventional cardiac enzymes and from the new parameters only the (first generation) cardiac troponin T was measured. For this latter marker a 90th-percentile value of 0.79 µg/l was reported, which is in good agreement with our reported 97.5th-percentile value of 0.7 µg/l. Gensini et al. (18) investigated the release patterns of cardiac troponin I in forty-two patients undergoing CABG. Eight patients experienced AMI and these patients had higher cardiac troponin I values than the remaining uncomplicated group of patients. Moreover, the discriminative power of troponin I was higher than that of the CKMB-isoenzyme. The results of this study cannot be compared with our results, since they use an other cardiac troponin I methodology. Alyanakins et al. (19) investigated forty-one (CABG, n=17; valve replacement, n=24) patients undergoing heart-surgery. Post-operatively these patients were subdivided into three groups. Group 1 consisted of five patients with Q-wave myocardial infarction, group 2 contained twelve patients with nonspecific ECG changes and/or need of inotropic support and group 3 (n=24) showed no complications. For group 1 patients significant higher values of cardiac troponin I, which methodology was different from the methodology we used, were reported than for group 3 patients. Group 2 patients showed peak cardiac troponin I values between those of group 1 and group 3. The investigators concluded that cardiac troponin I might be useful for the diagnosis of perioperative myocardial infarction.

In conclusion we report that the release patterns of cardiac markers after heart surgery depend on the type of and the circumstances during surgery. Highest values are reached 6-8 hours after start of the procedure. Patients undergoing CABG without CPB show the lowest levels, whereas patients undergoing CABG with CPB show higher levels, and patients undergoing valve replacements show the highest levels.

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CHAPTER 10.1

MONITORING OF IMPENDING MYOCARDIAL DAMAGE AFTER PLEURO-PNEUMONECTOMY AND INTRAOPERATIVE PHOTODYNAMIC THERAPY FOR MALIGNANT PLEURAL MESOTHELIOMA USING BIOCHEMICAL MARKERS

J.M. Klaase[1], J.C.J.M. Swaanenburg[2], H. Schouwink[3], M.N. Sosef[1], J.M.G. Bonfrer[4], F.A.N. Zoetmulder[1], E.J.Th. Rutgers[1], P. Baas[3]

Departments of [1]Surgery,
[3]Medical Oncology,
[4]Clinical Chemical Laboratory, The Netherlands Cancer Institute (Antoni van Leeuwenhoek Huis), Amsterdam,
[2]Pathology and Laboratory Medicine, University Hospital Groningen, Groningen.

Photochemistry and Photobiology 2000; 71: 351-354.

Summary
In five patients who were treated for malignant pleural mesothelioma (MPM) with pleuropneumonectomy and intraoperative photodynamic therapy (PDT), impending myocardial damage was monitored using ECG, the classical biochemical markers (Creatine Kinase (CK)-total activity, CKMB-mass, and myoglobin), and the new cardiac markers troponin I and troponin T. No severe myocardial damage could be encountered after pleuropneumonectomy and PDT. From this study in patients with MPM treated with pleuropneumonectomy and PDT it can be concluded that measurement of troponin I or T for the detection of myocardial damage is more suitable than measurement of the classical markers, because the classical markers are all elevated by the concomitant skeletal muscle damage.

Introduction

Malignant pleural mesothelioma (MPM) is in most patients in an advanced stage at the time of diagnosis. It is considered to be uncurable because at that time the tumor diffusely spreads into the pleural space (1). Therefore, microscopically radical resection can seldom be performed. In patients who are eligible for operation, adjuvant regimens consisting of chemotherapy or radiotherapy have been tested, without consistent survival benefit. Recently, intraoperative photodynamic therapy (PDT) was shown to be applied safely as an adjuvant therapy in a feasibility study from our institute (1).

One of the side effects of the PDT is damage to the normal intrathoracic structures like esophagus and heart. For the determination of (minor) myocardial damage, conventionally, the CKMB-isoenzym is used as the gold standard (2). However, CKMB is not heart-specific. Recently, cardiac troponin I (cTnI) and cardiac troponin T (cTnT) have been introduced as fully heart specific markers (2).

The troponins consist of a group of three subunits in the troponin-myosin complex on the thin filament of muscle myofibrils. They are involved in the regulation of muscle contraction and relaxation. Troponin T is the tropomyosin-binding subunit, which binds troponin I and troponin C to the tropomyosin. Troponin I is the actomyosin ATPase-inhibiting subunit and regulates the relaxation, whereas troponin C, the calcium-binding subunit, causes contraction. Only troponin I and troponin T have cardiospecific isoforms. Troponin T was introduced first; however, most studies indicate that cTnI is as specific as or even more cardiospecific than troponin T. In the sera of healthy volunteers both cTnI and cTnT are hardly detectable. Therefore, its appearance in blood is considered to be a clear signal of cardiac myocite damage (2,3).

For reasons of the already mentioned heart-specificity, the new cardiac markers are thought to be more suitable than the classical biochemical markers (CK-total activity, CKMB and myoglobin) for the detection of myocardial tissue damage. This is especially true in patients with concomitant chest walll muscle damage (2), which can be expected in patients treated with pleuropneumonectomy and PDT.

The aim of this study was to investigate impending myocardial damage in patients with MPM treated with pleuropneumonectomy and intra-operative PDT, using the classical biochemical markers and compare them with the new markers cTnI and cTnT.

Patients and methods

In the period october till december 1998, five consecutive patients with MPM, who were eligible for

surgery, were treated with pleuropneumonectomy followed by intra-operative PDT. Patient and treatment characteristics are given in table 1. Our treatment protocol for intraoperative PDT was published in detail before (1).
Blood samples were collected for the analysis of CK, CKMB-mass, myoglobin, cTnI and cTnT at the start of the operation, after finishing the pleuropneumonectomy, at the end of the PDT, and, subsequently, 2, 4, 8, 12, 20, 44 hours postoperatively. At the same time intervals also an electrocardiogram (ECG) was made. In case of suspicion of pericardial effusion, a cardiac ultrasound was performed. ECG and cardiac ultrasound were reviewed by a cardiologist.

Surgery

The operation consisted in all patients of a right pleuropneumonectomy. To reduce toxicity to the normal tissues, the theatre lights were out of focus and the normal skin was completely covered with sheets. The surgical goal was to achieve a macroscopically radical resection. In areas unsuitable for radical resection, a tumor reduction to less than 5 mm thickness was performed. The diaphragm and pericardium were spared as much as possible.

