The role of troponin T in the quantification of ischemic myocardial injury

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The role of troponin T in the quantification of ischemic myocardial injury / J.H.C. Diris
Thesis Universiteit Maastricht – With summary in Dutch.
ISBN 90-9017535-0

Printed by: Datawyse, Maastricht

The role of troponin T in the quantification of ischemic myocardial injury

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Maastricht, op gezag van de Rector Magnificus, prof. dr. A.C. Nieuwenhuijzen Kruseman, volgens het besluit van het College van Decanen, in het openbaar te verdedigen op donderdag 4 december 2003 om 16:00 uur.

Door
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Geboren op 26 juni 1976 te Helmond

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Dit proefschrift is tot stand gekomen mede dankzij een financiële bijdrage van Roche Diagnostics Nederland BV. Het verschijnen van dit proefschrift is mede mogelijk gemaakt door steun van de Nederlandse Hartstichting.

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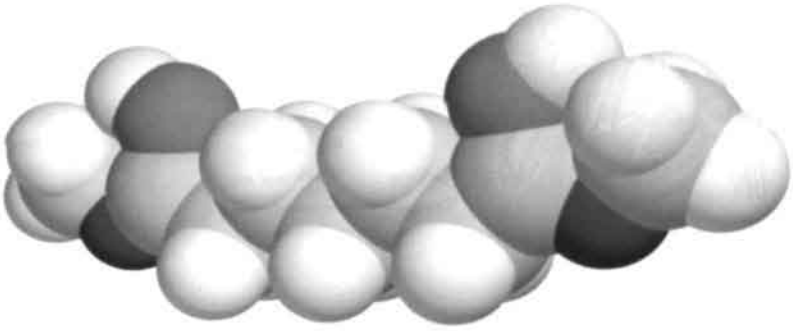
Introduction



Myocardial ischemia

Normal physiology and anatomy of the human heart

The human heart is an ingenious striated muscle with a complex electrophysiology. The right atrium of the heart receives the deoxygenated blood from the body (1) and via the right ventricle (2) this is pumped towards the lungs to be reoxygenated. Oxygen-rich blood from the lungs then enters the left atrium (3) and is pumped into the aorta by the left ventricle (4) (Figure 1-1). From the aorta the circulation is divided towards the brain, peripheral organs and the extremities. A small part is guided back to heart via the network of coronary arteries to provide the heart itself with the necessary nutrition and oxygen.

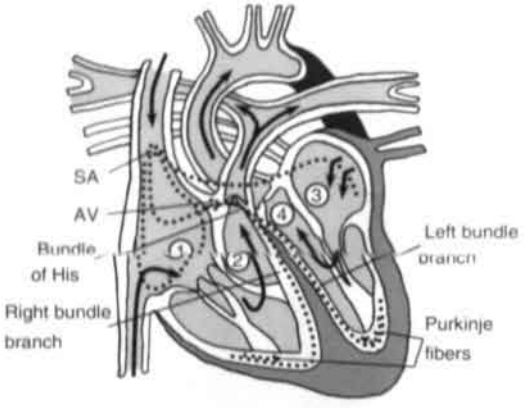


Figure 1-1, The human heart.
Blood flow and signal pathways in the heart. *Arrows* indicate direction of flow. *Dotted lines* indicate the electrical signal pathways. A signal is generated in the sino-atrial node (SA) and spreads over the right and left atrium towards the atrioventricular node (AV). From the AV-node the signal is transported through the Bundle of His towards the ventricles, this then guides through the left and right bundle branches, stimulating the ventricular muscles by the Purkinje fibres.

The cycle of muscle contraction and relaxation is regulated by a delicate system of sequential electrochemical events, guided by special nerves and conductive structures as described in Figure 1-1. This electrical activity of the heart can be measured and translated into the well-known electrocardiogram or ECG (Figure 1-2).

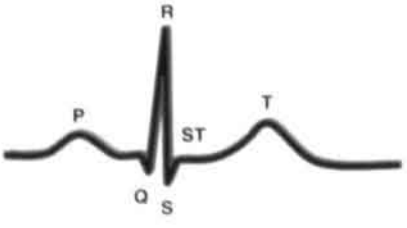


Figure 1-2, The electrocardiogram.
P-wave: Impulse from sino-atrial node through the right atrium to the atrioventricular node.
Q-wave: Activation of the interventricular septum, which activates from left to right.
R-wave: Activation of both ventricles, with the endocardial surface being activated before the epicardial surface.
S-wave: Activation of a few small areas of the ventricles at a rather late stage.
T-wave: Repolarisation of the ventricles.

Mechanisms of myocardial ischemia.

When the blood-flow through one of the coronary arteries is obstructed, part of the muscle receives less oxygen than required and will become ischemic. The obstruction can be caused by several mechanisms: (1) formation of stable plaques (Figure 1-3, *top*), causing narrowing of the arteries, (2) thrombus formation due to vessel wall injury or plaque rupture, causing an abrupt occlusion (whole or in part) of one of the coronary

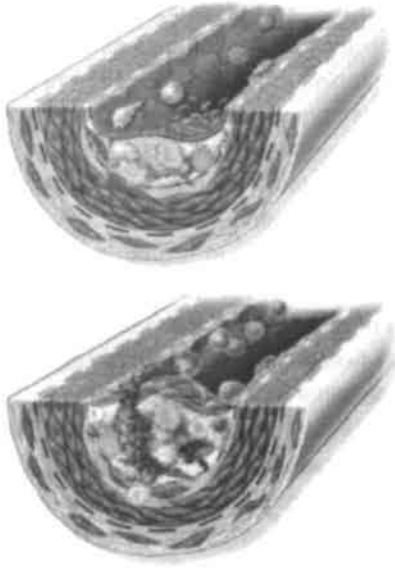


Figure 1-3, Occlusion of vessel.
Schematic representation of the occlusion of a vessel. *Top*: Plaque formation, stabilised by a thick fibrous cap. *Bottom*: Plaque rupture activated the coagulation cascade leading to thrombus formation. Image from: NEJM 1999;340(2):115-26.

arteries (Figure 1-3, *bottom*). Because the first process is a slow and gradual one, the decrease of blood supply can at first be compensated by the heart. A vessel can locally be occluded for more than 70% without any clinical symptoms due to these compensatory mechanisms. Eventually, part of the heart will become transiently ischemic which results in chest pain. Symptoms of this so-called angina pectoris are divided into four categories by the New York Heart Association.[1]

- Class I: Patients with no limitation of activities; they suffer no symptoms from ordinary activities.
- Class II: Patients with slight, mild limitation of activity; they are comfortable at rest or with mild exertion.
- Class III: Patients with marked limitation of activity; they are comfortable only at rest.
- Class IV: Patients who cannot carry on any physical activity without discomfort, symptoms occur at rest.

Due to some unknown triggers, a plaque can become inflamed causing the fibrous cap to thinnen. When this cap ruptures, the content of the plaque is exposed to the circulation, activating the coagulation cascade and a thrombus will begin to evolve. If it happens in a small vessel, only a minor part of the heart will become infarcted. However,

when a plaque in one of the coronary arteries ruptures and the artery becomes occluded, it causes massive downstream ischemia, which is called an acute myocardial infarction (AMI) or acute coronary event.

Events after AMI

When one of the coronary arteries is occluded, the tissue that is normally perfused by this artery becomes ischemic. If the circulation is not rapidly restored, the tissue cells at the centre of the ischemic area become necrotic and die. The presence of dead cells triggers an inflammatory reaction, leading to the activation of the complement cascade (see page 6). Penetration of the membrane attack complex into the cells initially results in small pores that eventually lead to rupture of the membrane, causing the cell content to be released into the interstitial space.[2,3] During diffusion through the interstitial space in order to reach the circulation, some proteins will diffuse faster than others, leading to differences in the appearance-time in the circulation after infarction.

Another consequence of myocardial ischemia is the change in signal conductive properties of the cell membrane, leading to changes in the ECG. Disturbances in the electrical properties can cause an altered sequence of muscle contraction, a diminished cardiac output or atrial or ventricular fibrillation. Scar tissue replacing the infarcted area can further decrease the cardiac output because of the stiffer mechanical properties.

Even if the circulation in an ischemic area is restored, the inflammatory reactions may cause the death of muscle cells (myocytes) that survived the anoxic period at the boundaries of the ischemic area.[4,5] Treatment and reduction of this reperfusion injury is one of the topics investigated in this thesis (Chapter 7).

Diagnostic parameters (ECG, cardiac markers)

The severity of acute coronary events demand treatment as soon as possible. When a patient with chest pain arrives at the emergency department of a hospital, one of the primary targets is to diagnose the underlying cause: myocardial, pulmonary or other. Besides the already mentioned ECG, the physician can also ask for measurement of cardiac markers at the department of clinical chemistry. Guidelines for the definition of recent myocardial infarction now include these cardiac markers.[6]

In earlier years, enzymes like creatine kinase (CK), α-hydroxybutyrate dehydrogenase (HBDH), lactate dehydrogenase (LDH), aspartate aminotransferase (AST) and myoglobin were used. More recently, new markers like CK-MB_{mass}, troponins and brain natriuretic peptide were added to this list. In addition to the use of cardiac markers for triage at the emergency department, they are also used for the monitoring of myocardial ischemia in AMI patients (Chapter 4 and 7) as well as perioperative myocardial ischemia in patients undergoing cardiac surgery (Chapter 6).

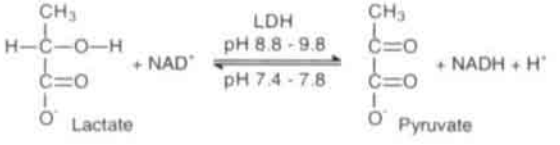
For the correct evaluation of these treatment effects and to avoid misinterpretation of data, it is crucial that underlying release mechanisms are known. Chapter 2, 3, 5 and 8 investigate these mechanisms with some surprising new insights.

Cardiac markers

Several enzymes and proteins have been and are used in the diagnostic process involving ischemic myocardial injury. The "old" enzymes like LDH, CK and HBDH, that lacked cardiac specificity, are more and more replaced by the more specific markers like CK-MB_{mass}, troponin T and troponin I. This paragraph provides some background information about the cardiac markers used throughout this thesis.[7-9]

Lactate dehydrogenase (LDH) and α-hydroxybutyrate dehydrogenase (HBDH)

LDH is a tetrameric molecule with a molecular weight of 134 kDa, that is composed of a combination of 2 subunits; H(eart) and/or M(uscle). The 5 possible combinations of these subunits result in 5 iso-enzymes that all function under slightly different conditions. LD1 (HHHH) is the dominant form present in cardiac tissue because of the aerobic



Equation 1-1

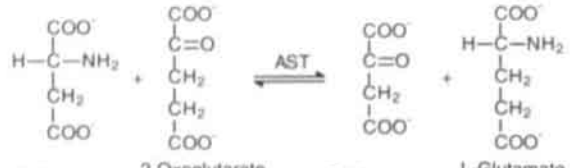
conditions, while LD5 (MMMM) dominates in the more frequent anaerobic skeletal muscles and the liver. The other iso-enzymes LDH2 (H_3M), LDH3 (H_2M_2) and LDH4 (HM_3) are also present in these tissues, albeit in different relative amounts. LDH is used in transient ischemic periods in most tissues of the human body and catalyses the conversion of a hydroxy- into a keto-group and vice versa, as shown in Equation 1-1. Normally, nicotinamide adenine dinucleotide (NAD^+) is used in the glyoclytic pathway and reduced to NADH. Anearobic regeneration of NADH to NAD^+ by lactate dehydrogenase is used when the normal, aerobic, regeneration of NADH via the Krebs cycle is not possible.

Enyzmatic activity of LDH is measured as the rate of NADH production and expressed as activity in Units per litre (U/L), where one unit corresponds to conversion of substrate in µmoles per minute by LDH at a given temperature. Total LDH activity will rise within 4–6 hours after the onset of myocardial infarction. The maximum is reached between 24 and 36 hours, and elevated LDH activity can still be measured more than 7 days after the onset of symptoms.

LDH not only catalyses lactate, but also various related 2-hydroxy acids and their counter 2-oxo acids. One of these acids, 2-hydroxybutyrate (or α-hydroxybutyrate), is the preferred target of LDH1 and LDH2. The overall dehydrogenation of 2-hydroxybutyrate is catalysed by so-called α-HBDH, which is therefore nothing more than a term for the combined activity of mainly LDH1 and LDH2.

Aspartate aminotransferase (AST)

AST is an enzyme present in the cytosol (40%) as well as in the mitochondria (60%) that catalyses the interconversion of an amino group between aspartate and 2-oxoglutarate (see Equation 1-2), favouring the formation of aspartate. Compared with other organs



L-Aspartate 2-Oxoglutarate Oxaloacetate L-Glutamate

Equation 1-2

such as the liver, kidneys and lungs, the heart has the highest concentration of AST, about 7800 times as much as the amount present in serum. AST is released from the heart 3–6 hours after the infarction, peaking at 12–24 hours and returning to normal after 3 to 4 days. The amount of AST released from the myocardium correlates with infarct size although it's long-term predictive value for mortality is low. Because of its presence in other tissues, AST is a non-specific cardiac marker and has therefore been replaced by other enzymes or proteins with higher tissue specificity.

Creatine Kinase (CK) and iso-enzymes (CK-MB mass / activity)

CK, a dimeric molecule with a molecular weight of approximately 81 kDa, regulates the phosphorylation of creatine by ATP (adenosine triphosphate) as shown in Equation 1-3. This process occurs in places of high energy demand like muscles. Like LDH, it is a cytosolic enzyme, although some of it is bound to myofibrilar structures. The two

Creatine Creatine Phosphate

Equation 1-3

subunits of CK can either be of B- (brain) or of M- (muscle) type, thus creating 3 possible iso-enzymes (BB, MB and MM) with the same function. All three iso-enzymes are present in tissue, but the composition differs between various tissues. Skeletal muscle contains predominantly CK-MM with about 1.1% CK-MB, while the heart contains the largest fraction (20%) of CK-MB. Because other tissue hardly contains any of the CK-MB iso-enzyme, this iso-enzyme is used as a cardiac marker. Significantly elevated serum CK-MB concentrations are reached within 3–6 hours after myocardial ischemia.

Depending on the method of measurement (substrate conversion or immunologically), either the activity ($CK\text{-}MB_{act}$) expressed in units per litre, or the concentration (CK-

MB_{mass}) in µg per litre is determined. Conflicting data about the presence or absence of CK-MB_{mass} in myocardial tissue as reported in literature is investigated in Chapter 5.
The usefulness of CK-MB_{mass} in the decision-making process involving patients that underwent coronary artery bypass grafting was investigated in Chapter 6. There, the sensitivity and specificity for the development of perioperative myocardial ischemia were determined for CK-MB_{mass}, troponin T and myoglobin at various time points after cardiac surgery.

Myoglobin

The function of myoglobin in the cytoplasm of human muscle cells is uncertain. Myoglobin resembles hemoglobin in its oxygen binding capacity, but unlike hemoglobin, it is unable to release oxygen at normal oxygen pressures. It might therefore act as a reserve oxygen pool for periods of extreme hypoxia as for instance might occur during extreme physical exercise.
When muscle tissue is damaged, the cytoplasmatic content of the myocytes, including myoglobin, is released into the circulation. Myoglobin, with its molecular weight of 17 kDa, is a cardiac marker that rapidly (within 2–4 hours) migrates from the interstitial space into the plasma after the onset of the infarction. Peak values are reached in 6–8 hours while plasma levels return to normal within 24 hours. Because both cardiac and skeletal muscle contain the same myoglobin its cardiac specificity is rather low. Serum myoglobin concentrations are expressed in µg/mL.

Troponin T and troponin I

Located on the thin filaments of striated muscles lay troponin complexes that consist of 3 subunits: troponin C with a molecular weight of 20 kDa, troponin I (26 kDa) and troponin

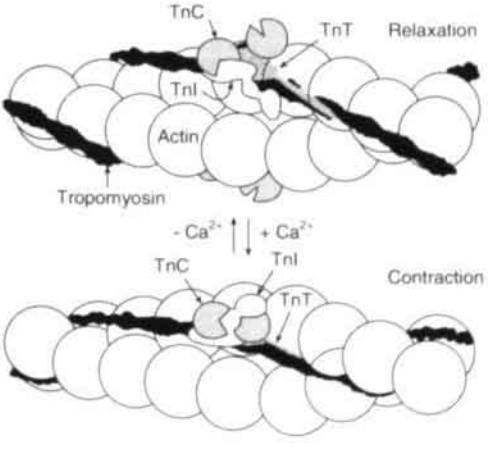


Figure 1-4, Troponin and muscle contraction. TnT = troponin T, TnC = troponin C and TnI = troponin I. Reprinted from Ann Clin Biochem 2001;38:423-49 with permission.

T (39 kDa). During depolarisation of the surrounding sarcolemma, initiated by a nerve stimulus, there is an influx of some Ca^{2+} ions into the cytoplasm of myocytes. This

triggers Ca^{2+} release from storage pools, thereby amplifying the signal. Troponin C binds the calcium, causing its conformation to change, pulling troponin I away from the actino-tropomyosin complex. The inhibition of actinomyosin ATPase by troponin I is then released, allowing ATP hydrolysis and muscle contraction. Troponin T enables the interaction of troponin C and I with tropomyosin and also stimulates the ATPase activity. Both troponin T and troponin I have cardiac specific isoforms, which are therefore used as a highly specific marker for myocardial damage.

Kragten *et al.* showed that the calculation of infarct size based on the "new" marker troponin T correlates with the "old" marker HBDH.[10] The release of cytosolic HBDH, and hence the calculated infarct size, is hardly influenced by the acute phase response after AMI, which is concluded from a total recovery of HBDH. However, the total amount of troponin T recovered after AMI is only a fraction of its tissue content, so whether this minor influence of the acute phase response also holds for the influence on the release of structural troponin T has remained an important question. In Chapter 4, this influence of the acute phase on calculated infarct size, quantified with HBDH or troponin T is investigated by means of acute phase protein C-reactive protein (CRP).

Chapter 2 describes the development of a highly sensitive method to purify and characterise troponin T compounds. This method is used to investigate the mechanisms of troponin T release from myocardial tissue (Chapter 2), and clearance from the circulation (Chapter 3). Answers are given to the question why serum troponin T concentrations are elevated in patients with severe renal failure, which has been discussed since the introduction of the troponin T assay.

The complement cascade

The human body is equipped with a complex mechanism of interacting proteins that mediate inflammation, opsonise foreign material for phagocytosis and induce direct cytotoxicity against various cells and micro-organisms. These proteins, when activated, form a cascade, in which they are cleaved, combined, converted and regulated. This cascade is known as the complement system. The complement cascade can be divided into the classical-, the alternative- and the mannan- or mannose-binding lectin (MBL) pathway, which all three converge via the common terminal complement pathway to the formation of the terminal complement complex. Figure 1-5 shows the cascade of these complement proteins.

The classical complement pathway

The classical pathway[11,12] is activated when one or more antigen-antibody (ag-ab) complexes bind to Complement protein 1 (C1). C1 is a tri-molecular complex of C1q, C1r and C1s, stabilised by Ca^{2+}. The ag-ab complexes binding to the C1q subunit induce internal changes, causing C1r to cleave C1s. This results in an esterase activity of C1s, which, in turn, can cleave C4 to produce C4a and C4b. C4a is a small peptide fragment and will be further discussed in the section about anaphylatoxins (page 6). If C2, another complement protein, binds to C4b, it can be cleaved by C1s into C2a and into C2b. The former remains attached to C4b to form the classical C3 convertase; the latter is released into the circulation.

C4b bound to an antigen protects nearby C1 complexes against inhibition by C1-inhibitor. It thus promotes further complement activation at the site of an immune complex while limiting the effects of non-specific C1 activation. Unbound C4b is readily inactivated by Factor I (FI) and Factor H (FH) into iC4b and then further cleaved by FI into C4c and C4d.[13] C4bc is used to indicate this latter group of iC4b and/or C4c and/or C4d.

In addition to the regulatory mechanisms of bound C4b and inactivation of unbound C4b by FI, other control mechanisms are present. The formation of a C3 convertase complex can be prevented by decay-accelerating factor (DAF), or the complex, once formed, can be destabilised thus enhancing its decay. C1-inhibitor (C1-INH) binding to the activated C1 complex is also able to inhibit further activation of the complement cascade. When bound to the C1 complex, C1-INH can induce its disassembly, leading to free $C1rC1s(C1\text{-}INH)_2$ complexes and a residual C1q molecule on the activating surface.

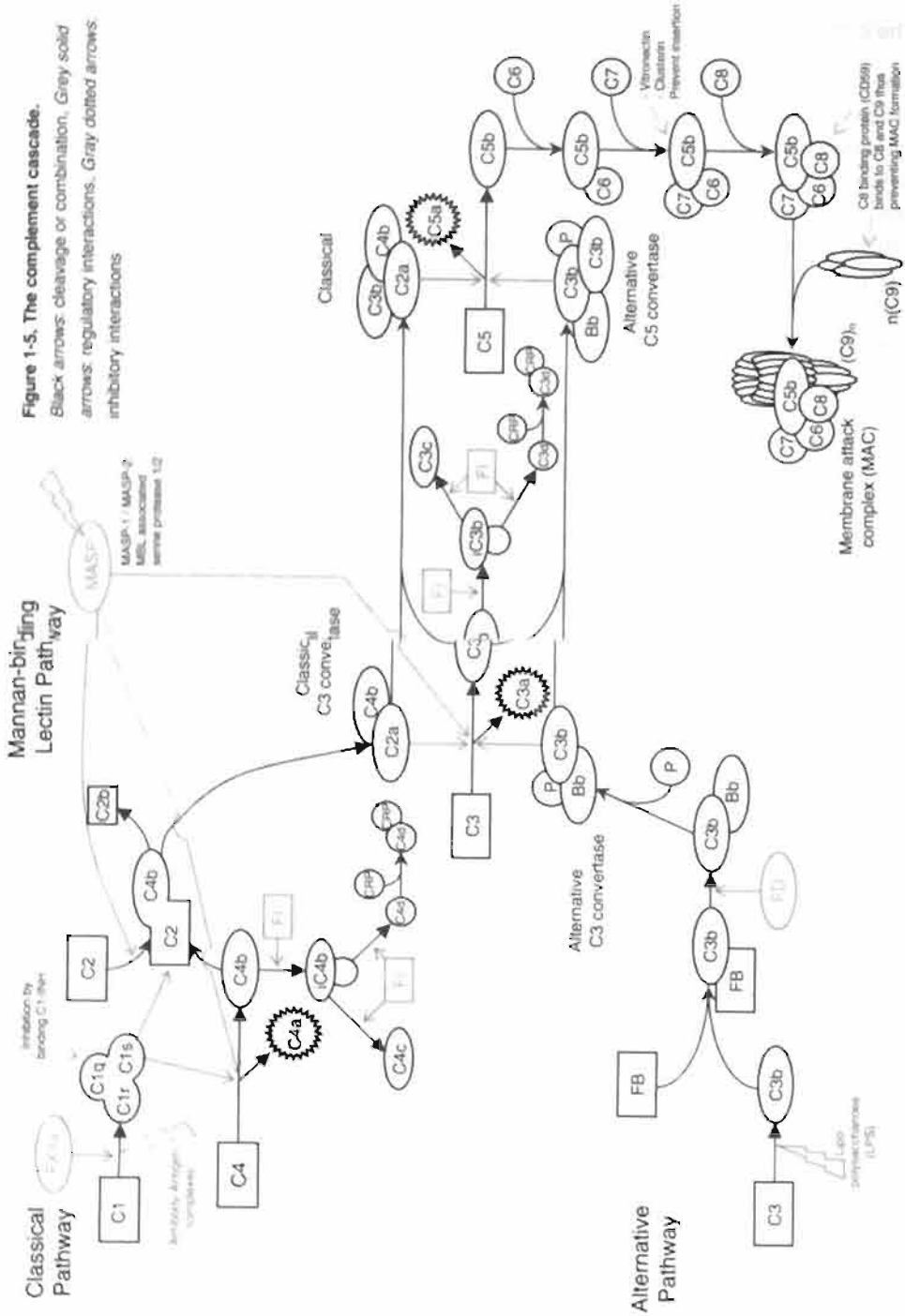


Figure 1-5. The complement cascade.
Black arrows: cleavage or combination. *Grey solid arrows*: regulatory interactions. *Gray dotted arrows*: inhibitory interactions

The alternative complement pathway

C3 plays an important role in the alternative pathway.[11] A small amount of C3 is continually hydrolysed to C3(H_2O) which, like C3b, is able to combine with factor B (FB) to further initiate the complement cascade. This continuous low-level C3b generation in serum is called C3 "tickover". The presence of C3b does not normally lead to activation, because it is rapidly cleaved and controlled by Factors H and I. If, however, this spontaneous C3b generation occurs near a foreign particle, binding of C3b to the surface enables it to bind Factor B and subsequently Factor D to form the unstable C3bBb: the alternative C3 convertase. Interaction of C3bBb with properdin leads to a more stable C3 convertase. C3 convertase will cleave more C3 into C3b, resulting in a considerable amplification of circulating C3b. Other initiators of the alternative pathway include lipopolysaccharides, trypsin-like proteases and cobra venom factor.

The mannan-binding lectin pathway

The mannan binding lectin (MBL) pathway is able to activate the complement system without C1 or the presence of antibodies.[14] The MBL pathway can be triggered by the binding of MBL to cell surface carbohydrates and is known to be activated after oxidative stress.[15,16] Binding of MBL activates the MBL-Associated Serine Proteases 1 and 2 (MASP-1 and MASP-2). Both proteases are able to cleave C4 and C2 to form the C3 convertase. After formation of the C3 convertase, the classical complement pathway is further activated up to the generation of the membrane attack complex. Because of the close resemblance of MASP-1 and -2 to the $C1r_2C1s_2$ complex, C1-inhibitor is also able to inhibit complement activation by the MBL pathway, as recently described by Petersen *et al.*[17]

Formation of C5 convertase

Both the classical and the alternative C3 convertases can combine with a C3b molecule to form C4b2a3b or C3bBbP3b *i.e.*, the classical and the alternative C5 convertase respectively. Cleavage of C3 by C3 convertase produces not only C3b, but also C3a, which, like C4a, is an anaphylatoxin. C5 convertase (classical as well as alternative) can cleave C5 into C5a, another anaphylatoxin, and C5b, the first component of the terminal complement pathway.

Terminal complement pathway and the lytic mechanism

C5b formed by cleavage of C5, subsequently combines with C6, C7, C8 and C9 to form C5b-9, the membrane attack complex (MAC). In order to combine with C6, C5b has to undergo conformational alterations, and because these alterations are short-lived, C6 has to be present for effective C5b6 complex formation. After its formation, C7 is rapidly attached to the C5b6 complex. The C5b67 thus formed, is highly hydrophobic and attaches itself to the lipid bilayer of the nearby target membrane. The hydrophobic C5b67 can be prevented from attaching to a membrane by a number of substances

during the time in which it is not yet bound to a membrane. These substances include low-density lipoproteins, serum protein S (formation of soluble SC5b-9), heparin, dextran sulphate and DNA. The next protein binding to C5b67 is C8 and, when it does, the γ-chain of C8 is inserted through the lipid bilayer, causing a small, unstable, hydrophilic lesion (0.4 to 3 nm).
The final step in the formation of a lytic complement complex is the addition of several C9 molecules to C5b6-8. The complete membrane attack complex (C5b-9) creates a lesion of 10 angström in diameter in the membrane, causing small molecules like ions and water to pass freely through the pore, while high molecular-weight macromolecules remain in the cell. The influx of water will cause the cell to swell and will eventually lead to rupture of the membrane and necrosis.

Anaphylatoxins
C3a, C5a and C4a are three small polypeptides released into the circulation when their native proteins (C3, C5 and C4) are cleaved upon activation.[11,18] These fragments are able to bind to their receptors on various cells like basophils, mast cells, lymphocytes and smooth muscle cells. All three peptides have anaphylatoxic activity: they induce degranulation of basophils and mast cells, cause smooth muscle cells to contract and stimulate neutrophils to release toxic oxygen species. These effects, combined with the release of histamine, induce vascular leakage.

Complement inhibition
Several studies have shown that the complement cascade is activated in ischemic myocardial tissue, and known to cause reperfusion injury.[19-21] This means that the complement proteins attack viable myocytes surrounding the necrotic tissue of an infarction, which leads to a further significant increase of the infarct size.
Because quality of life after AMI is directly related to infarct size,[22,23] it is preferred to reduce this to an area as small as possible.[16,24,25] Chapter 7 reports of a study we did to reduce the infarct size by inhibition of activated C1, thereby preventing the rest of the cascade to be activated.

Pharmacokinetic modelling

All kinds of proteins, hormones, peptides, metabolites, carbohydrates and electrolytes circulate in the human body. Ranging in size from small mononuclear ions to complex proteins of several hundreds of kilodaltons. These circulating compounds originate either from endogenous production or from exogenous sources.
Proteins are formed via the cellular route of DNA transcription into RNA, RNA translation into an early form of a protein, and post-translational modifications into the functional version. Another route for a protein to enter the circulation is absorption from food in the intestines. Although some proteins are synthesised at the same site where needed, many others have to be transported, which —in general— occurs by the circulation.
An alternative way for a compound to reach the circulation is by exogenous administration. This can be done orally, via absorption through the skin or by subcutaneous, intramuscular or intravenous injection. Each method of application has its own characteristics and mechanisms to which the compound is exposed.
Although the internal and the exogenous pathways are completely different, they both result in the introduction of the compound into the circulation at some point in time. While present in the blood, this compound will reach a certain concentration depending on the rates of introduction and removal. If removal is slower than introduction, concentrations will increase, thereby also increasing the total amount of circulating compound. If not, the concentration is the highest right after introduction and only decreases thereafter.
Knowledge about the mechanisms and rates at which these compounds are introduced and removed, is crucial for reliable estimation of various parameters, for example infarct size quantification (Chapter 2 and 5) or estimation of administration dose of a drug (Chapter 8).
A biological system to be modelled is divided into one or more compartments. Each compartment, with its own set of parameters for in- and out-flow, resembles a different environment to which the compound is exposed. In addition, mathematical rules for compound administration, production and removal can be defined. Initial and boundary conditions complete the model. Most modelling of physiological processes is done with the use of 1-, and 2-compartment models. These models will be discussed in the following paragraphs.

One compartment: modelling the plasma pool
Figure 1-6 shows a 1-compartment model with in- and out-flow of a certain drug. Because the inflow resembles the administration of the drug, its exact formulation

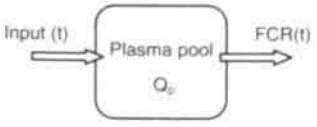


Figure 1-6. One-compartment model.
Q_p = amount present in the compartment

depends on the method of administration. A bolus injection directly into the circulation

gives a rather time-independent, immediate rise of the plasma content. In contrast, a drug orally administered, or given by infusion, penetrates the compartment with a certain speed. This speed will be dose dependent if the drug reaches the compartment via passive transportation. Active (enzymatic) transport most often occurs at a dose-independent rate.
The outflow or removal of the drug can involve several mechanisms like reaction with target molecules, renal clearance, diffusion or active transport out of the compartment. A model can contain equations for all these mechanisms individually, but these mechanisms can also be combined into one overall rate, which is called fractional catabolic rate (FCR).

$$\frac{dQ_p(t)}{dt} = Input(t) - FCR(t) \qquad \textbf{Equation 1-4}$$

The time-course of the amount of the drug present in the compartment (defined by Equation 1-4) depends on the difference between in-, and outflow. Changing these parameters in the model will also cause the calculated amounts to change, thereby increasing or decreasing the difference with the —in vivo— measured amounts.

Two compartments: modelling the plasma pool, adding an extravascular pool
When the administered drug has reached the circulation (*i.e.*, the plasma pool), it may have the opportunity to enter the extravascular space (Figure 1-7). Like the pathway of entering the plasma pool, the extravascular space can also be reached by active

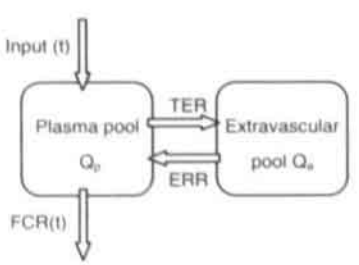


Figure 1-7, Two-compartment model.
Q_p = amount present in plasma, Q_e = amount present in the extravascular space.
FCR = Fractional Catabolic Rate constant, TER = Transcapillary Escape Rate constant and ERR = Extravascular Return Rate constant.

transportation or by passive diffusion, because of the concentration gradient. Both ways have their specific rates. Escape into the extravascular pool occurs predominantly from the capillary vessels, hence the name transcapillary escape rate (TER). The rate at which the drug returns from the extravascular pool into the plasma pool is called the extravascular return rate (ERR). Mass balances over the 2-compartments result in a set

$$\frac{dQ_p(t)}{dt} = Input(t) - (FCR + TER) \cdot Q_p(t) + ERR \cdot Q_e(t) \qquad \textbf{Equation 1-5}$$

$$\frac{dQ_e(t)}{dt} = TER \cdot Q_p(t) - ERR \cdot Q_e(t) \qquad \textbf{Equation 1-6}$$

of (dynamic) algebraic equations defined by Equation 1-5 and Equation 1-6.
Although even more compartments can be added to the model, this rarely increases the precision of the estimated parameters because of the biological variation and the limited accuracy of the measured data. However, if the physiology of the biological system is more complicated it still might be useful to add compartments, not for more accurate parameters, but for a better understanding of physical mechanisms.

Modelling algorithms

To determine the unknown parameters present in the equations, numeric algorithms are used that translate the equations into one so-called objective function. Specific algorithms then fit the objective function to the experimental data and try to minimise the difference between the experiment and the model. A minimal difference means that, given a certain model, the estimated parameters will approach reality as much as possible. However, using one or more wrong assumptions can (and in most cases will) result in complete nonsense.
An often-used algorithm is the efficient and robust Levenberg-Marquardt algorithm. In short, this algorithm is a hybrid between the Gauss-Newton algorithm and a grid search algorithm. For a rough determination of the global minimum, the parameter space is divided into a regular spaced grid. For each grid point the objective function is calculated. It is then assumed that the global minimum is in the vicinity of the grid point where the

Figure 1-8, Schematic representation of minimisation algorithm.
Left panel: Distribution space of all solutions of a 2-parameter model.
Centre panel: Search grid superimposed on distribution space for rough determination of local minimum.
Right panel: Steepest descend algorithm (*black arrow*) approaching the local minimum.

objective function is the lowest. At that point the Levenberg-Marquardt algorithm performs initially small, but robust steps along the steepest descent direction, and switches to more efficient quadratic Gauss-Newton steps as the minimum is approached. Figure 1-8 (*left panel*) shows a 2 dimensional parameter space (x and y) where the z-ordinate depicts the total sum of squares between the objective function and the measured data. The overlay of the search grid for the rough determination of the global

minimum is shown in the *centre panel* of Figure 1-8. The *right panel* of Figure 1-8, with the enlarged bottom part of the minimum, shows the final determination of the local minimum by the steepest descend algorithm.

In this thesis, 2-compartment pharmacokinetic modelling is used for the calculation of total protein release, and hence the infarct size, based on measured plasma values. This is only possible when both the tissue content, and the FCR of the cardiac marker are known. In Chapter 4, where the cumulative release curves of HBDH and troponin T are compared, these kinetic parameters are already known from previous studies[10]. The cause of some conflicting data regarding the tissue content of CK-MB is investigated in Chapter 5. A different approach was needed in Chapter 8 where the pharmacokinetic parameters of a 2-compartment model had yet to be determined using the iterative power of a computer program.

Renal clearance of circulating proteins

Waste products that enter the circulation have to be removed before dangerous concentrations are reached. The human body has two major recycle systems to deal as efficient as possible with those waste products. Large proteins and toxics are removed and catabolised by the liver while smaller proteins, molecules and ions are filtered by the kidneys. A schematic representation of one renal filtering unit (nephron) is shown in Figure 1-1. Blood containing the waste products flows through the glomerulus where the actual filtering takes place. The filtrate then passes through the proximal tubule and the loop of Henle via the distal tubule into the collecting duct.