Photodynamic Therapy

The photodynamic treatment was performed with light of 652 nm from a high power diode laser, and meta-tetrahydroxy phenylchlorin as the photosensitizer, which was injected intravenously 96 hours before. The light delivery to the thoracic cavity was monitored by in situ isotropic light detectors (6 watt Applied Optronics, USA). The light detectors were placed on strategic places in the thoracic cavity to enable an optimal light distribution from a pseudospherical light bulb which was placed in the center of the cavity. One probe was fixed on or near the pericardium. Using these calibrated isotropic light detectors both direct light and reflected light could be measured. The total hemithoracic surface was illuminated with 10 J/cm^2. On average the fluence rate varied from 5-25 mW/cm^2.
At the end of this PDT procedure, one drain was left in the thoracic cavity. Postoperatively all patients recieved oral anticoagulants and digoxin as standard procedure.

Biochemical Markers

CK-total activity (upper reference limit 70 U/L) was measured with a Vitros 750C analyser (Ortho Clinical Diagnostics, Beerse, Belgium).
Myoglobin (upper reference limit 70 ng/ml) measurements were performed using a BNII-nephelometer (Dade-Behring, Leusden, The Netherlands).
For cTnI measurements (upper reference limit 2.0 ng/ml) an AxSYM Analyser (Abbott Diagnostics Division, Hoofddorp, The Netherlands) was used.
CKMB-mass (url 5.0 ng/ml) and cTnT measurements (ureference limit 0.1 ng/ml) were performed on an Elecsys 2010 analyser (Roche, Almere, The Netherlands) using 'second generation' cTnT antibodies (4).

Table 1. Patient and treatment characteristics and postoperative cardiac events

no	ge	age	site	diagnosis	treatment	car-diac even

						ts
1	m	64	R	epitheloid mesothelioma	pleuropneumonectomy PDT 10 j/m^2	minor pericardial effusion
2	f	54	R	mixed type mesothelioma	pleuropneumonectomy PDT 10 j/m^2	minor pericardial effusion right bundle branch block
3	m	52	R	epitheloid mesothelioma	pleuropneumonectomy PDT 10 j/m^2	atrial fibrillation
4	m	52	R	mesenchimal mesotheliomal	pleuropneumonectomy PDT 10 j/m^2	minor pericardial effusion
5	m	60	R	mesothelioma unspecified	pleuropneumonectomy PDT 10 j/m^2	minor pericardial effusion

Results

The median duration of the operation was 5 (range 4-6) hours, including one hour PDT time (20 minutes of illumination and 20-40 minutes of preparation time). During the operation no complications occurred. The mean blood loss was 1 (range1-2) liter. No serious cardiac side effects occurred in the early postoperative period (day 1-2) (table 1). In 4 of the 5 patients a small amount of pericardial effusion was detected on the second postoperative day, but this did not result in therapeutic interventions. In 1 patient an atrial fibrillation was detected on the second post-operative day despite digoxine treatment. Conversion to a normal sinus rythm was achieved by treatment with cordarone. In one patient a right bundle branch block was detected on the ECG one day after the operation.

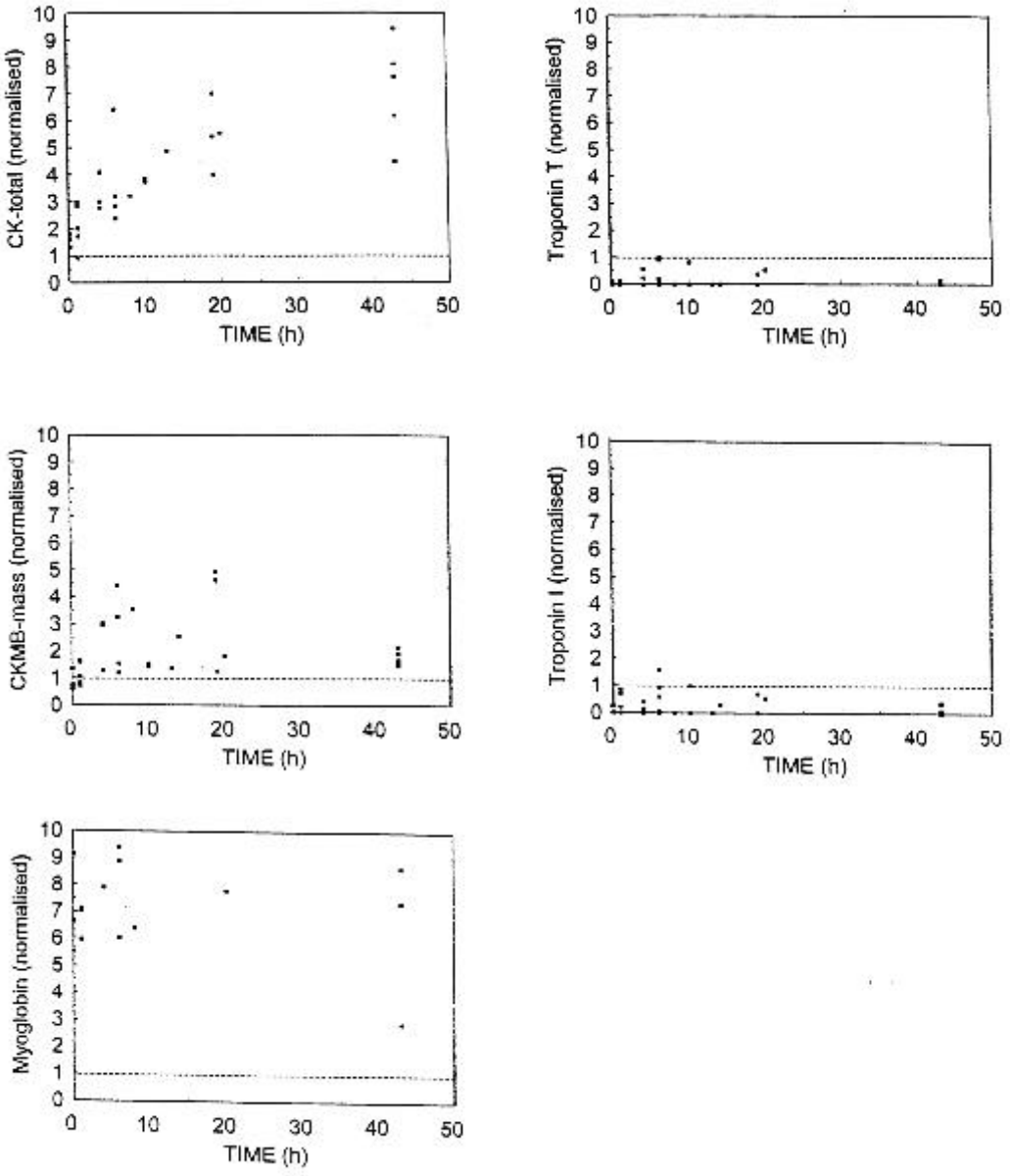

Figure 1. The release patterns of the normalised CK-total, CKMB-mass, myoglobin, cardiac troponin I and cardiac troponin T in 5 malignant mesothelioma patients undergoing pleuropneumonectomy and subsequent intraoperative photodynamic therapy.