Figure 1-9, Renal nephron.
Dark grey: Arterioles; *Light grey*: Venules; *White*: Renal tubuli converging into the urinary collecting duct.
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Most of the water and usable compounds present in the filtrate are reabsorbed in the tubuli to be further catabolised and recycled in the epithelial cells lining these tubuli. A closer look at the glomerulus (Figure 1-10) shows the basal membrane that separates blood from the filtrate. This membrane is perforated with many small pores through

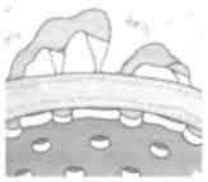

Figure 1-10, Close-up of glomerulus.
Schematic view showing from top to bottom: the epithelium with pores, the basal membrane and the endothelium with fenestrations.
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which plasma is pushed by hydrostatic pressure. Only compounds small enough can flow through the pores. The edges of the pores are covered with negatively charged carbohydrates that provide an extra barrier for ions and proteins that have an overall negative charge. Table 1-1 shows that there is no sharp molecular weight cut-off of the basal membrane, so a percentage of larger proteins like lactoglobulin will still be able to pass the membrane. Under normal circumstances reabsorption of these proteins is nearly complete, resulting in undetectable concentrations in the urine. However, in the diseased kidney the basal membrane will initially loose it's negative charge and the

pores will become wider. More proteins will be able to pass through the membrane into the filtrate. When this increased tubular load of proteins becomes too high, maximal renal reabsorption capacity is reached and proteins will be excreted via the urine (proteinuria).

Table 1-1, Renal filter properties.[26]

	Molecular weight (kDa)	% present in filtrate
Sodium	0.023	100
Urea	0.06	100
Myoglobin	16.9	96
Lactoglobulin	36.0	40
Albumin	69.0	< 1

In a later stage, when renal tissue becomes inflamed, antigen-antibody complexes will get trapped in the pores of the membrane and fibrinous tissue is deposited. These processes lead to a decrease of filter capacity and although the kidneys initially have a large reserve capacity, eventually this is not enough and waste products will build up in the circulation.

In Chapter 3 the role of renal failure and protein accumulation as a cause for elevated serum troponin T concentrations in hemodialysis patients is investigated. The data presented in that chapter will finally provide some detailed information about a topic that has been discussed by a lot of authors since many years.

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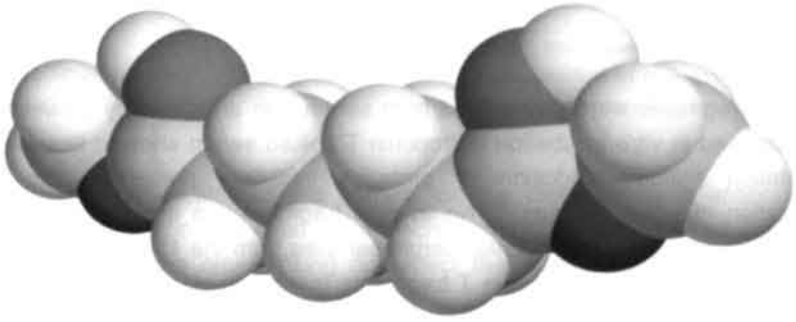
Chapter 2.

Fragmentation of troponin T in patients with acute myocardial infarction.



Diris JHC, Hackeng CM, Pinto YM, Boumans M-L, Hermens WTh, and Van Dieijen-Visser MP. *Article in preparation* 2003

These experimental findings can be explained by the fact that in a protein, only the N-terminal endings of peptide chains contain α-amines, while the remaining amines are ε-amines from the amino acids lysine or arginine. Looking at the different pK_a-values for lysine (Figure 2-1), one can see that at higher pH values first the α-amine gets

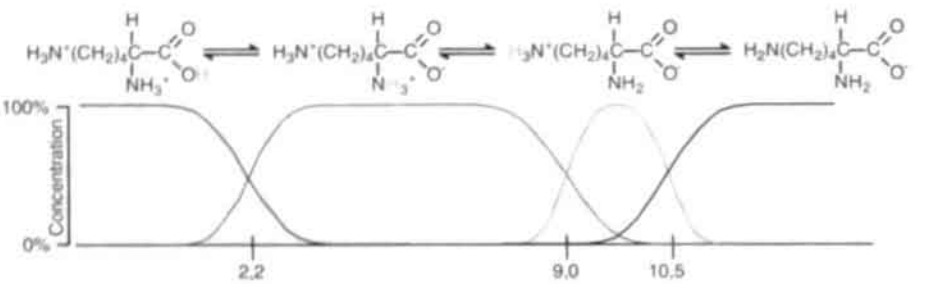


Figure 2-2, Protonation of Lysine.
Protonation of the lysine amino acid as a function of pH

deprotonated (pK_a = 9.0) and that with a further increase of pH the ε-amine (pK_a = 10.5) becomes available for reaction. In contrast, the ε-amine of arginine (pK_a = 12.5) is still protonated at a pH between 8 and 9 and thus unreactive towards imidoesters, which leaves only the ε-amine from lysine, and the relatively few α-amines of a protein available for reaction.

Cross-linking of antibodies

Antibodies are covalently cross-linked to solid surfaces or microparticles to prevent loss of antigen binding capacity due to unwanted desorption, caused by weak non-covalent binding, competitive inhibition with antibodies present in samples, or harsh elution conditions.

A typical cross-link protocol using imidoesters starts with the non-covalent capture of antibodies by immobilised protein-A or -G. The choice of either protein-A or protein-G depends on the subclass of the antibody and should be determined individually for each antibody. Because protein-A[‡] has a high preference for binding the Fc part of an antibody, an optimal surface orientation of the antibodies is achieved, leaving the antigen-binding sites free for antigen capture. This contrasts with coupling of antibodies using CNBr-activated sepharose, which binds antibodies at a random orientation, thereby reducing the total number of optimal available antigen-binding sites.

After antibody capture, DMP is added at a pH between 8 and 9 for covalent cross-linking of the antibody to protein-A. Because DMP is susceptible to hydrolysis even at basic pH, it is added in two or more portions with regular intervals to ensure complete reaction. Remaining free reactive groups are quenched with ethanolamine (a small molecule containing free α-amines) and all non-bound antibodies (if any) are eluted.

[‡] In this section, 'protein-A' is used where both protein-A and/or protein-G are applicable.

The reaction of DMP can occur at all α-amines and lysines of both the antibody and protein-A, so it is not only the desired covalent link between the two proteins that is formed. Loops between two amines at the same protein can be formed, and one-sided reactions leaving small "hairs" at various sites of the protein can also occur. When this happens at the antigen-binding site of the antibody it can result in a decrease in the antibody's affinity for the antigen. However, using Kabat's database[5] for a closer look at the antigen-binding sites (hypervariable loops) of mouse antibodies reveals that they are almost devoid of lysine (Figure 2-3).

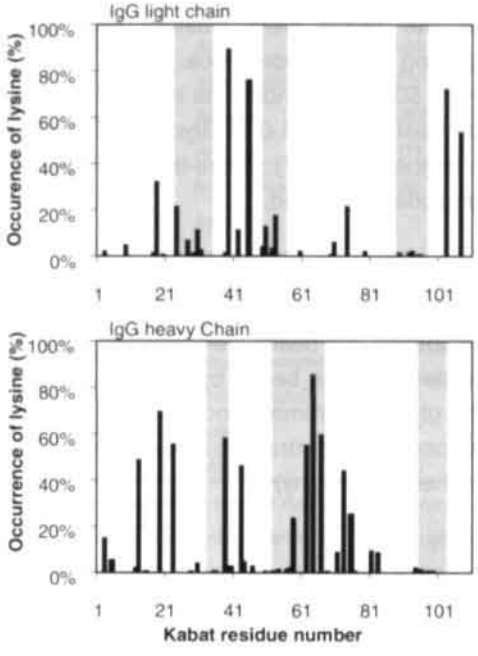


Figure 2-3. Occurrence of lysine.
Percentage of sequenced antibody-chains that contain a lysine amino acid at a specific Kabat position. Hypervariable antibody regions are shaded grey. A higher percentage bar indicates a more preserved lysine at the respective position. Based on Kabats database[5], total number of sequenced mouse IgG light chains = 2511, IgG heavy chains = 3346.

So there is only a small chance that cross-linked DMP significantly interferes with antigen binding capacity. Post-translational antibody modifications also have little influence on antigen binding because the only modifications that occur with antibodies is the attachment of unreactive carbohydrates. And even that is only 2–3% in the IgG antibody subclass.[6]

Application of immunoprecipitation: release characteristics of troponin T
With these immobilised antibodies it is possible to extract proteins from a difficult matrix like serum, with high specificity. We investigated time-sequential sera of patients with acute myocardial infarction for the presence of troponin T fragments.

Methods

Preparation of anti-troponin T sepharose beads

Protein-A sepharose (no. 17-0780-01, Pharmacia Biotech) was blocked with bovine serum albumin (BSA, no. A7030, SIGMA) in PBS. A mixture of anti troponin-T antibodies (9G6, 7F4, 1F11, 7A9, 1C11 from Research Diagnostics Inc. and 4C5 and 1A9 from Fortron) in PBS containing 0.1% BSA was added to the beads and incubated 1hr. After binding of the antibodies the beads were washed twice with PBS.

Prior to addition to the beads for cross-linking, dimethylpimelimidate (DMP, no. D8838 SIGMA) was added to 0.2 M triethanolamine in PBS to a final concentration of 20 mM, pH 8.6. After incubation for 30 minutes the beads were washed with 0.2 M triethanolamine in PBS. Cross-linking and washing was repeated twice.

Remaining reactive groups were quenched with 50 mM ethanolamine in PBS for 1 hour. Un-cross-linked antibodies were removed by washing with 1.0 M Glycine-HCl (pH 3.0) twice. All reactions were performed at room temperature (RT). Cross-linked beads were stored at 4°C in PBST to which 0.02% sodiumazide was added.

Precipitation of troponin T from serum

250 µL serum, 100 µL 6 M urea and 150 µL PBST were added to ±7.5 mg sepharose beads and incubated for 1.5 h at RT. After incubation, the beads were washed twice with PBST. Troponin T and its fragments were eluted from the beads by addition of 100 µL sample buffer (40mM Tris, 3.3% *SDS*, *50% glycerol*, bromephenolblue). Efficiency of precipitation was monitored by measuring troponin T concentrations on the Elecsys 2010 (third-generation troponin T test, Roche, Mannheim, Germany).

Electrophoresis and Western Blotting

Samples were loaded onto a 4–15% polyacrylamide gradient gel (Bio-Rad Ready Gel). After 15 min. stacking at 100 V and 40 min. separation at 150 V, the separated troponin fragments were transferred onto nitro-cellulose (60 min. at 100 V). Electrophoresis buffer: 2.5 mM Tris, 19.2 mM Glycine, pH 8.3. Blotbuffer: 2.5 mM Tris, 19.2 mM Glycine, 0.1% w/v SDS, pH 8.3 and 20% v/v Methanol.

Nitro-cellulose was blocked with 5% non-fat dry milk in PBS for 1h at RT. Prior to the addition of the first antibody the blots were washed with wash-buffer (0.02% Tween-20, 1% non-fat dry milk in PBS). After the first (over-night) antibody-incubation the blots were washed again and the second antibody labelled with horseradish peroxidase (goat-anti-mouse, Dako) was added for incubation during 1 h at 4°C. Western blots were developed using Enhanced Chemoluminescent Buffer (Perkin-Elmer Life Sciences) and captured on film (Kodak X-Omat Blue, Perkin-Elmer Life Sciences).

Patients

Serum was collected from 13 AMI patients that were sent in for routine analysis by the department of cardiology. Patients' characteristics are listed in Table 1-1. Troponin T-negative sera from healthy volunteers were collected and pooled and used for control purposes of the precipitation assay.

Table 2-1. Patients' characteristics.

Patient ID	Sex	Age (y)	Infarct location	Troponin T (µg/L)*	Kreatinin (µmol/L)*	Diabetes Mellitus	Treatment
1	M	74	Posterior	19.91	526	No	Thrombolysis
2	M	66	Anterior	39.03	168	No	PCI with stenting of main branch
3	M	42	Infero posterior	37.8	774	No	PCI of RCA
4	M	50	Anterior	23.93	118	No	PCI of LAD
5	M	54	Anterior	55.17	114	No	PCI with stenting of LAD
6	M	66	Infero- postero lateral	11.61	165	No	PCI with stenting of circumflex
7	M	76	Anterior	19.03	107	No	PCI with stenting of LAD
8	M	38	Anterior	29.13	256	No	Thrombolysis followed by PTCA
9	F	83	Antero lateral	88.68	403	No	PCI of LAD
10	M	63	Angina pectoris	0.46	108	Yes	PCI with stenting of LAD
11	M	56	Antero septal	18.31	539	No	PCI with stenting of LAD
12	F	78	Anterior	13.27	115	No	Thrombolysis
13	F	74	Anterior	12.14	86	No	Thrombolysis followed by PTCA

* Reported values are the highest measured concentrations.

PCI = percutaneous coronary intervention; RCA = right coronary artery; LAD = left anterior descendens; rTPA = recombinant tissue-type plasminogen activator; PTCA = percutaneous transluminal coronary angioplasty.

Analytical techniques

Anti-troponin T antibodies were covalently immobilised on protein-A sepharose as described on previous pages. Diluted serum from patients and controls was incubated on the sepharose beads to extract troponin T and its fragments. After troponin T precipitation, the beads were washed and incubated directly in sample buffer for application onto an SDS-polyacrylamide gel. Fragments were separated by electrophoresis and transferred to nitro-cellulose.

In vitro incubation of spiked serum samples

Troponin T-negative serum was pooled, and divided into 20 equal portions. A mixture of protease inhibitors in phosphate buffered saline with 0.01% Tween-20 (PBST) and sodium azide (0.02%) was added to 5 samples while the remaining samples received only the PBST with sodium azide. The same samples were prepared, but without the presence of sodium azide.

Purified human cardiac troponin T was diluted in 6 M urea and divided into 5 equal portions. Both serum samples and the troponin T solutions were frozen at -20°C. At time 0, 12, 24, 36 and 48 hours, one portion of troponin T was distributed over four serum samples (one with and one without protease inhibitors, with and without sodium azide) and all four samples were incubated at 37°C. After 48 hours, troponin T was measured in all samples and thereafter simultaneously precipitated as described below. After electrophoresis and western blotting, troponin T and its fragments were visualised using two different antibodies, directed against the N-terminal and C-terminal part of the molecule.

Influence of protease inhibitors

Two separate blood samples were collected from 5 patients of the dialysis department. After clotting, samples were centrifuged 12 min., 3500 rpm at 4°C. A cocktail of protease inhibitors was added to one of the two serum samples right after centrifugation. Both samples were then stored at -20°C until further fragment analysis.

Results

Development of the immunoprecipitation protocol

For the determination of the right conditions for the extraction of troponin T fragments from serum, various parameters have been checked. Protein-A was preferred over protein-G sepharose because use of the latter resulted in slightly more background signal in the developed Western blots.

Reducing agents like β-mercaptoethanol and dithiotreitol (DTT) combined with the use of Iodoacetamine (IAA) were also investigated. Because troponin T does not contain internal disulphide bonds, no significant differences were found, so these steps could therefore safely be omitted from the purification protocol.

Various temperatures (37, 56, 65 and 100°C) for the elution of precipitated troponin T fragments from the protein-A sepharose have been tried, resulting in most efficient elution at 56°C.

For the final step in the Western blotting, application of the secondary antibody, the difference between a rabbit-anti-mouse and a goat-anti-mouse labelled with horseradish peroxidase favoured the goat-anti-mouse antibody. The use of a goat-derived antibody resulted in better signal intensity on the developed film.

These optimised conditions heavily depend on the individual characteristics of the used antibodies against troponin T. A different use of this immunoprecipitation method using antibodies from other species could of course result in completely different conditions.

In vitro incubation

Prolonged incubation of human serum spiked with troponin T showed a significant decrease in the amount of intact troponin T (Figure 2-4), while 2 fragments (27 and 25

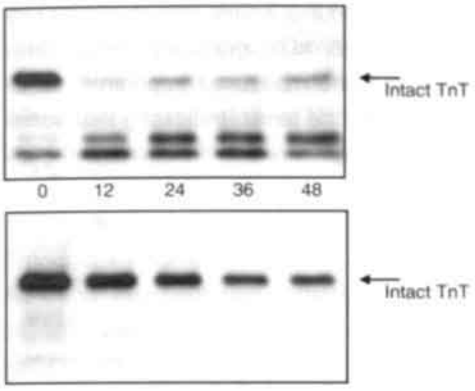


Figure 2-4, In vitro incubation of troponin T.
Upper panel: Spiked human serum incubated for 0, 12, 24, 36 and 48 hours at 37°C, without protease inhibitors.
Lower panel: Same samples but incubation in presence of protease inhibitors.

kDa) appear in time. The presence of protease inhibitors prevents the occurrence of fragments although some intact troponin T also seems to disappear. Two more fragments (16 and 19 kDa) are visible when a primary antibody against the C-terminus is used after Western blotting.

Influence of protease inhibitors

In vivo formed troponin T fragmentation patterns of samples to which, after blood collection, no cocktail of protease inhibitors was added did not differ from the patterns of samples with inhibitors as shown in Figure 2-5.

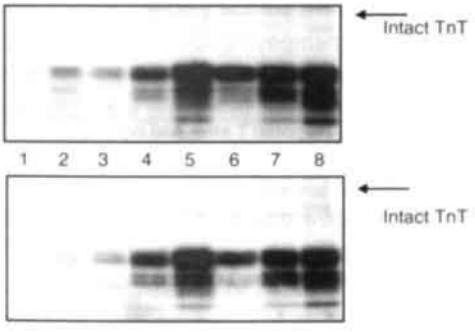


Figure 2-5, Influence of inhibitors.
Upper panel: Serum samples without protease inhibitors.
Lane 1: Negative control;
Lane 2–3: Dialysis patients troponin T = < 0.01 µg/L;
Lane 4–8: Dialysis patients troponin T = 0.092, 0.332, 0.112 and 0.140 µg/L respectively.
Lower panel: same samples but with protease inhibitors added directly after collection.