The test results of the biochemical markers are normalised by dividing the test result by the corresponding upper reference limit of that particular marker in order to be able to compare the results of the various markers. The normalised results of the biochemical parameters after pleuropneumonectomy and intra-operative PDT are shown in figure 1. From this figure it can be concluded, that the examined classical parameters CK-total, CKMB-mass and myoglobin are all elevated after operation, whereas only one troponin I measurement (patient 5) and no troponin T measurements exceed the upper limit of reference range (illustrated by the solid line at level 1).

Discussion

The use of PDT after pleuropneumonectomy has been shown to be feasible in MPM patients (1). Allthough there is a potential danger of damage by PDT to the normal intrathoracic structures (like esophagus and heart) on theoretical grounds, sofar, no serious cardiac side effects have been reported in the direct postoperative period. Only one study (5) is reported in the literature, which evaluates serial cardiac enzymes and ECG's after PDT for Barrett's esophagus in 12 patients. This study was carried out because atrial fibrillation after esophageal PDT had been reported in an earlier study. No significant or permanent abnormality was noted in the examined cardiac enzymes (CK-total, CKMB, LDH) or in the ECG's. Only one patient had transient atrial fibrillation during a follow-up endoscopy. However, in contrast to the present study the new markers cardiac troponin I and cardiac troponin T were not examined.

Because of their excellent cardiac specificity, troponin I and troponin T appear ideally suitable to discriminate between myocardial and skeletal muscle damage. This concerns for example non-cardiac surgery patients, like the patients in the present study, trauma patients or patients with chronic muscular disease, or after intense physical exercise (2,6,7).

The sensitivity of both troponins is at least equal to the known classical myocardial markers. The serum concentration of cTnI or cTnT seems to be the best biochemical marker for the detection of minor myocardial damage (8). Recently, for reasons of an insufficient sensitivity in the first hours following acute coronary syndromes, it was recommended to use another, more early myocardial marker, like myoglobin, in the cardiac panel for routine laboratory testing (9,10).

In the present study the examined classical markers CK-total activity, CK-MB mass and myoglobin were all elevated from the end of the PDT session on, whereas only one troponin I measurement was above the reference limit and no troponin T measurements were elevated. With the knowledge of the -at least- equal sensitivity of the classical markers and the troponins, it should be assumed that the classical markers were above their reference limits because of non-cardiac muscle damage. This can be explained by the trauma as a consequence of the operation and the intra-operative PDT. The finding, that only one troponin I concentration was minimally elevated and that no troponin T concentrations were elevated after operation and PDT, is in concordance with the fact that no serious cardiac side-effects were encountered in these patients. In only one patient there was a short period of atrium fibrillation, which may have been provoked by the right pneumonectomy itself instead of the PDT.

In conclusion, no serious myocardial damage could be detected as a consequence of pleuro-pneumonectomy and intra-operative PDT in patients with MPM. The performance of the new cardiac markers troponin I and troponin T seems to be more reliable for the detection of (minor) myocardial damage than the classical parameters CK-total activity, CKMB-mass and myoglobin,

because the classical markers were all elevated as a consequence of the concomitant skeletal muscle damage.

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CHAPTER 10.2

Troponin I, Troponin T, and Creatine Kinase-MB mass in patients with the carcinoid syndrome with and without heart failure.

W.G. Meijer[1], J.C.J.M. Swaanenburg[2], D.J. van Veldhuisen[3], I.P. Kema[2], P.H.B. Willemse[1], E.G.E. de Vries[1].

Department of Medical Oncology[1],
Pathology and Laboratory Medicine[2],
Cardiology[3], University Hospital Groningen.

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Carcinoid heart disease is a well known complication of longstanding carcinoid syndrome. It is characterized by the presence of "carcinoid plaques" on the mural endocardium (1). The carcinoid plaques are composed of smooth muscle cells, embedded in a stroma of acid mucopolysaccharides and collagen. In the plaques the elastine fibre content is decreased, the basal membrane of the endocardium is thickened and sometimes duplicated (2). Carcinoid plaques are predominantly found in the right heart (1), leading to pulmonary and tricuspid valve abnormalities. With echocardiography tricuspid valve regurgitation is found in 56% of patients with a carcinoid syndrome (3). A correlation of echocardiographic abnormalities with (high) serotonin secretion by the carcinoid tumour has been described (3). Heart failure was a cause of death in 41% of 63 midgut carcinoid patients (4). In patients with a carcinoid syndrome, median survival was significantly reduced by the presence of cardiac involvement (5).

Serotonin is a potent vasoconstrictor (6) and can lead to diminished myocardial blood flow (7). Plaque formation itself possibly affects the underlying myocardium. Furthermore, distention of the right heart, resulting from valve abnormalities, could lead to myocardial damage. Troponin I, troponin T, and the creatine kinase (CK) MB isoenzyme are released into the circulation after myocardial damage. Troponin I was reported to be a parameter with higher sensitivity and specificity than the conventional marker CKMB-mass for the detection of minor ischemic myocardial injury (8). Moreover, troponin I and troponin T are markers for risk stratification in patients with acute coronary syndromes. Furthermore, in chronic, non-ischemic cardiac conditions, such as idiopathic dilated cardiomyopathy, increased troponin T concentrations were found to correlate with a short term unfavourable prognosis (9), and cardiac troponin T has been reported to be progressively released in advancing stages of heart failure (10).

We, therefore, we analysed troponin I, troponin T, and CKMB-mass to detect myocardial damage in patients with carcinoid syndrome, who are exposed to increased concentrations of circulating serotonin. The outcomes of the troponin I, troponin T and CKMB-mass measurements were compared between the patients with and without heart failure, and between echocardiographic subgroups.

We investigated 20 consecutive patients (9 men, 11 women) with histologically confirmed midgut carcinoid tumours, and a clinical carcinoid syndrome. The median age was 57.5 years (range 43-74 years). In all 20 patients systemic carcinoid symptoms had been present for 9-154 months (median 72 months) and all had metastatic carcinoid disease. A standardized questionnaire was used for assessment of cardiovascular symptoms. Ten of the 20 patients had symptoms of heart failure (dyspnea (n=4), ankle edema (n=4), orthopnea (n=1) and nycturia (n=5)); 6 of these patients were classified as New York Heart Association class II, and 4 as class III heart failure. None of the patients had a history of precordial pain and electrocardiography revealed no signs of myocardial ischemia in any of the patients. Echocardiography was performed using a two-dimensional technique with color flow imaging. All echocardiographic investigations were interpreted by one experienced cardiologist. The patients were divided into three groups, according to the echocardiographic results. Group I consisted of patients with a normal echocardiogram. Patients were placed in group II if they met one of the following criteria: tricuspid regurgitation, right atrial enlargement, or inferior caval vein collapsing to < 50% of maximal

diameter during inspiration. Patients in group III fulfilled two or three of these criteria. Echocardiography was normal in six patients (group I). In eight patients slight abnormalities were detected (group II), and five patients showed overt carcinoid heart disease (group III). In one patient, transthoracic echocardiography was not feasible.

Urinary 5-hydroxyindoleacetic acid concentrations were determined in ether extracts by HPLC with fluorometric detection and expressed in mmol/mol urinary creatinine (11). All 20 patients showed an increased excretion of urinary 5- hydroxyindoleacetic acid (median 16.5 mmol/mol creatinine, upper limit of reference range, 3.8 mmol/mol creatinine).

Troponin I was measured with an AxSYM(TM) analyser (Abbott Diagnostic Division). Troponin T and CKMB mass analyses were performed using an Elecsys 2010(TM) analyser (Roche). Troponin T was measured with both second and third generation troponin T reagents. In the third-generation procedure, the calibrators are of human origin, leading to more accurate results (12). The cut-off values were 2.0 µg/L for troponin I, 0.1 µg/L for troponin T, and 5.0 µg/L for CKMB mass.

The results of the troponin I and troponin T measurements for all carcinoid patients were below the detection limits of the AxSYM (<0.2 µg/L) as well as the Elecsys 2010 (<0.01 µg/L) analyzers. The CKMB mass concentrations (0.3 - 2.4 µg/L) were also within the reference limits. The 10 patients with clinical heart failure and the 5 patients with overt carcinoid heart disease on echocardiography (group III) also showed no detectable troponin I and troponin T concentrations. In these subsets of patients CKMB-mass ranged from 0.7 to 1.3 µg/L.

From these findings we conclude that patients with carcinoid syndrome have no detectable signs of myocardial damage, if the new and sensitive markers are used. Even patients with *(a)* prolonged exposure to high serotonin levels, *(b)* clinically observable heart failure, and *(c)* echocardiographic evidence of carcinoid heart disease, show no detectable troponin I and troponin T concentrations. This might be explained by the following. In carcinoid syndrome, there is right ventricular failure attributable to the involvement of the pulmonary and tricuspidal valve, whereas the myocardium itself is not primarily involved. Furthermore, the mass of the right ventricle is small compared with the left ventricle. Therefore, myocardial damage may be too small to lead to increases in troponin concentrations in the general circulation.

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CHAPTER 11

Cardiac markers: state of the art and future perspectives.

Current state of the art

Conventionally, the cardiac markers play an important role in the (sub)acute detection of myocardial damage. For diagnostic purposes, they are commonly used, especially, in patients with acute ischemic syndromes. However, the 'gold standard' CKMB-isoenzyme, whether measured as activity or mass, has appeared to be non-heart specific. Subsequently, an acute myocardial infarction (AMI) may be "under-" or "over-" diagnosed.

By the end 1980's the highly cardiac specific troponin T has become available as a biochemical utility to detect myocardial tissue damage. However, it took several years before cardiac troponin T was accepted as a specific cardiac marker. First, because it was a new parameter, whereas CKMB had been widely accepted and used already for several decades ('unknowned means unbeloved'). Second, the first generation cardiac troponin T reagent was not completely heart specific, as the label-antibody used in the ELISA methodology showed 20% cross reactivity with skeletal muscle tissue. Several years after the introduction of cardiac troponin T, cardiac troponin I was introduced as a possibility for the detection of cardiac damage, because the use of cardiac troponin T was patented.

The interpretation of cardiac troponin I levels from different reagent manufacturers is complicated, because the various manufacturers of this marker use both different antibodies as well as different calibration standards. The standards vary in their compositions regarding the various troponin I-C-T related complexes. Furthermore, uniformity of troponin I standards is complicated, because, after cell necrosis, troponin I exists of several forms in blood circulation (most of them are the complexes troponin I-C and troponin I-C-T). Moreover, the troponin I molecule is not stable in circulation as it is susceptible to proteolysis, resulting in degradation of the molecule. To overcome the standardisation problem, an AACC-subcommittee was installed to make recommendations for uniformity of the cardiac troponin I analysis.

During the first part of the last decade attention was focussed on the performance of cardiac troponin T and cardiac troponin I as marker for the detection of AMI. Although both markers have a relatively low molecular weight, and, subsequently, may fast released from damaged cells, it turned out that both cardiac troponin T and cardiac troponin I were not the best 'early' biochemical markers for the detection of AMI. The reason is that both troponins are myofibril bound, and, therefore, it takes more time than for the cytosol linked parameters (such as myoglobin) to be elevated in circulation after myocardial cell necrosis. As is reported in chapters 4 and 5 the CKMB2/CKMB1 isoform ratio is more suitable as cardiac marker for early detection of AMI. However, the analytical procedure is more complex than it is for troponin analysis.

Besides the assessment as marker for the detection of myocardial cell damage, also studies were carried out to investigate the performance of cardiac troponin T and cardiac troponin I as markers for prognostic purposes in patients with ischemic syndromes including (un)stable angina pectoris ('risk stratification'). As the first reported results look very promising more attention was focussed on this subject. Moreover, more or less at the same time, the cardiac troponin I and cardiac troponin T methodologies were improved. The troponin T methodology was improved, because, first, the label-antibody was further developed towards a 100% heart-specificity, and, second, calibration standards of human instead of bovine origin were introduced. Furthermore, the sensitivities of the cardiac troponin T and cardiac troponin I assays, especially in the low detection region, were enhanced. Consequently, this resulted in both a better accuracy and precision, which is, concerning the low concentration area, the most important for risk stratification.