Precipitation of serum from AMI patients

A time-dependent process is visible in Figure 2-6 where a series of 9 samples from patient 1 are displayed. The first sample was taken about 3 hours after the onset of symptoms. While the initially present fragment of 25 kDa gradually disappears, fragments of 23, 20, 16 and 9 kDa appear. Note that the measured troponin T value does not directly correlate with the cumulative density of the lanes, indicating that some fragments might not be recognised by the troponin T assay.

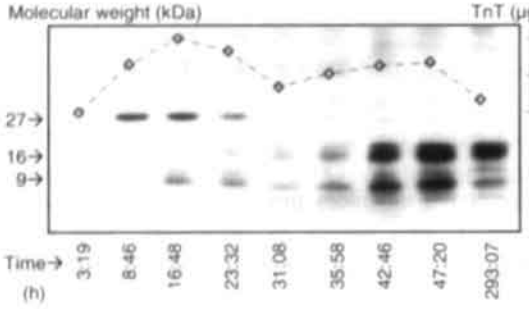


Figure 2-6, Troponin T after AMI (1).
Precipitated serum samples from AMI patient 1. Serum troponin T concentrations are: lane 1: 0.02; 2: 12.9; 3: 19.9; 4: 16.3; 5: 6.97; 6: 10.4; 7: 12.7; 8: 13.7 and 9: 3.46 µg/L respectively. Time (h) after the onset of symptoms.

Figure 2-7 shows the release pattern of AMI patient 2. Similar as in Figure 2-6, no intact troponin T can be identified. The first sample of this patient was taken later after the onset of symptoms because of hospital transfer.

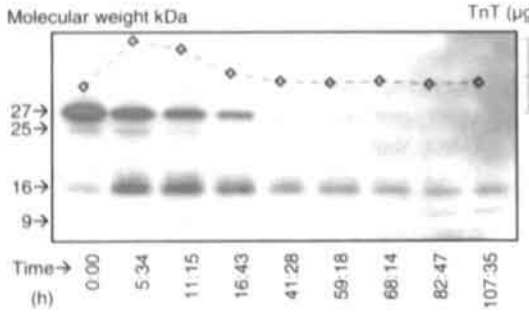


Figure 2-7, Troponin T after AMI (2).
Precipitated serum samples from AMI patient 2. Troponin T concentrations are: Lane 1: 14.4; 2: 39.0; 3: 34.4; 4: 22.0; 5: 17.1; 6: 16.4; 7: 17.1; 8: 15.8; 9: 16.1 µg/L respectively. Time (h) after obtaining the first sample.

Fragment analysis of samples from patient 3 to 11 showed the presence of similar fragments as in patient 1 and 2. Like in Figure 2-6 and Figure 2-7, the patterns change during the hours after infarction. A tendency towards more and smaller circulating fragments was present in patients with a decreased renal function (based on serum creatinin concentrations) in the days following infarction.

Discussion

Background of cross-linking

We now have provided a clear overview into the underlying mechanisms of cross-linking with imidoesters. Although several cross-link protocols using imidoesters were available, none had been thoroughly described in the literature. With the information provided in this paper it is possible to trace the causes of possible problems occurring in immunoprecipitation assays with cross-linked antibodies when using different antibodies and/or other solid matrices.

Troponin T fragments

Although fragmentation patterns change during the time-course after infarction, all patients display in general the same fragments. Intact troponin T is only visible in the early hours after infarction, in a later stage, fragments ranging in size from 9 to 27 kDa are found. Based on the present data, no definitive conclusions can be drawn about the quantitative relations between the different fragments. A direct link between the disappearance of one fragment and the appearance of another has not been found yet.

Influence of protease inhibitors

When using an analytical method that takes several incubation steps, it is important to verify that fragments that are found originate from in vivo processes and are not formed during the sample preparation. With the in vitro incubation experiment we have shown that the addition of protease inhibitors prevents the fragmentation of troponin T (Figure 2-4) and intact troponin T is present on the Western blot. Combining this information with the fragmentation patterns from the sera of the dialysis patients (Figure 2-5), we can conclude that although fragmentation can occur in vitro (at 37°C), the sample preparation process (at room temperature) without the presence of protease inhibitors does not influence the pattern of fragments already present in the sera. Therefore, post-sampling addition of protease inhibitors to serum is not needed.

Cell-death of the ischemic myocyte

The typical biphasic troponin T release curve of AMI patients was thought to be caused by the early release of cytosolic troponin T, followed by the slower release of structural troponin T.[7] As shown in Figure 2-6, the fragmentation pattern is different for the two phases of the curve. The absence of intact troponin T, even in the earliest sample with an elevated troponin T concentration, suggests a rapid fragmentation mechanism. It is uncertain whether this initial fragmentation occurs already within the ischemic —but intact— myocyte or after rupture of the cell membrane. Either way, at least two different proteolytic processes have to be involved to cause the later shift in fragmentation pattern. Possibly, the early-formed troponin T fragments are further cleaved in the circulation and/or the sustained proteolytic activity in the myocardium itself further degrades the troponin T that is still bound to myofibrillar structures. However, more

research has to be done to determine the exact mechanisms of the existing troponin T fragmentation.

Infarct size quantification

Previous studies have compared infarct sizes based on classic proteins like CK and HBDH with the results of infarct size quantification based on CK-MB[7] and troponin T.[8] One of the presumptions made for these calculations is the clearance rate of the protein. For troponin T, a protein with a molecular weight of 39 kDa, this clearance rate was estimated at $0.11 \pm 0.05\ h^{-1}$.[8] With this speed of clearance, the cumulative amount of troponin T found after myocardial infarction was only 6–7% of the tissue content of the diseased myocardial tissue. However, this rate was based on a non-renal clearance mechanism. We showed that troponin T is readily fragmented in ischemic myocardial tissue and that these fragments are cleared from the circulation by the kidneys[9]. The clearance rate of this mechanism is about ten times higher ($1.2\ h^{-1}$) than the rate that was used for the infarct size quantification. This means that with the novel mechanistic insights not 6–7% is recovered, but 60–70% based solely on the differences in clearance rates. It is likely that some troponin T is no longer recognised by the assay because of fragmentation in to too small peptides. This will add to the total amount of troponin T released from the myocardium, possibly increasing the recovery even further.

Conclusions

With the presented method of immunoprecipitation we are able to visualise a changing pattern of several troponin T fragments in serum of AMI patients. We address various aspects of the troponin T release mechanism and provide a valuable tool for further research into basic kinetic mechanisms, not only for troponin T but also for other proteins.

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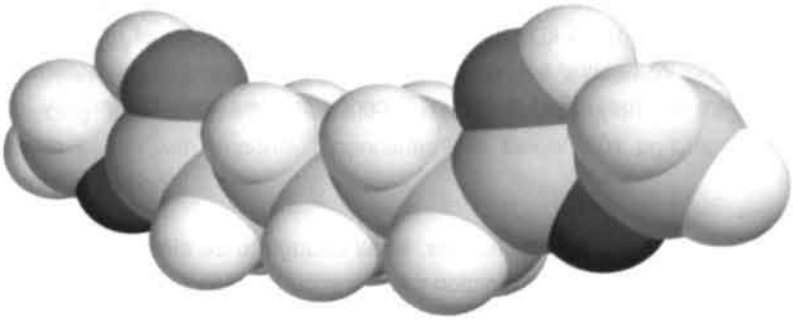
Chapter 3.

Elevated troponin T concentrations in renal failure patients explained



Adapted from Diris JHC, Hackeng CM, Kooman JP, Pinto YM, Hermens WTh, Van Dieijen-Visser MP: Elevated troponin T concentrations in renal failure patients explained. *Submitted* 2003.

Abstract

Background
Patients with severe renal dysfunction and no clinical symptoms of myocardial ischemia often have unexplained elevated serum concentrations of cardiac troponin T. We investigated whether in vivo fragmentation of troponin T could explain the increases found in these patients.

Methods
Troponin T, creatine kinase isoenzyme MB ($CK\text{-}MB_{mass}$) and myoglobin serum concentrations were measured in all 63 dialysis patients of our in-hospital dialysis department. A highly sensitive immunoprecipitation assay, followed by electrophoresis and Western blotting was used to extract and concentrate troponin T and its possible fragments from serum of these 63 hemodialysis patients.

Results
Although $CK\text{-}MB_{mass}$ values excluded ischemic myocardial events, troponin T fragments ranging in size from 8 to 25 kDa were present in serum samples of all dialysis patients, and not in serum of healthy subjects. A statistically significant positive correlation was found between the measured troponin T concentrations and the history of dialysis therapy.

Conclusions
Troponin T is fragmented into molecules, small enough to be cleared by the kidneys of healthy subjects. Impaired renal function causes accumulation of these fragments and is very likely the cause of the unexplained elevations of serum troponin T, found in patients with severe renal failure.

Introduction

Elevated concentrations of cardiac troponin T in patients with renal failure and no signs of myocardial damage have been point of confusion and discussion in recent years.[1-4] Various groups have reported these findings, but no satisfying explanation has been given yet. In the mid-nineties some hypothesised that foetal expression of cardiac troponin T in skeletal muscle was the cause of the increase, but others have proven this hypothesis to be wrong and suggested it to be caused by cross-reactivity of skeletal troponin T in the first-generation troponin T assay.[5-7] However, the second and third-generation troponin T assays still demonstrate the unexplained elevated troponin T concentrations in patients with renal failure. A third option for the unexplained elevations of troponin T concentration has also been mentioned: fragmentation of troponin T and subsequently (impaired) renal clearance.[3,8] Van Eyk *et al.* have published several reports[9,10] mainly about the fragmentation of troponin I, but some figures also show a fragment of troponin T of 25 kDa. However, these findings have not been discussed in the light of renal function impairment. Recently, Ziebig *et al.* reported findings that strongly suggest significant renal contribution to the elimination of plasma troponin T, but no definitive answer was given.

We used a highly sensitive immunoprecipitation assay to isolate and concentrate troponin T fragments, subsequently separated by gel-electrophoresis and visualised by western blotting. With this method we are able to visualise troponin T fragments (8–25 kDa) even in serum with troponin T concentrations below 0.01 µg/L according to the third-generation assay.

Methods

Patients

After obtaining informed consent, serum samples of 63 patients from the in-hospital dialysis department of the University Hospital Maastricht were collected prior to dialysis and stored at -20°C. Troponin T, creatine kinase isoenzyme MB ($CK\text{-}MB_{mass}$) and myoglobin (Roche Diagnostics GmbH, Mannheim, Germany) were measured in separate serum samples, sent in for routine analysis. All 63 samples were also subjected to troponin T fragmentation analysis. Patients' characteristics are listed in Table 3-1.

Pooled serum samples from healthy subjects were used as a negative control. Spiked with purified human cardiac troponin T (Advanced Immunochemical, USA), they were used as positive control.

Analytical techniques; Immunoprecipitation of serum samples

A mix of 5 anti-cardiac troponin T antibodies (clones 9G6, 7F4, 1C11, 1F11, 7A9, Research Diagnostics Inc, USA) directed against specified epitopes throughout the troponin T molecule, were used to catch as many fragments as possible. These

been discussed, but recent results from a large population (n = 1951) suggest that even a slight increase in troponin T, with measurable plasma values between 0.010 and 0.10 µg/L, might indicate the presence of minor subclinical myocardial events.[13,14]
Löwbeer *et al.* recently reported a high association between troponin T serum concentrations (> 0.1 µg/L) and poor outcome in patients starting dialysis treatment.[15] However, the prognostic value of serum troponin T concentrations between 0.01 and 0.1 µg/L, often found in hemodialysis patients is less clear.
We found that CK-MB_{mass} values were normal in 56 out of 63 patients and the seven remaining patients had only slightly increased concentrations. With these normal concentrations of a relatively specific cardiac marker, ischemic myocardial events are unlikely. This was confirmed by the results listed in Table 3-2, which strongly indicate that slowly increasing troponin T levels (medians of quartiles still below 0.1 µg/L) are not caused by increased release from the myocardium due to a deteriorating cardiovascular status (indicated by constant CK-MB_{mass} medians), but are solely the result of accumulating troponin T fragments.
Although myoglobin concentrations are elevated in all patients, earlier reports have indicated that this is caused by the decrease in renal clearance capacity.[16,17] Differences between the quartile median myoglobin concentrations are statistically significant (see Table 3-2), but due to the absence of a trend, it is evident that these differences merely reflect a large variance of the measured concentrations.
We believe that the plasma value for troponin T (0.0002 ± 0.0001 µg/L)[12] in healthy subjects is the result of a continuous micro-loss of cardiomyocytes during normal life. This results in an estimated loss of 27 mg cardiac tissue per year based on a renal clearance rate of 1.2 h^{-1}, a plasma volume of 3 L and a cardiac tissue content of 234 µg/g.[18] Clearance of troponin T normally happens with such a speed that serum concentrations are below the current detection limit of 0.010 µg/L. However, impaired renal function could lead to a diminished clearance rate that can cause the measurable increase in basal troponin T concentrations. So although intact troponin T is too large to be effectively cleared, because of fragmentation, actual clearance rates are much higher than previously estimated.[18] These findings are supported by the reports of a rapid decrease of troponin T concentrations in renal transplantation patients.[1,19]
The prognostic factor of increased troponin T concentrations in patients with renal failure has been discussed in several articles.[20-22] A recent article showed that the short-term predictive value of troponin T concentrations remains useful despite decreased renal function.[23] A large study, recently conducted amongst 224 dialysis patients,[4] showed that higher levels of troponin T are associated with long-term increased risk of death. However, the major difference between earlier studies and our results are based on the fact that in the present study the duration of dialysis therapy, instead of troponin T concentration, was used for group classification. This way, individually occurring ischemic myocardial events are evenly distributed among the quartiles of therapy duration.

With our findings regarding the relation between duration of dialysis therapy and increasing troponin T concentrations we do not question the predictive value of troponin T, but we show the presence of an additional underlying phenomenon attributing to increased troponin T concentrations in patients with renal failure.
The present study proposes an explanation for the unexplained troponin T elevations in dialysis patients by showing that accumulated fragments are present in their serum, even in samples with measured troponin T concentrations below 0.010 µg/L. These findings clarify the cause of the unexplained elevations of serum troponin T, found in patients with severe renal failure.

Limitations of the study
We now have demonstrated that troponin T is split into fragments ranging in size from 8 to 25 kDa, small enough to be cleared by the kidneys of healthy persons. Unfortunately, because of the unavailability of the unlabeled antibodies from the third-generation Roche troponin T assay, no comparison could be made between the intensity of the detected fragments and the measured concentrations. Only when fragmentation analysis can be performed using the same antibodies as used in the Roche assay, the single fragments contributing to the measured troponin T concentrations can be identified, quantified by band intensity, and then compared.

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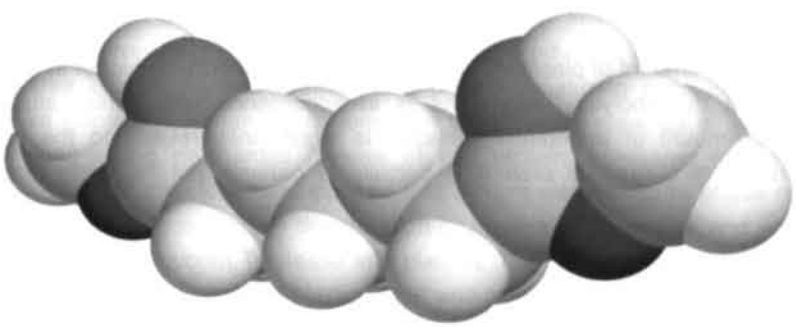
Chapter 4.

Effect of acute phase response on cumulative troponin T release



Adapted from Diris JHC, Kragten JA, Kleine AH, Hermens WTh and Van Dieijen-Visser MP: Effect of Acute Phase Response on Cumulative Troponin T Release *Clin Chem Lab Med* 2000; 38(10):955–959.

Abstract

Background

We studied a possible effect of the extent of the acute phase response after acute myocardial infarction on the cumulative release of troponin T. The height of the acute phase response might influence the cumulative release of troponin T, bound to the myofibrillar structures of the heart, in a different way compared to the free cytoplasmic cardiac marker hydroxybutyrate dehydrogenase.

Methods

The study was performed in patients receiving (n = 16) and in patients not receiving (n = 6) thrombolytic therapy. The cumulative amount of C-reactive protein in plasma, *i.e.* the quantified acute phase response, was related to the cumulative plasma release of hydroxybutyrate dehydrogenase (an established method for infarct sizing) on the one hand and to that of troponin T on the other hand. The cumulative protein release was calculated using a 2-compartment model for circulating proteins

Results

The cumulative amount of plasma C-reactive protein is significantly higher in the patients not receiving thrombolytic therapy, as is in accordance with earlier studies. The cumulative amount of troponin T released is significantly related to the cumulated concentration of C-reactive protein, especially in patients not receiving thrombolytic therapy.

Conclusions

The intensity of the acute phase response, estimated from cumulative plasma C-reactive protein response, has no effect on the relative proportions of troponin T and hydroxybutyrate dehydrogenase released into plasma.

Introduction

Acute myocardial infarction induces an acute phase response. The acute phase response is a well-known clinical phenomenon consisting of leukocytosis, fever and changes in the plasma concentrations of the so-called acute phase proteins, like for instance C-reactive protein (CRP).

Before the introduction of thrombolytic therapy several studies were published, indicating a relation between acute phase response, measured from the area under the plasma concentration curve of several acute phase proteins, and enzymatic infarct size.[1,2] It was also found that coronary recanalisation variably reduces the infarct associated rise in CRP. A weaker association between CRP and infarct size, estimated from cumulative hydroxybutyrate dehydrogenase (HBDH) release, was found in the patients receiving thrombolytic treatment, when compared to patients not treated with thrombolytic drugs.[3]

In a recent study we found that the cumulative troponin T release in gram equivalents of cardiac tissue varied between 4 and 13% (range) of the cardiac enzyme release.[4] As troponin T is bound to myofibrillar structures, differences in acute phase response might influence the release of this protein in a different way, when compared to the total plasma release of a free cytosolic cardiac marker like HBDH. Post-infarction inflammation involves recruitment of leukocytes and production of free radicals in the infarcted area and could thus create local, protein-denaturing conditions. Structural proteins like troponin T remain much longer in the infarcted area, as is also apparent from the long-lasting myocardial release of this protein, compared to cytoplasmic enzymes.[4] It could therefore be supposed that a more intense acute phase response might cause more extensive local denaturation or release of troponin T and would thus alter the cumulative troponin T release into plasma. The aim of the present study is to quantify the cumulative amount of plasma CRP and compare it to the cumulative plasma HBDH and troponin T release in patients receiving and in patients not receiving thrombolytic therapy.