The goal of measuring cardiac troponin T and cardiac troponin I has been extended from a particular marker for AMI to a marker of prognostic value for patients with acute coronary syndromes presenting with acute chest pain. Typically for this category of patients is the introduction of the so-called 'Observation Unit Department'. The purpose of this department is to improve and shorten the Triage-process of patients who are presenting with chest pain complaints at the Emergency Department, but for whom, at presentation to the hospital, a reliable diagnosis can not be made according to established criteria. Conventionally these patients were directly admitted to the Coronary Care Unit (CCU), where they were observed for at least 24 hours. About 75% of these patients did not experienced AMI and, therefore, they might unnecessarily be admitted to the CCU. This resulted not only in an unnecessarily emotional experience for the patient, but also in an increase in costs. With the introduction of the Observation Unit, it becomes possible to postpone the decision about admission to the CCU for maximally several hours until the final diagnosis has been established. In daily medical practice this includes, that, after arrival at the hospital, the patient will stay in the Observation Unit. Here, the ECG of the patient is continuously registered and during this several hours period also the troponin level is measured. At least 8 hours after the onset of anginal complaints an AMI is ruled out when the ECG has not been changed and the troponin concentration is still below the upper limit of the reference range or has not changed significantly. Subsequently, the patient does not have to be admitted to the CCU and may be discharged. This procedure will yield to a more efficient use of the CCU. In this respect it is important that the turn around times (TATs) of cardiac marker results are within one hour after blood collection. If the central laboratory cannot fulfil this requirement, point-of-care (POC) testing might be implemented according to recommendations of the National Academy for Clinical Biochemistry (NACB). If POC-testing is implemented it should be equipped to report cardiac marker results quantitatively. This is a necessity, as, most of the time, serial measurements will be performed, so that trend analysis may be used for interpretation purposes. As is reported in chapter 6, the Stratus CS and Triage Cardiac Panel are such POC-testing devices. After completing the study described in chapter 6, the Stratus CS was installed at the CCU for daily clinical practice. It turned out that non-analytical educated personnel can reliably perform the cardiac marker tests. Moreover, the troponin I results were more useful for classifying patients with acute chest pain than the "conventional" CKMB results.

Besides the application of measuring cardiac troponin in patients with myocardial ischemia, it appeared that both cardiac troponin I and cardiac troponin T were more sensitive and more specific markers than CKMB for the detection of myocardial damage in patients with accompanying skeletal muscle damage. This category of patients includes patients after (blunt) trauma, after surgery, and after other invasive procedures causing myocardial tissue damage.

In chapter 7 the results are reported of a study concerning patients experiencing blunt trauma. From this study it was concluded that the cardiac troponin I and cardiac troponin T are more reliable for the detection of myocardial damage than the conventional CKMB parameter. In particular, the conventional CKMB was more frequently elevated than the cardiac troponins were. This may be explained, because, on the one hand, CKMB is also part of skeletal muscle, whereas cardiac troponin I and cardiac troponin T are unique for myocardial muscle tissue. On the other hand, both new markers are more sensitive for minor myocardial damage, because per gram heart tissue the content of troponin is higher than that of CKMB. Moreover, cardiac troponin is, in contrast to CKMB, in healthy human beings hardly detectable in circulation. This means that minimal detectable concentrations of cardiac troponins in circulation are suspicious for myocardial tissue damage.

Therefore, it can be concluded that the role of CKMB as "gold standard" for myocardial damage has to be replaced by cardiac troponin I or cardiac troponin T. In this respect, the role of CKMB may have been become more or less obsolete and, consequently, this parameter should be removed from the request forms for blood investigation in routine (biochemical) patient care.

Future perspectives

Patients with an increased risk on cardiac events may be identified by (slightly) elevated concentrations of cardiac troponin I or cardiac troponin T. Therefore, efforts should be taken to prevent that they experience an irreversible ischemic event. This strategy may be executed either by treatment with drugs or by making use of invasive techniques.

A promising marker for reversible myocardial ischemia may be glycogen phosphorylase BB (GP-BB). This non-heart specific enzyme plays a role in the glycolytic pathway. In contrast to all other biochemical markers, GP-BB may be already released into the circulation during periods of reversible ischemia. In addition, like the other markers, GPBB can also be measured in increased levels in circulation following cell necrosis.

Another line of investigation may be the role and use of cardiac markers together with coagulation parameters for patients with acute coronary syndromes. However, it is crucial, that all indices can reliably and quantitatively be measured 24 hours a day, 7 days a week, with a turn-a-round time of preferably less than 30 minutes, but definitely less than 60 minutes. In this respect, it will probably turn out that the best way to fulfill this requirement will be the use of point-of-care equipment.

Another issue to investigate may be the content of cardiac markers within the human heart in relation to heart failure. The marker brain natri-uretic peptide (BNP) may, in respect to heart failure, also be a promising parameter for diagnostic purposes.

Which cardiac troponin parameter (I or T) should be used in clinical practice? So far, only in patients with end-stage renal disease different results have been reported for cardiac troponin I compared with cardiac troponin T. The levels of cardiac troponin T are more frequently elevated compared with cardiac troponin I. To date, however, there is no consensus which parameter is more reliable in this respect. Several explanations have been reported to explain the difference between the troponin I and troponin T results in patients with end-stage renal failure, varying from real myocardial tissue damage to reexpression of cardiac troponin T in skeletal muscle. In this respect, important evidence has been provided by Apple et al. (Clin Chem December 1999). They concluded that cardiac troponin T mRNA was reexpressed in skeletal muscle of end stage renal disease patients.

As mentioned previously, the use of cardiac troponin T has been patented by Roche™ (the former Boehringer Mannheim™). So, this marker can only be measured with equipment of this particular manufacturer. In contrast, cardiac troponin I can be measured on (binding-)analysers from most of the other equipment manufacturers. But the compatibility among cardiac troponin I results from the different manufacturers is complicated by the lack of cardiac troponin I standardisation.

In conclusion, clinically (except for end-stage renal disease patients) there is no preference for cardiac troponin I or cardiac troponin T. Therefore, whether cardiac troponin I or cardiac troponin T should be performed, depends usually on the manufacturer of the equipment used in the laboratory.

Finally, it should be taken into consideration that the role of CKMB as “gold standard” for myocardial tissue damage has been become more or less obsolete with the acceptance of the troponins as 100% specific markers for myocardial tissue damage. The use of cardiac troponin I or cardiac troponin T in combination with CKMB should be avoided, because this procedure may induce confusion, especially, if only one of the markers is beyond the upper limit of reference range. Moreover, it is more expensive to measure CKMB in conjunction with cardiac troponin I or cardiac troponin T.

Epilogue

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CHAPTER 12

Samenvatting voor de leek

Van oudsher is bloedonderzoek een belangrijk hulpmiddel om hartspierschade vast te stellen. Dit berust op het feit, dat tot het hartweefsel behorende stoffen (ook wel 'cardiac markers' genoemd) in de bloedbaan terecht komen na het afsterven van hartspiercellen. In het algemeen is het zo, dat er een continu proces is van hartspiercellen, die delen en afsterven. Dit leidt ertoe, dat er constant cardiac markers aanwezig zijn in het bloed. Schade aan de hartspier leidt tot een verhoging van de concentratie van cardiac markers in het bloed, omdat door de schade een verhoogd aantal hartcellen afsterft. Na verloop van tijd verdwijnt deze verhoogde hoeveelheid cardiac markers weer uit de bloedbaan, doordat deze stoffen verder worden afgebroken, worden opgenomen in de lever of het lichaam verlaten via de nieren door middel van uitscheiding in de urine.
Verhoogde schade aan de hartspier kan het gevolg zijn van meerdere oorzaken. De belangrijkste oorzaak is hartspierschade, die ontstaat door vernauwingen in de kransslagaderen. Dit kan tot gevolg hebben, dat er te weinig zuurstof aan de hartspier wordt aangeboden (dit noemen we 'ischemie'). Bij een totale afsluiting van één van de kransslagaderen sterft er hartspierweefsel af en is er sprake van een hartinfarct. Deze vorm van hartspierschade is een veel voorkomende aandoening in de westerse wereld en is een belangrijke doodsoorzaak. In de loop van de vorige eeuw zijn de inzichten en de behandeling duidelijk verbeterd. Het is van groot belang gebleken, dat de ziekte in een vroeg stadium wordt herkend, zodat zo snel mogelijk een juiste behandeling kan worden ingesteld. Hartspierschade kan ook optreden tengevolge van een stomp letsel (bijv. een verkeersongeval of een val van een hoogte) of tengevolge van een scherp letsel, zoals bij operaties aan het hart. Tevens kan hartspierschade veroorzaakt worden door sommige behandelingen met medicijnen ter bestrijding van kwaadaardige ziekten.
Een veel gebruikte cardiac marker voor het vaststellen van hartschade is het enzym Creatine Kinase (CK) en dan met name het iso-enzym CKMB. Dit iso-enzym komt niet alleen in hartweefsel voor maar ook in andere weefsels zoals skeletspierweefsel. De concentratie van CKMB in hartspierweefsel is echter veel hoger dan in skelet-spierweefsel. Het CKMB iso-enzym is in de loop van de tijd alom geaccepteerd als "gouden standaard" voor het aantonen van hartschade in bloed. Andere stoffen, die gebruikt kunnen worden voor het aantonen van hartschade in bloed, zijn de enzymen lactaat dehydrogenase (LD) en aspartaat amino transferase (ASAT). Het nadeel van al deze stoffen is echter, dat ze niet hartspecifiek zijn. Ze komen namelijk ook in andere weefsels voor zoals met name in het al eerder genoemde skeletspierweefsel.
Tot voor kort was het niet mogelijk om stoffen in bloed te bepalen, die specifiek voor hartschade zijn. Een aantal jaren geleden (begin jaren 90) verschenen de eerste resultaten van studies waaruit bleek, dat het mogelijk was geworden om een stof in het bloed te bepalen, die wel specifiek is voor hartschade: het hart-specifieke troponine T. Kort daarna werd hetzelfde gerapporteerd over hart troponine I. Troponine I en troponine T zijn stoffen, die behoren tot het troponine complex. Dit complex komt alleen in de dunne filamenten van de dwars gestreepte spier voor. Het troponine complex bestaat uit troponine C, troponine I en troponine T en speelt een rol bij het samentrekken en het ontspannen van de spier. Hoewel dit troponine complex zowel in hart- als in skeletspierweefsel voorkomt, is het toch mogelijk onderscheid te maken tussen de hart- en skeletspier vormen van troponine I en troponine T. De verklaring hiervoor is, dat de samenstellingen van de hart troponine I en hart troponine T moleculen verschillend zijn van die van de skeletspier troponine I en troponine T moleculen. In tegenstelling tot de samenstelling van de hart en skeletspier troponine I en troponine T moleculen, is de samenstelling van het hartspier troponine C molecule

exact gelijk aan dat van het skeletspier troponine C molecule. Daarom speelt troponine C geen rol van betekenis in de specifieke herkenning van hartspierschade.
Het doel van de in dit proefschrift beschreven onderzoeken is het vergelijken van de toepasbaarheid van 'standaard' cardiac markers t.o.v. de 'nieuwere' cardiac markers voor het vaststellen van hartschade met verschillende oorzaken. De volgende oorzaken van hartschade worden in de verschillende hoofdstukken besproken: ischemie van het hart, hartschade na stomp letsel, hartschade na grote (niet hart-) operaties, hartschade na hartoperatie en hartschade gerelateerd aan kwaadaardige ziekten.
In **hoofdstuk 1** wordt aangegeven aan welke voorwaarden een cardiac marker moet voldoen om ideaal te zijn. Vervolgens worden de moleculaire bouw en eigenschappen en de meetmethodieken van de meest gebruikte cardiac markers beschreven. Het betreft met name de cardiac markers creatine kinase, lactaat dehydrogenase, myoglobine en de troponines.
In **hoofdstuk 2** worden de resultaten weergegeven van een studie, die als doel heeft te onderzoeken of de cardiac markers hart troponine I, hart troponine T, myoglobine en hydroxyboterzuur dehydrogenase (HBD) homogeen verdeeld zijn over het hart en daarnaast vaststelt, wat de inter-individuele variatie van deze markers is. De markers blijken niet homogeen verdeeld te zijn over het hart. De concentratie van alle onderzochte markers is hoger in de ventrikels (de 'hartkamers') dan in de atria (de 'boezems') uitgedrukt in zowel per gram weefsel als per gram weefsel-eiwit. Hieruit volgt, dat de serum concentratie van een marker afhangt van de plaats in het hart, waar de schade heeft plaats gevonden. Daarnaast is er inter-individueel een veelvoud in concentraties van de markers in het hartweefsel gevonden. Dit betekent dus, dat een hogere concentratie in bloed niet per definitie ook een grotere hoeveelheid weefselschade inhoudt.
In **hoofdstuk 3** wordt met behulp van immuun-histochemische meetmethoden bestu-deerd hoe snel hartweefselschade aan te tonen is na een infarct m.b.v. hart troponine I- en hart troponine T-weefselkleuringen. Tevens is onderzocht in hoeverre de hart troponine I en hart troponine T specifieke markers voor het hart zijn. Voor deze studie zijn hartweefsels verkregen van patiënten, die met verschillende intervallen zijn overleden aan een acuut myocard infarct. Uit de studie is gebleken, dat het minimaal 24 uur duurt, voordat infarcerings gebieden in het hart aangetoond kunnen worden. Ter vaststelling van de weefselspecificiteit zijn hart troponine I en hart troponine T weefselkleuringen toegepast op zowel hart- als ander spierweefsel. Uit de resultaten van dit onderzoek blijkt, dat hart troponine I en hart troponine T alleen aan te kleuren zijn in hart spierweefsel en niet in ander spierweefsel.
In de **hoofdstukken 4 en 5** worden verschillende cardiac markers getest in een onder-zoek, dat tot doel heeft om vast te stellen welke markers als eerste verhoogde waarden in bloed te zien geven na een acuut myocard infarct. Hart troponine I en hart troponine T zijn niet de eerste markers, die verhoogde waarden in het bloed te zien geven na hartschade. Een verhoogde CKMB2/CKMB1 isovormen ratio en een verhoogde myo-globine concentratie in bloed zijn als eerste verhoogd na hartschade. CKMB2 is het volledig in tact zijnde enzym CKMB (de zogenaamde 'weefselvorm'). CKMB1 (de zogenaamde 'serumvorm') verschilt van CKMB2, doordat het eindstandige aminozuur lysine afgesplitst is van het CKMB2 molecule. CKMB1 is het produkt na de eerste stap van het afbraakproces van het iso-enzym CKMB. Analoog hieraan is het CKMM3 (de 'weefselvorm') hetzelfde als het volledig in tact zijnde iso-enzym CKMM molecule. Het CKMM1 (de 'serumvorm') is het restant van het iso-enzym CKMM, nadat van beide subeenheden, waaruit het iso-enzym CKMM is opgebouwd, het eindstandige aminozuur lysine is afgesplitst. Verder blijkt uit