Methods

Patients

Patients (12 male and 10 female) presenting at the Department of Cardiology of the Atrium Medical Centre Heerlen, within 6 hours after the onset of symptoms typical for acute myocardial infarction (chest pain and ST-segment elevation) were included in this study.

Of these patients 16 (11 men and 5 women) received thrombolytic therapy. First 160 mg of acetylsalicylic acid (aspirin) was given (unless already given by the general practitioner or ambulance nurses), followed by 1.5 million units of streptokinase, administered by intravenous infusion over a period of 40 minutes.

Table 4-1 Patient data

Patient group	S⁺ (n = 16)					S⁻ (n = 6)					Median S⁺ vs. S⁻
				Percentiles					Percentiles		
Parameter	Mean	SD	Median	25th	75th	Mean	SD	Median	25th	75th	
Treatment delay (h)	2.26	1.31	1.79	1.43	3.06	2.21	1.09	1.79	1.41	3.25	ns
Age (years)	60	9.4	60	55	64	72.7	8.6	70	66	81	0.007
Q_{HBOH} (72) (g-eq/L)	5.06	3.77	3.51	2.61	65.4	6.44	4.97	5.50	2.17	11.5	ns
$Q_{Troponin\ T}$ (168) (g-eq/L)	0.398	0.300	0.244	0.196	06.49	0.499	0.382	0.404	0.202	0.820	ns
Q_{CRP} (168) (mg/L)	149	173	68	33	204	405	287	344	220	504	0.021
$Q_{Troponin\ T}$ (168) / Q_{HBOH} (72) x 100 (%)	7.82	2.83	6.74	5.54	9.68	8.62	2.41	8.99	6.17	10.54	ns
Q_{CRP} (168) / Q_{HBOH} (72) (mg/g-eq)	32.6	32.1	21.7	91	43	89.5	77	60.6	38.3	141	0.027
Q_{CRP} (168) / $Q_{Troponin\ T}$ (168) (mg/g-eq)	487	500	317	106	693	995	695	834	629	1273	0.077 ns

S⁺ are patients treated with thrombolytic thrombolytic therapy and S⁻ patients not treated with thrombolytic therapy.

ns = not significant.

Four hours after the start of thrombolytic therapy, a bolus of 12,500 units of heparin was administered by subcutaneous injection, which was repeated every 12 hours for a period of 5 days. Only six patients not receiving thrombolytic therapy could be included. The patient groups were unfortunately heterogeneous with respect to sex and age, which is caused by the difficulty of including patients not receiving thrombolytic therapy.
All patients included in the study had given informed consent. Excluded were patients with increased risk of bleeding, previous coronary bypass surgery or Q-wave infarction in the same location, severe hepatic or renal disease, or inability to give informed consent.
Venous blood samples of 10 mL each were obtained just before starting thrombolytic therapy and 1, 2, 3, 4, 5, 6, 8, 10, 14, 18, 22, 28, 34, 46, 58, 70, 82, 94, 118, 142 and 166 hours later. Exact sampling times were recorded and expressed as time after the onset of symptoms. Most samples were taken from indwelling catheters and care was taken to prevent hemolysis. Blood was collected in pre-distributed and labelled tubes, containing dry heparin to prevent clotting, and was taken to the laboratory immediately. Plasma samples, obtained after routine centrifugation, were stored at -70°C until analysis. The Medical Ethical Committee of the Hospital approved all procedures followed. Patient data are presented in Table 4-1.

Enzyme and troponin T assays
Enzymatic activity of HBDH was measured spectrophotometrically at 37°C using a centrifugal analyser (Cobas Bio System, Hoffmann La Roche, Basel, Switzerland). A commercially available test kit was used for HBDH (Deutsche Gesellschaft für Klinische Chemie recommendation, optimised test from Boehringer). The HBDH test is based on preferential catalytic activity of the myocardial isoforms of lactate dehydrogenase (LDH1 and LDH2) in the conversion of 2-hydroxybutyrate. Activities were expressed in micromoles of substrate converted per minute (U), either per litre of plasma (U/L) or per gram wet weight of myocardial tissue (U/g).
Troponin T was measured using the troponin T immunoassay from Boehringer Mannheim.[5] The assay (first-generation) was carried out in coated tubes, using the Boehringer Mannheim ES22 photometer.
CRP was measured by turbidimetry on a Cobas Fara analyser from Roche (Roche, Mijdrecht, The Netherlands). For CRP measurement, the antiserum was obtained from Roche, using a calibrator T-standard CRP, also from Roche.

Cumulative release of cardiac markers and quantification of acute phase response
Q(t), the cumulative release of enzyme activity and troponin T concentration per litre of plasma from the onset of acute myocardial infarction (time zero) up to time t, was calculated with Equation 4-1 for a 2-compartment model for circulating proteins:

$$Q(t) = C(t) + TER \cdot \int_0^t \exp^{-ERR(t-\tau)} \cdot C(\tau)d\tau + FCR \int_0^t C(\tau)d\tau \qquad \text{Equation 4-1}$$

The three terms are the enzyme activity in plasma, the extravasated activity and the activity eliminated from plasma, all three expressed per litre of plasma. The parameters TER, ERR and FCR represent the fractional rate constants for transcapillary escape, extravascular return and fractional catabolism, respectively. This 2-compartment model has been validated in the dog[6-9] and parameter values estimated in man [10] are:
$FCR_{HBDH} = 0.015\ h^{-1}$, $FCR_{CK} = 0.20\ h^{-1}$, $TER = 0.014\ h^{-1}$ and $ERR = 0.018\ h^{-1}$.
For troponin T, a value of $FCR = 0.11 \pm 0.05\ h^{-1}$ (mean ± SD) was obtained from a fitting procedure on troponin T and HBDH plasma curves.[9-11] Knowledge about the clearance and extravasation rates of proteins once entered into the circulation is sufficient for calculation of the cumulative release into the plasma compartment.[12] The two pools used in the 2-compartment model are the intra- and extravascular pool and not the free and bound proteins. So with the 2-compartment model for circulating proteins the amount of protein entering the circulation can be calculated. The model has been extensively verified both in animal and in experimental studies.[10,12-14]
For CRP, a value of $FCR = 0.040 \pm 0.05\ h^{-1}$ (mean ± SD) was obtained from a fitting procedure on CRP and α_1-antitrypsin plasma curves, using the same method as described for troponin T. For α_1-antitrypsin a FCR of $0.013\ h^{-1}$ was used.[12,15]
Plasma concentrations measured at time t, C(t), were corrected by subtraction of the normal steady-state activities C_s. If the first plasma sample was obtained within 4 hours after the onset of symptoms, the corresponding enzyme activities were used for C_s. In the remaining cases, fixed mean values of 112 U/L, 0 µg/L and 4 mg/L were used for respectively HBDH, troponin T and CRP.

Infarct size in gram equivalents of myocardial tissue per litre of plasma
In order to express myocardial injury in gram equivalents (g-eq) of healthy myocardium per litre of plasma, the cumulative release of enzymes per litre of plasma was divided by the respective enzyme content per gram wet weight of cardiac tissue. For HBDH 156 U/g wet weight and for troponin T 234 µg/g wet weight was used.[11,16] Estimation of cumulative HBDH is an accepted method for myocardial infarct sizing.

Statistical analysis
The timed plasma curves are presented as medians with 25 and 75 percent quartiles. Pearson's correlation coefficient was calculated to show relations between cumulative releases of the different parameters. The Mann Whitney U-test was used for non-parametric comparison of data of two independent groups. The level of significance was set at $p < 0.05$. For comparison of slopes and intercepts of regression lines a method equivalent to analysis of covariance (ANCOVA) was used ($p < 0.05$). The 95% confidence intervals of the slopes are indicated in the Figures.

Results

Median and cumulative plasma concentration after acute myocardial infarction

Median plasma CRP concentrations as a function of time after the onset of symptoms for the two patient groups are shown in Figure 4-1.

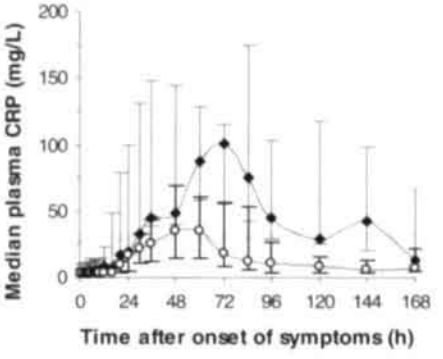


Figure 4-1, Plasma CRP after AMI.
Median plasma concentrations of C-reactive protein (mg/L) as a function of time in patients who received thrombolytic therapy (*circles*, S⁺, n = 16) and in patients who received no thrombolytic therapy (*diamonds*, S⁻, n = 6). Median and 25th and 75th percentiles are indicated.

Cumulative release of the different proteins into plasma

For troponin T and HBDH, median and mean cumulative release, expressed in gram equivalents of myocardial tissue per litre of plasma, are indicated in Table 4-1. As has

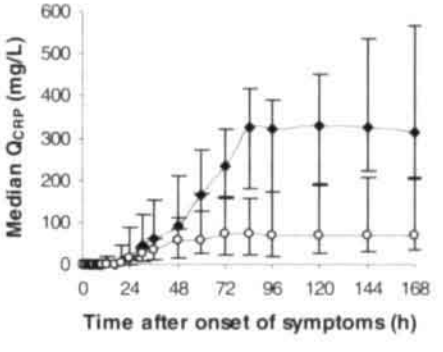


Figure 4-2, Cumulative CRP after AMI.
Median cumulative amount of C-reactive protein expressed (mg/L) as a function of time in patients who received thrombolytic therapy (*circles*, S⁺, n = 16) and in patients who received no thrombolytic therapy (*diamonds*, S⁻, n = 6). Median and 25th and 75th percentiles are indicated.

been reported earlier,[4,11] cumulative troponin T, in contrast to HBDH release is still increasing after 72 hours and is therefore computed over 168 hours. Compared to the free cytoplasmic protein HBDH, troponin T is only fractionally released after acute myocardial infarction.[11]

The median calculated cumulative amount of CRP as a function of time is shown in Figure 4-2. After 168 hours, a significantly higher amount of CRP (Q_{CRP}) was found in

patients who had not received thrombolytic therapy, compared to patients who had received thrombolytic therapy ($p < 0.021$) (Figure 4-2).

Correlation between infarct cumulative acute phase release

Figure 4-3 shows a significant correlation between Q_{CRP} and Q_{HBDH} (*top*), between Q_{CRP} and $Q_{troponin\ T}$ (*centre*) and between Q_{HBDH} and $Q_{troponin\ T}$ (*bottom*) especially in patients

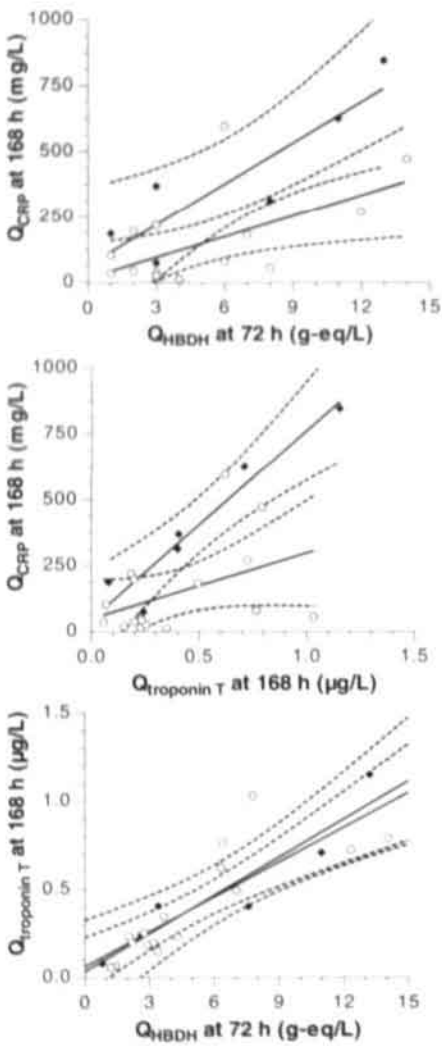


Figure 4-3, Correlations between cumulative protein releases.

Correlation between (*top*) cumulative hydroxybutyrate dehydrogenase release at 72 hours (infarct size) and the cumulative amount of C-reactive protein after 168 hours, between (*centre*) cumulative troponin T release and cumulative C-reactive protein and between (*bottom*) cumulative hydroxybutyrate dehydrogenase and cumulative troponin T in patients receiving thrombolytic therapy (*circles*, S⁺, n = 16) and in patients not treated with thrombolytic therapy (*diamonds*, S⁻, n = 6). The dotted lines indicate the 95% confidence limits of the regression lines.

(*top*) *circles*: $y = 26.8\ x + 12.9$, $r = 0.58$; $p < 0.02$; *diamonds*: $y = 52.6\ x + 66.1$, $r = 0.91$; $p < 0.011$; (*centre*) *circles*: $y = 248\ x + 50$, $r = 0.43$; ns; *diamonds*: $y = 717x + 47$, $r = 0.96$; $p < 0.003$; slopes significantly different ($p < 0.04$); (*bottom*) *circles*: $y = 0.072\ x + 0.036$, $r = 0.95$; $p < 0.003$; *diamonds*: $y = 0.066x + 0.68$, $r = 0.82$; $p < 0.003$

not receiving thrombolytic therapy. For the correlations between CRP and HBDH or troponin T, the slopes are lower in the patients who received thrombolytic therapy, indicating a lower CRP response (Figure 4-3, *top* and *centre*).

Between the cumulative release of troponin T and HBDH, a good correlation was found and here the slopes of the two patient groups were not significantly different. This indeed suggests a lower CRP response in patients receiving thrombolytic therapy, which is in accordance with the literature.[3]

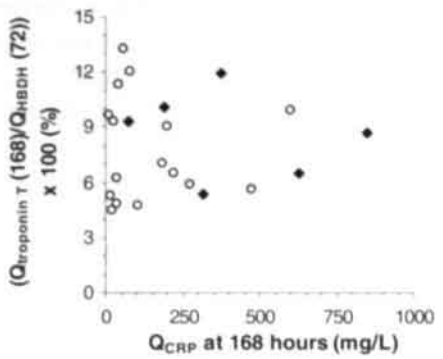


Figure 4-4, Fraction of troponin T release.
Ratio of cumulative troponin T and hydroxybutyrate dehydrogenase release, ($Q_{troponinT}$ (168) / Q_{HBDH} (72) x 100, *i.e.*, the fraction of troponin T released, related to the cumulative amount of C-reactive protein after 168 hours (Q_{CRP} (168)) for the different groups (*circles*, S', n = 16), (*diamonds*, S', n = 6)

Figure 4-4 shows that the fraction of troponin T released, compared to HBDH release, is not influenced by the height of the CRP response.

Discussion

Patient groups

Since the introduction of thrombolysis, this therapy has proven to be very effective in patients suffering from acute myocardial infarction. For that reason every patient with signs of acute myocardial infarction will be stratified for thrombolysis, unless there is a significant contra-indication to do so. It goes without saying that these factors influence the composition of the two different groups in the present study. It is very difficult to include a group of patients receiving no thrombolytic therapy and matching the other group. In the present study, treatment delay was comparable between the groups.

The aim of the present study was to investigate the cumulative release of troponin T in relation to the acute phase response in the two patient groups. As troponin T is bound to myofibrillar structures, thrombolytic therapy and the height of the acute phase response might influence the cumulative release of this protein in a different way compared to the free cytosolic enzymes. As it is known that cumulative cytoplasmic enzyme release, *i.e.* enzymatic infarct size, is not influenced by vessel patency, HBDH release was used for estimation of infarct size.[17] As we studied cumulative release, plasma time-concentration curves and cumulative release patterns were calculated from the onset of symptoms and not from the onset of treatment.

Acute phase response, C-reactive protein response

In previous studies it has been indicated that coronary recanalisation affects the CRP response in acute myocardial infarction.[2,3,18] Patients with an open coronary artery after treatment with plasminogen activator or combined with angioplasty had a smaller CRP response in relation to infarct size than patients in whom the treatment failed or who did not receive thrombolytic therapy. If thrombolytic therapy is not given, there is a stronger association between serum CRP and infarct size.[3] Although the mechanism is not clear, it seems that rapid reperfusion of the myocardium changes the infarct-related inflammatory response. Subsequently, the cytokine-stimulated synthesis of CRP does not take place to the degree expected from infarct size.

In the present study, the cumulative amount of plasma CRP over a period of 168 hours was estimated from a highly frequently sampled plasma curve and related to infarct size (Q_{HBDH}). Although the groups are small, the results obtained for CRP are in accordance with the study by Pietilä *et al.*[2,3,18]

Recovery of the different markers

Quantitative recovery of cytosolic cardiac proteins into plasma after ischemic myocardial damage has been demonstrated in experimental studies. The total activities of free cytosolic creatine kinase and HBDH lost from dog heart after permanent coronary occlusion[6] equalled the total release of these proteins into plasma. In man, conditions could be different. However, the fact that similar estimates of infarct size are obtained for different enzymes indicate that, also in man, these cytoplasmic proteins are recovered completely in plasma within 72–100 hours after the onset of symptoms.[4,19,20]

In a previous study, we found that in patients receiving thrombolytic therapy, cumulative troponin T release is only a fraction of cumulative cytoplasmic creatine kinase or HBDH release.[11] The structural proteins like troponin T remain much longer in the infarcted area and differences in acute phase response might influence its release. However, from the present data it becomes clear that the intensity of the acute phase response, estimated from cumulative plasma CRP, has no effect on the relative proportions of troponin T and HBDH released into plasma.