deze studie, dat de CKMB2/CKMB1- en de CKMM3/CKMM1-isovormen ratio's de enige markers zijn, die met één bloedafname en één analyse een betrouwbare indicatie kunnen geven in de tijd, die verstreken is tussen het moment van hartschade en het moment van bloedafna-me.
In **hoofdstuk 6** worden twee nieuwe apparaten uitgetest, die ontwikkeld zijn om als bepalingsmethodiek te dienen buiten het centrale laboratorium in de nabijheid van de patiënt zoals bijvoorbeeld 'aan het bed' van de patiënt. We spreken dan van 'point of care' (POC) of 'near patient testing' analyse. Het bijzondere van deze apparatuur is, dat zij gemaakt zijn met de optie, dat ook personeel zonder analytische opleiding in staat moet zijn om op betrouwbare wijze analyses uit te voeren. Bovendien moet de kwaliteit van de resultaten van deze apparatuur vergelijkbaar zijn met de resultaten van de apparatuur, die binnen het centrale laboratorium gebruikt wordt voor het bepalen van dezelfde cardiac markers. De beide POC-apparaten (met name de Stratus Cardiac Status™ en de Triage Cardiac Panel™) kunnen de concentraties van hart troponine I, CKMB-massa en myoglobine in bloed meten. De CKMB-massa en myoglobine blijken een goede tot redelijk goede correlatie te zien te geven. De correlatie is minder goed voor hart troponine I. Dit laatste heeft te maken met het gebrek aan overeenstemming tussen de verschillende leveranciers van het troponine I reagens. Het betreft zowel het antilichaam, dat gebruikt wordt voor de herkenning van hart troponine I in bloed tijdens de bepaling als het gebrek aan uniforme standaarden, waarmee de bepaling geijkt moet worden. Momenteel is er een internationale werkgroep geïnstalleerd om aanbevelingen te doen voor de standaardisering van de hart troponine I bepaling. Dit probleem ligt anders voor de bepaling van hart troponine T. Deze bepaling is namelijk voorzien van een patent, waardoor er maar één leverancier op de markt is, die reagens voor deze bepaling levert. Na de testfase is de Stratus CS geïnstalleerd als POC-analyser op de afdeling Hartbewaking in het AZG. De testen zijn uitgevoerd door verpleegkundigen van deze afdeling. De geproduceerde resultaten bleken betrouwbaar, omdat zij bij vergelijking overeen bleken te komen met de resultaten, die bepaald waren in het centraal laboratorium. Op grond van deze bevindingen lijkt het verantwoord, dat niet-analytisch geschoold personeel de cardiac marker testen uitvoert. Tevens is gebleken, dat het resultaat van de hart troponine I bepaling beter dan het resultaat van de CKMB bepaling overeen komt met de klinische toestand van de patiënt.
In **hoofdstuk 7** wordt een studie beschreven, die uitgevoerd is bij patiënten, die een stomp letsel hadden opgelopen als gevolg van een ongeval. De belangrijkste oorzaken van dit soort ongevallen waren een verkeersongeval en een val van hoogte. De patiënten werden onderverdeeld in twee categorieën. De eerste categorie bestond uit 51 patiënten, die een ongeval hadden doorgemaakt zonder borstletsel. Bij deze patiënten waren geen aanwijzingen, dat ze hartschade hadden opgelopen. De tweede categorie bestond uit 38 patiënten, die als gevolg van het ongeval ook borstletsel (en mogelijk hartschade) hadden ondergaan. Uit de onderhavige studie blijkt, dat de jarenlang als "gouden standaard" gebruikte CKMB bepaling in ongeveer de helft van de patiënten uit de eerste categorie verhoogde waarden te zien geeft, terwijl er geen (klinische) reden is om aan te nemen, dat deze patiënten hartletsel hebben opgelopen. Deze bevindingen gelden niet voor de hart troponine I en de hart troponine T bepaling. Voor deze beide laatste parameters geldt, dat bij de patiënten, waar geen verdenking is op hart-letsel, de concentraties in bloed ook niet verhoogd zijn. De conclusie is daarom, dat de 'nieuwe' cardiac markers hart troponine I en hart troponine T betrouwbaarder zijn voor het diagnosticeren van hartschade na stomp letsel dan de van oudsher in gebruik zijnde cardiac marker CKMB. Een tweede conclusie is, dat, indien een patiënt hartletsel