Addendum

New insights in the troponin T release mechanisms as described in Chapter 2, and the renal removal of troponin T described in Chapter 3, have revealed a much larger FCR for troponin T. Although this significantly changes the calculations made for the estimation of total cumulative troponin T release, as discussed at page 6, the main conclusion of this chapter —the intensity of the acute phase does not influence troponin T release— remains unaltered.

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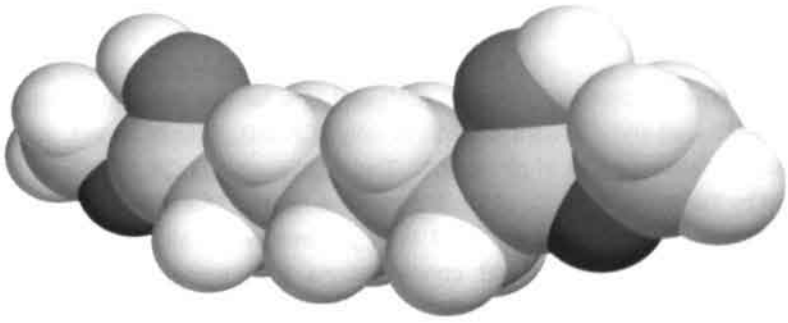
Chapter 5.

CK-MB content and tissue acidosis



Adapted from Boumans ML, Diris JHC, Nap M, Muijtjens AMM, Maessen JG, Van Dieijen-Visser MP, Hermens WTh: Creatine Kinase Isoenzyme MB controversy: perimortal tissue acidosis may explain the absence of CKMB in myocardium at autopsy. *Clin Chem* 2001;47(9):1733–5.

Abstract

Background

The amount of creatine kinase isoenzyme MB (CK-MB) in plasma of patients with myocardial infarction has been found to be closely related to the total CK activity. In contrast, reported values for the human cardiac tissue content of CK-MB have differed widely. This discrepancy between tissue and plasma content was investigated in the present study.

Methods

Creatine kinase isoenzymes, aspartate aminotransferase (AST) and α-hydroxybutyrate dehydrogenase (HBDH) were measured in human cardiac autopsies obtained from 20 patients who died from non-cardiac causes, 6 autopsies from patients who died after acute myocardial infarction (AMI), and 9 biopsies from patients who underwent open heart surgery. In addition CK and CK-MB were measured in plasma samples of 164 patients suffering from AMI, taken six hours after the onset of symptoms. Human cardiac tissue samples were experimentally exposed to pH values between 5 and 7.5.

Results

Extremely high coefficients of variation (CV) of 131% for the tissue content of CK-MB mass ($CK\text{-}MB_{mass}$) and 79% for CK-MB activity ($CK\text{-}MB_{act}$) were found, whereas the other enzymes showed CVs of about 25%. The large scatter in CK-MB content could be explained by the virtual absence of CK-MB in some hearts. This effect was not related to autopsy delays. In the hearts obtained from patients with AMI, endocardial samples showed consistently lower CK-MB concentrations than epicardial samples. In vitro exposure to acidosis demonstrated instability of CK-MB for pH values below 6.

Conclusion

This study shows that the low and highly variable CK-MB content of myocardial tissue is not related to sample location or to autopsy delay. However, a variable degree of perimortal myocardial acidosis may explain our results as well as the controversial findings in the literature.

Introduction

In patients with AMI it has been shown that the activity of CK-MB in plasma consistently accounts for about 15% percent of total CK activity,[1-3] but published data on CK-MB content in cardiac tissue, both absolute and as a percentage of total CK, have been conflicting. Values in accordance with 15% plasma activity of CK-MB have been published,[3-6] while virtual absence of CK-MB from healthy myocardium has also been claimed.[7-9] It was speculated that low levels of CK-MB in normal hearts and high levels of CK-MB in coronary heart disease could be explained by a mechanism of cellular adaptation.[7-9] However, CK-MB is a rather labile enzyme with a limited thermostability[10-12] and susceptible to pH.[10,12] So low CK-MB tissue content in autopsies could be explained by inactivation of CK-MB as well.

The objective of the present study was to examine whether inactivation of CK-MB in autolysing myocardium, either post-mortem or during perimortal tissue acidosis, could explain the absence of CK-MB in cardiac tissue. Therefore, the protein mass of CK-MB ($CK\text{-}MB_{mass}$) and the activity of CK-MB ($CK\text{-}MB_{act}$) were measured both in myocardial autopsies and in biopsies taken from the atrial appendices of patients who underwent open-heart surgery.

Regional variation in CK-MB tissue content was measured in myocardial autopsies. The influence of tissue acidosis was verified from in vitro exposure of heart tissue to a pH range of 5.0–7.5. Finally, since in patients with AMI the ischemia and hence the tissue acidosis is located predominantly in the endocardium, the differences in CK-MB content between endocardial and epicardial samples were studied in heart slices of patients who died after AMI.

Methods

Autopsies

Myocardial autopsies from 20 patients who died from non-cardiac causes and without a known history of cardiac complaints were obtained from the Department of Clinical Pathology of the Atrium Hospital in Heerlen. A slice of 1 cm thickness was excised from the heart, about midway between the apex and the base, and coloured with a standard tetrazolium assay, to check for absence of abnormalities. After colouring the slice was stored at -70°C. Similar heart slices were obtained from 6 patients who died from a recent AMI.

For the first 9 of the 20 non-AMI heart slices, the left ventricle was cut circumferentially into 2 anterior, 2 lateral, 2 posterior and 2 septal transmural samples (see Figure 5-1), and each sample was divided into epicardial and endocardial parts, as described by Van der Veen.[13] The right ventricle was divided into 2 transmural samples, and 2 additional samples were taken from the left and right atrial appendices. Left ventricular samples were numbered counter-clockwise, starting with the first anterior sample just to the right of the LAD (endocardial sample 1 and epicardial sample 2), up to the last septal sample

(endocardial sample 15 and epicardial sample 16). So, odd and even numbers indicated endocardial and epicardial samples, respectively. Initial analysis of hearts 1–9 showed that enzyme content did not vary significant for samples obtained at different locations.

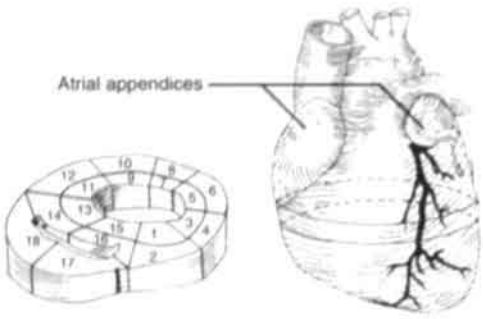


Figure 5-1, Sampling of heart slice.
Reprinted from Cardiovascular Research, V22:611-19, van der Veen *et al.*, Myocardial enzyme depletion in infarcted human hearts: infarct size and equivalent tissue mass. Copyright (1988) with permission from European Society of Cardiology.

Therefore, only a single sample was randomly taken from the left ventricle of the remaining 11 heart slices.

Patient plasma

Because of approximately equal disappearance rates of CK and CK-MB from plasma, the ratio of these proteins in plasma of patients suffering from AMI remains the same during the initial phase after AMI. CK and $CK\text{-}MB_{mass}$ were therefore measured in venous blood samples of 164 AMI patients, taken about six hours after onset of symptoms. Clotting was prevented by dry heparin and samples were centrifuged immediately. The supernatant plasma was stored at -70°C.

Biochemical analysis

Tissue samples of 90–200 mg of wet weight were homogenised and sonicated in 0.05 M Tris-HCl buffer, pH = 8.5, containing 3 mM mercapto-ethanol and 15 µM pyridoxal phosphate as described.[14] From the thoroughly mixed homogenate a sample was taken to determine dry weight. The remaining homogenate was centrifuged for 10 min. at 3000 rpm and the supernatant was assayed. Dry weight was determined by lyophilisation in a Leybold Heraeus vacuum freeze drier (Type GT2) after previous freezing of the samples. All data were expressed per milligram dry weight of tissue.

Creatine kinase (CK), aspartate aminotransferase (AST) and α-hydroxybutyrate dehydrogenase (HBDH) were determined spectrophotometrically on a centrifugal analyzer (Cobas Fara II System, Roche Diagnostics, Mijdrecht, The Netherlands), using commercially available test kits (CK-NAC-activated test from Merck Diagnostics, Darmstadt Germany, Granutest 25; AST Granutest 25 also from Merck; HBDH optimised test from Roche Diagnostics). Activities were determined in duplicate and expressed in micromoles of substrate converted per minute and per litre (U/L). CK was assayed at 37°C and HBDH and AST at 25°C.

$CK\text{-}MB_{mass}$ was measured with the Immulite Automated Analyzer (detection limit, 0.42 µg/L; Diagnostic Product Corporation). In addition, $CK\text{-}MB_{mass}$ was measured with the fully automated Chiron ACS:180 (Bayer, Houten, The Netherlands). The capture antibody of the ACS method is an anti-CK-B, whereas it is the "Conan-type" anti CK-MB, used in most commercial $CK\text{-}MB_{mass}$ assays[15] in the Immulite assay.
$CK\text{-}MB_{act}$ was measured with the Isomune assay of Roche Diagnostics (immunoinhibition), using a centrifugal analyser (Cobas Fara, Roche Diagnostics, Mijdrecht, NL). Tissue-samples with total CK activities exceeding 1000 U/l were diluted.
Mitochondrial CK (CK_{mit}) activity was also measured with the Isomune assay. This assay involves a separate step by which all CK-MM and CK-MB is bound to an anti-M antibody and removed from the sample. The remaining activity, corrected for background conversion by Adenylate Kinase, was taken as CK_{mit}. It was checked by electrophoresis (Creatine Kinase isoenzyme electrophorese kit, Beckmann Instruments, Inc.) that no CK-BB was present in the tissue. CK-MM activity was obtained by subtraction of $CK\text{-}MB_{act}$ and CK_{mit} from total CK activity.

Analysis of data
Data analysis was performed with standard software (SPSS Inc., Chicago, Illinois, USA, version 10.0). Analysis of variance was performed to investigate the effect of several factors on the enzyme content of a sample. Three factors were investigated: heart, location and side. The factor heart has 9 levels for the 9 different hearts, the factor loc has 4 levels (anterior, lateral, posterior and septal) for the circumferential location, and the factor side has 2 levels (endo- and epicardial) for the transmural location. The model used was:

$$C_{ijkl} = \mu + \delta_i + \delta_j + \delta_k + \delta_{ij} + \delta_{ik} + \delta_{jk} + \delta_{ijk} + \varepsilon_{ijkl} \qquad \text{Equation 5-1}$$

with:
- C_{ijkl} = observed activity in heart i at loc j and side k in sample l.
- μ = true general mean of C_{ijkl}.
- δ_i = deviation in heart i (i = 1 to 9).
- δ_j = deviation in loc j (j = 1 to 4).
- δ_k = deviation in side k (k = 1 or 2).
- δ_{ij} = interaction of heart i and loc j (some hearts may have larger differences between locations than others).
- δ_{ik} = interaction of heart i and side k.
- δ_{jk} = interaction of loc j and side k.
- δ_{ijk} = interaction of heart i, loc j and side k.
- ε_{ijkl} = residual deviation in C_{ijkl} due to remaining experimental scatter.

Variance components corresponding to the deviations (main effects) and interaction effects in the above model were estimated and tested for significance ($p < 0.05$).

The influence of sex, autopsy delay, heart weight and age on tissue protein content was investigated by means of linear regression equations.

Influence of tissue acidosis.
Tissue samples of 60–150 mg of wet weight were incubated at 37°C in a phosphate-citrate buffering solution, containing 2% bovine serum albumin (BSA, fraction V, A-4503, Sigma Chemical Company, USA), for eight different incubation periods. After incubation the buffered solution and the tissue sample were separately frozen at -70°C. Tissue samples were homogenised and CK, CK-MB_{act}, CK-MB_{mass}, AST and HBDH were determined in the homogenates as well as in the incubation buffers.

Results

Patient characteristics and tissue data.
Table 5-1 presents patient characteristics and experimental data for 20 patients who died from non-cardiac causes and patient characteristics for 6 patients, who died from AMI. In the 6 patients who died from AMI presented in Table 5-1, only HBDH, CK and CK-MB_{mass} were determined. Overall inter-individual variation (CV) in tissue content for heart 1–20 was 22% for HBD, 23% for AST, 26% for CK_{mit}, 37% for CK and 45% for CK-MM, with extremely large values of 131% and 79% for CK-MB_{mass} and CK-MB_{act}, respectively. As shown in Figure 5-2, this large variation of CKMB is explained by virtual absence of this protein in some hearts (patient number 1,3,4,6 and 7).

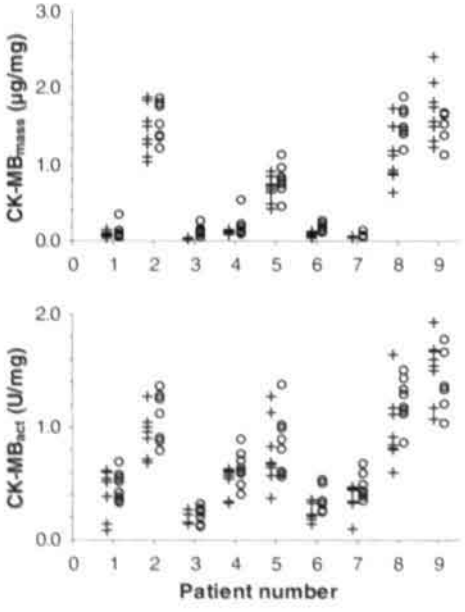


Figure 5-2, CK-MB content in 9 hearts.
Left ventricular tissue content of CK-MB_{mass} and CK-MB_{act} in 16 samples obtained in each of 9 hearts of patients who died from non-cardiac causes (first 9 hearts from Table 5-1). Endocardial samples are indicated by + and epicardial samples by o.

Table 5-1, Patient characteristics and experimental data of tissue sampling.

ID	Sex	Age (yr)	Autopsy delay	Length (cm)	HW (g)	Cause of death	CK Total	CK-MB_{act}	CK-MB_{mass}	CK-$_{rel}$	HBDH	AST
Patients died from non-cardiac causes												
1	M	83	48	165	450	Bronchoalveolar carcinoma	6.52	0.32	0.05	1.89	0.56	0.69
2	M	65	51	181	450	Sepsis	10.75	0.95	1.57	2.19	0.53	0.77
3	M	79	46	167	310	Pneumonia, sepsis	5.44	0.11	0.05	1.69	0.47	0.60
4	F	78	3	172	360	Cerebral hemorrhage, bronchopneumonia	6.06	0.59	0.17	1.43	0.40	0.70
5	M	77	16	178	450	Lung embolism	7.55	1.02	0.77	1.48	0.49	0.61
6	F	87	21	150	560	Respiratory insufficiency	4.16	0.24	0.12	1.34	0.39	0.51
7	F	71	9	172	500	Aspiration pneumonia (icterus)	5.40	0.43	0.05	1.65	0.48	0.71
8	M	41	19	177	410	Cerebral hemorrhage, bronchopneumonia	8.11	1.28	1.40	1.69	0.50	0.75
9	F	88	63	162	500	Sepsis, lungfibrosis, apnoe	11.38	1.50	2.07	2.58	0.65	0.79
10	F	69	12	157	600	Respiratory insufficiency (pneumonia)	4.93	0.22	0.05	2.29	0.44	0.75
11	F	83	8	162	430	Disturbance of blood chemicals	4.04	0.26	0.06	1.47	0.54	0.73
12	M	79	69	168	500	Sepsis	4.75	0.53	0.52	1.26	0.42	0.58
13	F	83	51	134	330	Respiratory insufficiency	6.92	1.25	1.88	2.05	0.50	0.74
14	M	65	22	170	470	Sepsis (hepatogenic shock)	13.21	1.60	2.14	3.11	0.77	1.21
15	F	70	67	170	540	Cerebral hemorrhage	8.54	0.27	0.05	2.82	0.72	0.93
16	M	72	17	168	450	Respiratory insufficiency, bronchopneumonia	8.00	0.57	0.07	1.98	0.54	0.59
17	M	71	17	170	450	Stomach hemorrhage, shock	6.42	0.29	0.07	2.27	0.70	0.84
18	M	28	24	186	440	Lung embolism	8.48	0.26	0.09	1.90	0.39	0.57
19	F	67	12		480	Stomach lymphoma	3.06	0.30	0.02	1.67	0.42	0.47
20	M	65	6	161	650	Respiratory insufficiency	6.00	1.10	0.42	2.12	0.62	0.76
						Mean	6.99	0.66	0.58	1.94	0.53	0.72
						CV (%)	37.2	72.9	132.0	25.5	21.5	22.8

Table 5-1. (continued): Patient characteristics and experimental data of tissue sampling.

ID	Sex	Age (yr)	Autopsy delay	Length (cm)	HW (g)	Location of recent infarction	CK Total	CK-MB_{act}	CK-MB_{mass}	CK-$_{mit}$	HBDH	AST
Patients died from a recent myocardial infarction												
21	M	68	48	167	600	L, Ap, S						
22	M	78	48	171	520	P						
23	M	76	15	165	700	A, L						
24	M	71	10	180	550	P	No random sampling because of recent infarction					
25	F	56	24	159	500	A, S, Ppap						
26	M	65	19	166	490	A, P, S						

CK-total, CK-MB_{act}, CK_{mit}, HBDH and AST are expressed in Units per mg dry weight (U/mg dw). CK-MB_{mass} is expressed in µg/mg dw.
HW = Heart Weight; A = anterior; L = lateral; P = posterior; S = septal; Ap = apex; Ppap = posterior papillarymuscle
Patients 1-9 were used to determine the variation within the heart.

Analysis of variance for nine left ventricles

Variance component estimation showed that the factor heart accounted for the major part of total variance: 65% for CK, 62% for CK-MM, 42% for CK_{mit}, 79% for $CK\text{-}MB_{act}$, 90% for $CK\text{-}MB_{mass}$, 55% for HBDH and 49% for AST. These contributions were all statistically significant, while all other main and interaction effects were not. The contribution of experimental scatter to total variance was 21% for CK and CK-MM, 39% for CK_{mit} and HBDH, 51% for AST, 14% for $CK\text{-}MB_{act}$ and 5% for $CK\text{-}MB_{mass}$. Differences between sampling sites did not contribute systematically to total variation in tissue protein content.

The influence of sex, autopsy delay, heart weight and age

Linear regression analysis for 20 patients who died from non-cardiac causes showed no significant effects of sex, age, autopsy delay and heart weight on cardiac protein content. Longer autopsy delays were not associated with lower CK-MB values. On the contrary, a tendency to lower CK-MB in hearts for short autopsy delays was noted (Figure 5-3), but was not statistically significant.

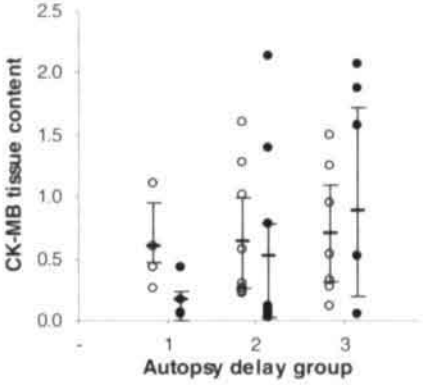


Figure 5-3, Autopsy delay and tissue content. Influence of autopsy delay on left ventricular $CK\text{-}MB_{mass}$ (*black circles*, µg/mg dw) and $CK\text{-}MB_{act}$ (*white circles*, U/mg dw) content of 20 patients who died from non-cardiac causes. Medians and the 25th and 75th percentiles are presented. The three groups present: (1) Autopsy delay < 10 hours (n = 4), (2) Autopsy delay 10–25 hours (n = 9), (3) Autopsy delay > 45 hours (n = 7).

$CK\text{-}MB_{mass}$ versus $CK\text{-}MB_{act}$

Figure 5-4 presents correlations between CK, $CK\text{-}MB_{mass}$ and $CK\text{-}MB_{act}$ in 6 hour plasma samples from AMI patients and left ventricular tissue samples from the first 9 hearts of Table 5-1. As shown in Figure 5-4 (*left panel*), $CK\text{-}MB_{mass}$ correlated well with CK in plasma samples. In contrast, almost one third of the tissue samples contained no $CK\text{-}MB_{mass}$ at all (Figure 5-4, *centre panel*), whereas in the remaining samples ($CK\text{-}MB_{mass}$ > 0.2 µg/mg dw) the ratio $CK\text{-}MB_{mass}$/CK was approximately equal to the ratio in plasma (0.20 and 0.25, respectively). Figure 5-4 (*right panel*) presents the correlation between $CK\text{-}MB_{mass}$ and $CK\text{-}MB_{act}$. A large cluster of data points on the ordinate shows that CK-

MB_{mass} was more often depleted than CK-MB_{act}. For the remaining samples (CK-MB_{mass} > 0.2), the regression equation showed a slope of 610 U/mg, equal to the specific activity.

High correlation coefficients (r > 0.98) were found between the two different CK-MB_{mass} assays used in the present study and no systematic difference was noted. Therefore, only the CK-MB_{mass} values determined with the Immulite assay are shown in the present study.

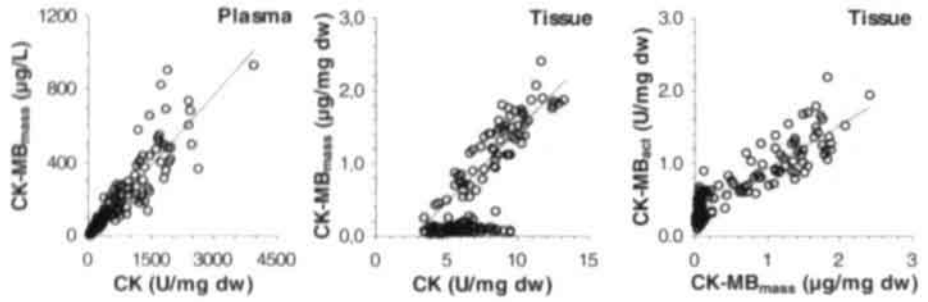


Figure 5-4. Relations found in plasma and tissue samples.

Relation between CK-MB_{mass}, CK-MB_{act} and CK in plasma samples and in tissue samples obtained at autopsy. Linear regression equations are: CK-MB_{mass} vs CK in plasma samples (*left panel*): y = 0.25x + 1.33 (n = 164). CK-MB_{mass} vs CK in tissue samples (*centre panel*): y = 0.20x - 0.52 (CK-MB_{mass} > 0.2 µg/mg dw; n = 70). CK-MB_{act} *vs* CK-MB_{mass} in tissue samples (*right panel*): y = 0.61x + 0.32 (CK-MB_{mass} > 0.2 µg/mg dw; n = 70).

Effect of pH changes

Figure 5-5 presents the results for CK-MB_{mass} of the incubations of tissue samples at different pH values. CK-MB, both activity and mass, almost completely disappeared within two hours of incubation at pH = 5.0 and 5.5. In contrast, HBDH and AST remained stable on all four different pH values during the complete interval of 24 hours (data not shown). CK-MM diminished at pH = 5 to 60% in 4.5 hours and to almost 10 % at 24 h.

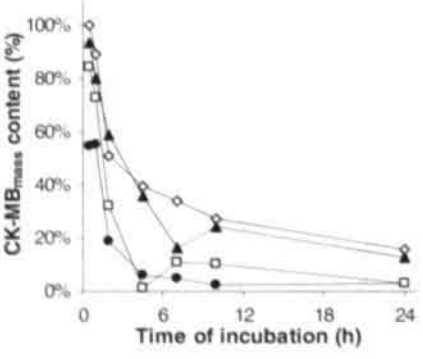


Figure 5-5, Influence of pH.

Influence of pH-changes on CK-MB_{mass} in heart tissue. Data are expressed as percentage of the tissue content obtained at time = 0. *circles* = pH 5, *squares* = pH 5.5, *triangles* = pH 6.5, *diamonds* = pH 7.5.

CK_{mit} remained constant for the first 10 hours, and at pH = 5.0 and 5.5 diminished to 60% in the following 14 hours. In conclusion, CK-MB is highly susceptible to acidosis in contrast to the other cardiac markers.

Influence of ischemia on transmural CK-MB differences

The transmural ratios (endocardial/epicardial) of HBDH and CK-MB_{mass} were computed in only 4 of the first 9 non-AMI hearts and in 4 of the 6 AMI hearts due to the very low CK-MB_{mass} content in the remaining hearts (< 0.2 µg/mg). Infarcted areas in the AMI hearts had normal physiologic HBDH content (> 0.4 U/mg dw) and endocardial/epicardial ratios of HBDH were equal in non-AMI and AMI hearts (1.08 ± 0.04 and 0.98 ± 0.05, respectively; mean ± 95% confidence interval). However, for CK-MB_{mass}, the ratios between non-AMI and AMI hearts differed significantly: 0.92 ± 0.12 and 0.53 ± 0.16, respectively ($P < 0.005$; Student t-test). This shows that CK-MB_{mass} disappears from AMI hearts before significant leakage of proteins from the infarcted tissue has started.

Biopsies versus autopsies

Tissue content of CK-MM, CK-MB_{mass}, CK-MB_{act} and HBDH in left ventricular, right ventricular and atrial samples of the first 9 autopsies of Table 5-1 and in 9 atrial biopsies were measured. The results confirm a study[16] reporting a significantly higher HBDH content in the left ventricle, compared to the right ventricle and the atrial appendices. No significant differences were found between autopsies and biopsies of the atrial appendices. CK-MM was significantly lower in atrial than in ventricular autopsies. Average differences of CK-MB (both activity and mass) between the 4 groups were not statistically significant, but CK-MB_{mass} was virtually absent (< 2%) in 4 of the 9 left ventricle samples. This tendency was less for CK-MB_{act}.

Discussion

Possible effects of sampling location, autopsy delay, sex, age and heart weight on CK-MB content of human myocardium

Studies of CK-MB content in human hearts[3-9,17] and reviews of myocardial CK isoenzyme composition[18-20] have shown a variation between 1% and 50% of total CK. In these studies a large variety of samples (autopsies or biopsies, left- or right ventricular or atrial samples) and test methods (electrophoresis, column chromatography, immunoassays or activity assays) was used. In contrast, consistent percentages of about 15% were found for CK-MB content in serial plasma or serum samples of patients suffering from AMI. To date no satisfactory explanation for this discrepancy has been offered.

Roberts[21] suggested that sampling at different sites in the heart could influence the scatter in the CK-MB content of human myocardium, but the present study shows no significant influence of the transmural or circumferential position in the heart. Therefore

the sampling location could not explain the large variation between the hearts, as confirmed in other studies.[13,22,23]
Another factor could be the autopsy delay. A decrease of CK-MB with increasing autopsy delay has been reported,[17] but other studies found no such effect,[13,22] in accordance with the present study. Age, heart weight and sex also had no significant influence.

The "fetal shift" hypothesis
It has been suggested that CK-MB would be almost absent in healthy human hearts and that the increased myocardial CK-MB in hypertrophied hearts or hearts of patients with a history of AMI was caused by expression of the foetal form of CK, the B subunit.[7-9] As mentioned by Roberts,[21] however, such a mechanism could not explain the good correlation between CK-MB and CK in serum of patients suffering for AMI, as demonstrated in Figure 5-3.
An alternative explanation is suggested by the fact that the "healthy" hearts in these studies were obtained from victims of traffic accidents. Although the autopsy delays were very short, between 15 and 180 min., and these patients were used as organ donors, a perimortal effect such as a short period of severe myocardial tissue acidosis may have caused inactivation of CK-MB. This might explain why these authors, to their own surprise, found high (>15%) CK-MB values in donor hearts obtained from patients on circulatory support.[9] In such hearts intracellular pH is regulated by cardioplegic buffer solutions, preventing tissue acidosis.[24]

Acidosis and loss of CK-MB
Because sampling location and autopsy delay are not responsible for the large difference between the hearts, another explanation would be a perimortal artefact. The instability of CK-MB to pH-values below 6 has been shown in earlier studies[10,12] using purified CK-MB. As seen in Figure 5-5, acidosis could result in the unexpected absence of CK-MB in myocardium. Because of the narrow range of pH values at which inactivation occurs, between 5.5 and 6.0, it could explain the 'all or none' aspect of this phenomenon. Data of the time course of myocardial pH after AMI[24,25] show that intracellular pH may reach values below 5.5. After death, acidosis will soon become less due to termination of lactate production and diffusion of the lactate already produced. The tendency to lower $CK\text{-}MB_{mass}$ values for short autopsy delays, as shown in Figure 5-3, suggests that part of acidosis-induced inactivation may be reversible.
The lower endocardial CK-MB content is consistent with the predominantly endocardial location of ischemia in AMI. This transmural effect is much more pronounced for $CK\text{-}MB_{mass}$ than for HBDH, and is also larger for AMI patients, than for patients who died of a non-myocardial cause. Due to a larger degree of metabolic acidosis in the left ventricle than in the right ventricle, CK-MB could be absent from left ventricular samples, while being present in the right ventricle. Nascimben and Sylven[8,9] also reported almost zero values for CK-MB in the left ventricle in contrast to normal values in the right ventricle and atria.

Conclusions

The effects of a variable degree of perimortal myocardial acidosis may not only explain the controversial findings in the literature, but also the findings of the present study. Lack of CK-MB in apparently healthy hearts from traffic accident victims[7,9] could be explained by serious tissue acidosis in the prolonged agonal phase of these victims who survived long enough to die after hospital admission.

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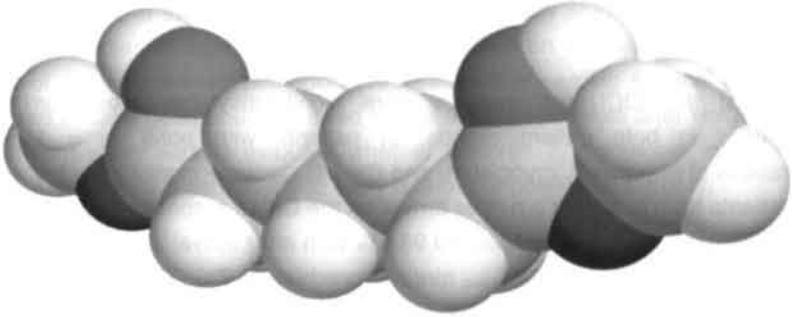
Chapter 6.

Early diagnosis of myocardial infarction after CABG



Adapted from Fransen EJ, Diris JHC, Maessen JG, Hermens WTh, Van Dieijen-Visser MP: Evaluation of "New" Cardiac Markers for Ruling Out Myocardial Infarction After Coronary Artery Bypass Grafting *Chest* 2002;122(4):1316–21.

Abstract

Background

This retrospective study was conducted to evaluate the value of serum troponin T, myoglobin, and creatine kinase isoenzyme MB mass ($CK\text{-}MB_{mass}$) concentrations for ruling out perioperative myocardial infarction (poMI) early after cardiac surgery.

Methods

One hundred eighty-one patients undergoing coronary artery bypass grafting (CABG) with cardiopulmonary bypass were included at the cardiothoracic surgery department in our university hospital. Serum concentrations of troponin T, myoglobin, and $CK\text{-}MB_{mass}$ were measured preoperatively (baseline), on arrival at the cardiosurgical intensive care unit (CICU), and at 2, 4, 8, 12, 16, and 20 h after arrival at the CICU. The strength of the markers studied for ruling out poMI was studied using receiver operating characteristics curves. Based on the sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) for each marker at every time point, optimal cut-off values were calculated.

Results

On arrival at the CICU, all markers were significantly increased from baseline concentrations in both patient groups. In patients with poMI (n = 14), serum concentrations of troponin T, myoglobin, and $CK\text{-}MB_{mass}$ were significantly higher than in control patients from 8, 2, and 0 h after arrival on the CICU, respectively. $CK\text{-}MB_{mass}$ was the earliest marker, and its NPV reached 98.6% 12 h after arrival at the CICU. On arrival at the CICU, the NPV for $CK\text{-}MB_{mass}$ already reached 96.7%. The NPV for myoglobin reached 98.4% 12 h after arrival at the CICU. Troponin T was not an early marker for ruling out poMI, with an NPV reaching 98.6% 12 h after arrival on the CICU. During the first 8 h after arrival at the CICU, sensitivity, specificity, PPV, and NPV of $CK\text{-}MB_{mass}$ exceeded those of myoglobin and troponin T. In later measurements (until 20 h after arrival at the CICU), troponin T gave the most sensitive definition of poMI.

Conclusions

For ruling out poMI on the CICU after CABG, $CK\text{-}MB_{mass}$ is a better marker than myoglobin and troponin T during the first 12 h after arrival on the CICU. Using these markers, postoperative treatment of cardiac surgical patients might be further improved.

Introduction

In patients undergoing coronary artery bypass grafting (CABG), early diagnosis of perioperative myocardial infarction (poMI) is important because it remains a serious complication.[1,2] Currently, the diagnosis of poMI is based on changes in the ECG and increased release of biochemical markers. Previously, several biochemical markers for detection of myocardial damage have been proposed. We showed that cardiac marker proteins (fatty acid-binding protein (FABP) and myoglobin) release can be used to determine myocardial tissue loss due to the surgical procedure.[3] In addition, we showed that these proteins can be used to discriminate surgery-related myocardial injury from tissue loss caused by poMI. FABP was shown to allow diagnosis of poMI as soon as 4 h after removal of the aortic cross-clamp.

However, next to early diagnosis, markers used for the detection of poMI should also be sensitive and specific. In this respect, FABP and myoglobin do not fulfil these recommendations. Troponin T and creatine kinase isoenzyme MB mass (CK-MB_{mass}) have been shown to be promising candidates.[1,4-9] As being part of the tropomyosin complex of myocardial tissue, troponin T is highly cardiac specific, which could improve the diagnosis of poMI in cardiac surgical patients. In many studies, the emphasis of the diagnostic properties of biochemical markers has been on the detection rather than the ruling out of poMI. However, postoperative treatment of cardiac surgical patients could be improved in case poMI could be ruled out as early as possible after surgery. The aim of the present study was to evaluate whether troponin T, myoglobin, and CK-MB_{mass} measurements enable a sensitive and early rule-out of poMI after surgery.

Methods

Patients

One hundred eight-one adult patients undergoing elective CABG with the use of cardiopulmonary bypass (CPB) were enrolled. Age boundaries were set between 35 years and 80 years. Exclusion criteria were as follows: (1) ongoing infarction; (2) treatment with fibrinolytics within 48 h prior to surgery; (3) hepatic disease as indicated by aspartate aminotransferase (ASAT) and alanine aminotransferase levels > 2 times the upper limit of normal, or by bilirubin levels > 1.5 times the upper limit of normal; and (4) severe coagulation abnormalities. The study was performed according to the rules of the local medical ethical committee.

Intraoperative patient management

Standard anesthetic (lorazepam, fentanyl citrate, sufentanil citrate, alfentanil hydrochloride, midazolam hydrochloride, pancuronium bromide) and monitoring techniques (ECG, central venous/pulmonary and arterial pressure monitoring, urinary output, rectal and skin temperature monitoring) were used in all patients. Before

connection of the extracorporeal circuit for CPB, 300 IU/kg body weight heparin was administered, (Heparin Leo; Leo Pharmaceutical Products BV; Weesp, the Netherlands) in order to achieve an activated coagulation time > 480 s (Hemochron 400; International Technidyne Corporation; Edison, NJ). Specifications on the extracorporeal circulation circuit, CPB procedures and surgical procedures have been described previously.[10] Postoperative patient treatment in the cardiosurgical ICU (CICU) was standardised and similar for both patient groups. None of the patients received thrombolytic agents.

Blood sampling

Blood samples were obtained preoperatively (baseline), on arrival at the CICU, and at 2, 4, 8, 12, 16, and 20 h after arrival at the CICU. All samples were collected in 10 mL integrated serum separator tubes (Corvac; Sherwood Medical; St. Louis, MO). Immediately after sampling, blood was cooled, routinely centrifuged, and serum samples were stored at -70°C until analysis.