tijdens het ongeval oploopt, het een aantal uren kan duren, voordat de hart troponine I of de hart troponine T concentratie in bloed verhoogd is.
In **hoofdstuk 8** worden de resultaten weergegeven van een studie, die tot doel had om de conventionele diagnostische hulpmiddelen voor het aantonen van hartschade, zoals de CKMB bepaling en ECG veranderingen, te vergelijken met de nieuwe cardiac markers hart troponine I en hart troponine T bij patiënten, die grote niet-cardiale chirurgie hebben ondergaan. Het betreft o.a. grote buikoperaties, vaatoperaties en operaties om kwaadaardige gezwellen te verwijderen. Uit de resultaten blijkt, dat, wanneer een verhoogde CKMB waarde als criterium wordt gebruikt, bij ongeveer de helft van de 60 geïncludeerde patiënten er geconcludeerd zou worden, dat er sprake zou zijn van hartspier-schade. Slechts bij 4 van de 60 patiënten zijn de hart troponine T resultaten verhoogd en bij 5 patiënten de hart troponine I concentratie. Dit komt veel beter overeen met de 2 patiënten, waarbij de definitieve diagnose acuut myocard infarct is gesteld. Uit deze studie is geconcludeerd, dat het bepalen van de hart troponine I of hart troponine T concentratie in bloed betrouwbaarder informatie levert dan het gebruik van de conventionele markers.
In **hoofdstuk 9** worden het verschijn- en verdwijn patroon beschreven van cardiac markers in bloed van patiënten, die verschillende vormen van hartchirurgie hebben ondergaan. Geen van de geïncludeerde patiënten heeft na de operatie complicaties gekregen. Het betreft patiënten, die een coronaire bypass operatie ondergaan, waarbij bij een groep van 36 patiënten tijdens de operatie de hart-long machine wordt gebruikt en een tweede groep van 23 patiënten, waarbij geen gebruik wordt gemaakt van de hart-long machine. Bij de eerste groep patiënten worden na de operatie in vergelijking met de tweede groep patiënten veel hogere hart troponine I en hart troponine T concentraties in bloed vastgesteld. De hoogste concentraties worden gemeten in de periode 6-8 uur na de start van de operatie. De waarden van de tweede groep patiënten vertonen nauwelijks een stijging ten opzichte van de uitgangswaarden, die worden bepaald bij de start van de operatie. Naast de patiënten met coronaire bypass operatie worden ook patiënten gevolgd, die een aorta- of een mitralis klep vervanging moeten ondergaan. De hart troponine I en hart troponine T concentraties in bloed van deze patiënten vertonen een patroon, dat vergelijkbaar is met die van de patiënten die een coronaire bypass operatie met gebruik van de hart-long machine ondergaan. Overigens met dien verstande, dat de hoogst gemeten concentraties ongeveer een factor twee hoger zijn. Op grond van deze resultaten is geconcludeerd, dat de concentraties van cardiac markers in bloed van patiënten na hartchirurgie afhankelijk zijn van de soort operatie, de omstandigheden tijdens de operatie en het tijdstip van bloedafname na de operatie.
In **hoofdstuk 10** worden de resultaten van twee studies weergegeven van patiënten, die mogelijk hartschade hebben opgelopen naar aanleiding van therapie, die is ingesteld ter behandeling van een kwaadaardige ziekte. De eerste studie betreft patiënten, waarbij de diagnose kwaadaardig mesothelioom is vastgesteld. Bij deze patiënten is de rechterlong verwijderd en aansluitend tijdens de operatie zijn zij intra-thoracaal nabestraald. De vraagstelling hierbij is of deze nabestraling ook hartschade tot gevolg heeft. De van oudsher gebruikte marker CKMB vertoont bij alle patiënten verhoogde waarden. De concentraties in bloed van hart troponine I en hart troponine T vertonen nauwelijks verschillen t.o.v. de uitgangswaarden, die bepaald zijn voor de start van de bestraling. Dit komt goed overeen met andere onderzoeken, waarvan de resultaten erop duiden, dat er geen aantoonbare hartschade is vast te stellen bij deze patiënten. Uit deze studie is de conclusie getrokken, dat de nieuwe cardiac markers betrouwbaarder zijn voor het vaststellen van hartschade bij deze categorie patiënten dan de al langer in gebruik zijnde cardiac markers. De tweede studie in

hoofdstuk 10 betreft patiënten, waarbij de diagnose carcinoïd syndroom is gesteld. Uit ervaring is bekend, dat een deel van de patiënten, die aan deze ziekte lijdt, hartfalen ontwikkelt. Bij geen van de onderzochte patiënten zijn verhoogde hart troponine I, hart troponine T of CKMB waarden in bloed gevonden. Hoewel bij een aantal patiënten hartfalen is vastgesteld, kan dus met behulp van de verschillende cardiac markers geen hartschade worden aangetoond. Mogelijkerwijs kan dit verklaard worden, doordat de snelheid van weefselverval zo laag is, dat deze niet aangetoond kan worden via het bepalen van een toename van cardiac marker concentraties in bloed.

In **hoofdstuk 11** wordt een overzicht gegeven van de diagnostische mogelijkheden van de cardiac markers op dit moment en van de te verwachten toekomstige mogelijkheden. Momenteel is duidelijk, dat de cardiac markers hart troponine I en hart troponine T onmisbaar geworden zijn voor het aantonen van hartschade met behulp van bloedonderzoek. Het jarenlang gebruikte CKMB is met de introductie van deze nieuwe markers eigenlijk overbodig geworden. Een zeer veel belovende toepassing van de nieuwe cardiac markers is die bij patiënten met pijn op de borst, waarbij (nog) geen acuut myocard infarct kan worden vastgesteld. Uit onderzoek is namelijk gebleken, dat de kans, dat zij een acuut myocard infarct kunnen krijgen binnen ongeveer een maand na het doormaken van de klachten groter is naarmate de hart troponine I of hart troponine T concentraties in bloed hoger zijn. Toekomstig onderzoek zal moeten uitwijzen op welke manier deze patiënten het best behandeld kunnen worden, opdat zij geen acuut myocard infarct zullen gaan doormaken.

Ten slotte kan als conclusie getrokken worden, dat de criteria van de ideale cardiac marker het meest benaderd worden door de hart troponine I en de hart troponine T. Met de invoering van deze cardiac markers in de reguliere patiëntenzorg is de jaren-lang gebruikte CKMB bepaling overbodig geworden. Om verwarring te voorkomen en onnodige kosten tegen te gaan dient deze bepaling dan ook uit het pakket van alledaagse aanvragen verwijderd te worden.