Myocardial infarction diagnosis

Diagnosis of poMI was established by a cardiologist based on ECG changes (new persistent Q waves and ST-segment deviations; ≥ 1 mm ST-segment elevation in two or more limb leads and/or ≥ 2 mm ST-segment elevation in two or more precordial leads), and a typical rise and fall in the serum CK, CK-MB activity, and ASAT curves. This resulted in two patient groups: patients in whom poMI developed (poMI group), and patients without poMI (no-poMI group).

Analytic techniques

The serum concentrations of CK-MB_{mass}, myoglobin, and cardiac troponin T (third-generation) were all analysed on the Elecsys 2010 (Roche Diagnostics GmbH; Mannheim, Germany). Routine clinical chemistry parameters ASAT, alanine aminotransferase, bilirubin, and CK-MB activity were determined on the Beckman Synchron CX7 System (Beckman Coulter; Fullerton, CA).

Data analysis

All data are presented as mean ± SEM. A Mann-Whitney *U* test was used for comparisons between two variables at the same time point. A Wilcoxon matched-pairs, signed-ranks test was used for comparisons of values from one variable between two time points. A X^2 test was used to test nonnumeric variables. Receiver operating characteristics (ROC) curves were used to compare the performance of the biochemical diagnostic methods of poMI and to determine the appropriate cut-off values for the different cardiac markers. Sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were calculated to analyse the diagnostic value of each marker. The level of significance was set at $p < 0.05$.

Results

Clinical characteristics

The perioperative characteristics of all patients are shown in Table 6-1. Fourteen patients (7.7%) showed evidence of poMI according to ECG changes and routine laboratory data. These patients had longer CPB times and a longer postoperative hospital stay than the patients without poMI.

Table 6-1, Clinical characteristics of patients with or without poMI*

Variables	No-poMI (n=167)	poMI (n=14)	p value
Age (yr)	63.8 ± 1	67.1 ± 3	ns
Male / female gender (%)	74.9/25.1	78.6/21.4	ns
Distal anastomoses (No.)	3.7 ± 0.1	3.9 ± 0.3	ns
CPB duration (min.)	80 ± 3	108 ± 13	< 0.05
ACC duration (min.)	50 ± 1	65 ± 8	ns
Post operative hospitalisation (days)	7.2 ± 0.3	15.5 ± 4	< 0.05

*Data are presented as mean ± SEM.

CPB = cardiopulmonary bypass; ACC = aortic cross clamping; ns = not significant.

Cardiac marker concentrations

Preoperative CK-MB_{mass} concentrations in the no-poMI group and the poMI group were 1.5 ± 0.3 µg/L and 1.4 ± 0.2 µg/L, respectively (Figure 6-1, *top*). CK-MB_{mass} concentrations in the no-poMI group and the poMI group each increased until 4 h after arrival at the CICU, and then further increased only in the poMI group. From arrival at the CICU until 20 h thereafter, CK-MB_{mass} concentrations were significantly higher in the poMI group ($p < 0.05$). At the first postoperative day, CK-MB_{mass} concentrations were 13.3 times higher in poMI patients compared to no-poMI patients. Maximal CK-MB_{mass} serum concentrations were 20.1 ± 1.3 µg/L in the no-poMI group at 4 h after arrival at the CICU, and 204.2 ± 50 µg/L in the poMI group at 20 h after arrival at the CICU.

Preoperative troponin T concentrations in the no-poMI group and the poMI group were 0.027 ± 0.014 µg/L and 0.017 ± 0.012 µg/L, respectively (Figure 6-1, *centre*). Postoperative troponin T concentrations in the no-poMI group and poMI group each increased above preoperative concentrations, and were similar until 4 h after arrival at the CICU. From this time point on, postoperative troponin T in the no-poMI group steadily decreased toward the first postoperative day (20 h after arrival at the CICU), whereas the troponin T concentration in the poMI group persistently increased until 20 h after arrival at the CICU, at this time being 6.8-fold higher than in the control subjects. Thus, troponin T concentrations in the poMI group were significantly higher than in the no-poMI group from 8 h to at least 20 h after arrival at the CICU. Maximal troponin T serum concentrations were 0.90 ± 0.05 µg/L in the no-poMI group at 4 h after arrival at the CICU, and 3.3 ± 0.7 µg/L in the poMI group at 20 h after arrival at the CICU.

Preoperative myoglobin concentrations in the no-poMI group and the poMI group were 48 ± 4 µg/L and 44 ± 4 µg/L, respectively (Figure 6-1, *bottom*). Myoglobin concentrations increased from preoperative to the time of arrival at the CICU in both no-poMI and poMI patients. In poMI patients, myoglobin concentrations continued to increase, reaching peak levels at the first postoperative day (20 h after arrival at the CICU). At the latter time point, myoglobin concentrations were 3.5 times higher compared to no-poMI patients. A significant difference in myoglobin plasma concentrations between no-poMI and poMI patients was reached at 2 h after arrival at the CICU. Maximal myoglobin serum concentrations were 337 ± 13 µg/L in the no-poMI group at arrival on the CICU, and 1,067 ± 355 µg/L in the poMI group at 20 h after arrival on the CICU.

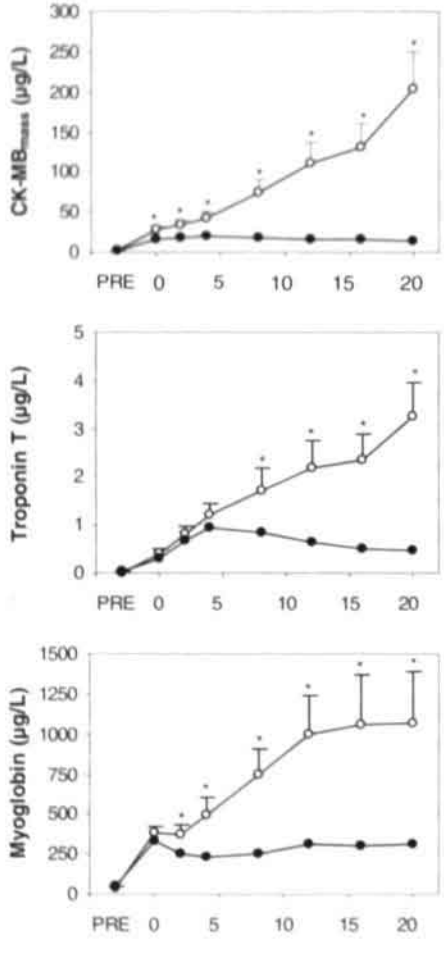


Figure 6-1. Serum concentrations.
Mean serum concentrations of CK-MB$_{mass}$ (*top*), troponin T (*centre*), and myoglobin (*bottom*). Preoperatively (PRE), on arrival (0), and at 2, 4, 8, 12, 16 and 20 h after arrival at the CICU in poMI (*white circles*, n = 14) and no-poMI (*black circles*, n = 167) patients.
*$p < 0.05$.

Threshold values and test characteristics

The strength of correlation between standard criteria (ECG and routine laboratory CK-MB activity) and serum troponin T, myoglobin, and CK-MB_{mass} was studied using ROC curves. The area under the curve for each marker at every time point is shown in Table 6-2. CK-MB_{mass} and myoglobin showed a close correlation between standard criteria for

Table 6-2. Area under the ROC curves and optimal cut-off.

	CK-MB_{mass}		Troponin T		Myoglobin	
Hours after arrival at the CICU	AUC*	Optimal Cut-off‡	AUC	Optimal Cut-off	AUC	Optimal Cut-off
0	0.74	17.9	0.59	0.25	0.65	331
2	0.76	20.0	0.57	0.65	0.78	261
4	0.79	21.1	0.60	0.84	0.85	255
8	0.82	22.0	0.73	1.0	0.90	297
12	0.90	28.9	0.83	1.07	0.89	433
16	0.88	22.2	0.91	0.75	0.89	396
20	0.96	29.2	0.94	0.81	0.89	359

*AUC = area under the curve, ‡ cut-off values are given in µg/L

poMI diagnosis and the cut-off values calculated using the ROC curves. For troponin T, this close correlation became evident later in the postoperative period. Cut-off values were derived from the corresponding coordinates between the intersection of the right bottom to left top diagonal and the ROC curve (Figure 6-2). Cut-off values for each marker at every time points are shown in Table 6-2. Serum levels of troponin T > 1.0 µg/L 8 h after arrival at the CICU confirmed the presence of poMI with a sensitivity of

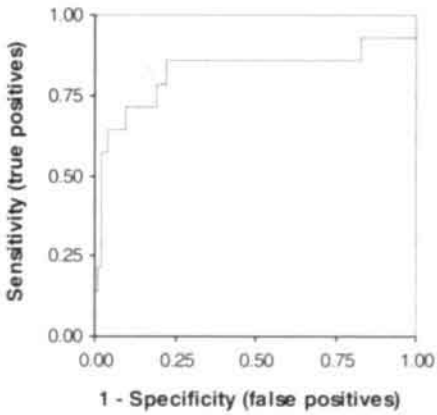


Figure 6-2. ROC curve.
Receiver operating characteristics curve of CK-MB_{mass} at 8 hours after arrival at the CICU. Optimal cut-off = 22.0 µg/L

76.9%, specificity of 72.7%, PPV of 18.2%, and NPV of 97.6%. At the same time point, CK-MB_{mass} serum levels > 22.0 µg/L confirmed the presence of poMI with a sensitivity of

78.6%, specificity of 77.6%, PPV of 22.9%, and NPV of 97.7%. Serum myoglobin levels > 297 µg/L 8 h after arrival at the CICU confirmed the presence of poMI with a sensitivity of 80%, specificity of 80.4%, PPV of 22.2%, and NPV of 98.3%. For each marker and at every time point, the sensitivity, specificity, PPV, and NPV of a single sample were calculated (Table 6-3).

Discussion

poMI is a serious complication after cardiac surgery, with a reported incidence of up to 26%, depending on the criteria used to select the patient groups.[2,11] Previously, we showed that using cardiac marker proteins instead of enzymes theoretically enabled a poMI to be diagnosed earlier after surgery.[3] Furthermore, we showed that in case CPB is used, myocardial tissue injury is inevitable.[3,12] However, both routinely used enzymatic markers (CK and CK-MB) and the proteins (FABP and myoglobin) we tested thus far either failed cardiac specificity or did not enable a diagnosis of poMI early after surgery.[3] The ideal marker for the diagnosis of poMI early after cardiac surgery would however possess both before mentioned characteristics.

Table 6-3, Test characteristics of CK-MB_{mass}, Moyglobin and troponin T.

	Hours after arrival at the CICU						
Variables	0	2	4	8	12	16	20
CK-MB_{mass}							
Sensitivity	71.4	71.4	71.4	78.6	85.7	78.6	64.3
Specificity	71.8	71.9	71.3	77.6	86.8	80.8	89.7
PPV	17.9	17.5	17.2	22.9	35.3	25.6	34.6
NPV	96.7	96.8	96.7	97.7	98.6	97.8	96.7
Myoglobin							
Sensitivity	60.0	70.0	70.0	80.0	80.0	75.0	66.7
Specificity	59.3	67.6	74.6	80.4	84.0	78.0	73.9
PPV	9.5	13.2	16.3	22.2	25.8	17.1	14.8
NPV	95.4	97.0	97.2	98.3	98.4	98.1	97.0
Troponin T							
Sensitivity	53.8	61.5	53.8	76.9	84.6	84.6	76.9
Specificity	53.3	61.1	54.9	72.7	84.2	73.7	69.5
PPV	8.2	11.0	8.6	18.2	29.7	20.0	16.4
NPV	93.7	95.3	93.8	97.6	98.6	98.4	97.5

Troponins are highly cardiac specific, a characteristic that could improve the diagnosis of poMI in cardiac surgery. In the present study, we studied the value of serum troponin T, myoglobin, and CK-MB_{mass} concentrations in patients undergoing CABG. Plasma concentrations of the cardiac markers show that in case a patient has undergone CABG

with the use of CPB, some myocardial damage occurs in all patients. These relatively moderate elevations of the markers studied, in comparison to concentrations found in patients with acute myocardial infarction (AMI), may reflect minimal myocardial damage. Based on the plasma curves (Figure 6-1, *bottom*) and its fast appearance and clearance features, myoglobin may be used to estimate ischemia-reperfusion injury early postoperatively. However, based on the test characteristics (Table 6-3), myoglobin is not the most suitable marker for ruling out poMI in patients undergoing CABG. Although the cut-off values of cardiac markers have been reported elaborately for patients presenting with acute chest pain,[13-15] these values are not well established for patients during and after cardiac surgery. Based on the data of the patients in the present study, we found optimal cut-off values for each marker at every time point (Table 6-2). Carrier *et al.*[1] recently showed acceptable test characteristics for troponin T in CABG patients 24 h after surgery, which increased toward 48 h after surgery. In the present study, however, we found optimal test characteristics for troponin T already at 12 h after arrival at the CICU. In addition, troponin T concentrations in the patients reported by Carrier *et al.*[1] were approximately twofold the concentrations found in the patients of the present study. The latter finding can be explained by the fact that Carrier *et al.* used the first-generation reagents for troponin T determinations as opposed to the third-generation reagents we used in the present study. The use of the linear regression formula provided by others,[16] and the instructions for use of the Troponin T STAT Immunoassay (Roche Diagnostics Gmbh, Germany), results in similar troponin T concentrations in the patients described by Carrier *et al.* and our patients. Nevertheless, the cut-off values in the patients of Carrier *et al.* were higher than the ones we found in our patients. Also, Swaanenburg *et al.*[17] recently showed that the release patterns of cardiac markers after uncomplicated heart surgery depend on the type of surgery and the circumstances during surgery. Therefore, because of insufficient analytical standardisation of the various cardiac marker methodologies, we strongly recommend that each institution should determine its own release patterns of cardiac markers for cardiac surgical procedures, and subsequently calculate the corresponding optimal cut-off values. Previously it was shown by de Winter *et al.*[9] that in patients with AMI, the size of the infarction influences the sensitivity and specificity in the early hours after onset of symptoms for CK-MB_{mass} and troponin T, while this effected myoglobin less. Troponin T, CK-MB_{mass}, and myoglobin levels increased earlier in patients with large infarcts. The latter finding may be one of the reasons for the fact that it is hard to find proper markers for poMI early after surgery. Our previous findings[3] and present findings show a large dispersion in postoperative cardiac marker plasma levels. Consequently, calculating optimal cut-off values based on the mean plasma levels in the control patients plus two times the SD results in cut-off values that rule out patients with small poMIs. Furthermore, the test characteristics of the marker studied will not reach appropriate values. In the present study, the test characteristics of the markers used were calculated using ROC curves. This resulted in relatively high values of particularly the NPV. The lowest NPV calculated for each marker at every time point was 93.7 (Table 6-3), indicating that in the worst case poMI could be

ruled out with 93.7% certainty using any of the markers studied. The relatively high values of sensitivity, specificity, and NPV at all postoperative time points may be explained by the fact that in our patients the "onset of symptoms," as it is usually called in patients with AMI, is the same for all patients, supposing that the poMIs in the present study have a perioperative aetiology.

Conclusions

Although the measurement of serum troponin T eventually might give the best definition of poMI, CK-MB_{mass} is the preferred marker for ruling out poMI. The data of the present study show that in patients undergoing CABG, troponin T and CK-MB_{mass} should be measured during the first 8 h after arrival on the CICU not to detect, but rather to exclude poMI. Whether this will lead to a better management of these patients from current postoperative treatment protocols remains to be evaluated in ongoing studies.

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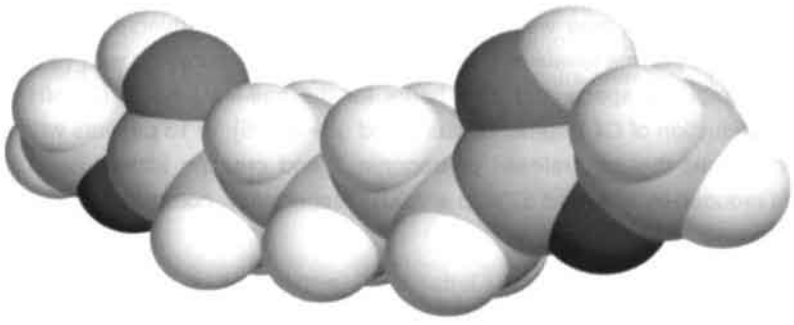
Chapter 7.

The Cetor study



Adapted from De Zwaan C, Kleine AH, Diris JHC, Glatz JFC, Wellens HJJ, Strengers PFW, Tissing M, Hack CE, Van Dieijen-Visser MP, Hermens WTh: Continuous 48-hours C1-inhibitor treatment, following reperfusion therapy, in patients with acute myocardial infarction. *Eur H J* 2002;23(21):1670–7.

Abstract

Background

Complement inhibition by C1-inhibitor (C1-INH) has been shown to reduce myocardial ischemia-reperfusion injury in animal models. We therefore studied the effects of intravenous C1-INH, following reperfusion therapy, in patients with acute myocardial infarction (AMI).

Methods

C1-INH therapy was started not earlier than 6 hours after AMI, in order to prevent interference with thrombolytic therapy. A loading dose of C1-INH was followed by continuous infusion for 48 hours, using 3 escalating dosage schemes. Efficacy of complement inhibition was estimated from C4 activation fragments. Plasma concentrations of cardiac markers were compared to values measured in matched control patients.

Results

In 22 patients, C1-INH was well tolerated and drug-related adverse events were not observed. Target plasma levels of C1-INH were reached accurately, with values of 48.2 mL/kg for distribution space and 35.5 hours for the half-life time of C1-INH. A dose-dependent reduction of C4 fragments was found ($p = 0.005$). In 13 patients who received early thrombolytic therapy, release of troponin T and creatine kinase isoenzyme MB mass was reduced by 36% and 57% ($p = 0.001$) respectively, compared to 18 controls.

Conclusions

Continuous 48-hours treatment with C1-INH provides safe and effective inhibition of complement activation after AMI and may reduce myocardial injury.

Introduction

Acute myocardial ischemia induces activation of the complement system. Activation products, formed through the 'classic' and 'alternative' pathways, promote neutrophil invasion into the ischemic tissue and the formation of cytolytic transmembrane complexes, leading to cell death.[1-3] Such local complement activation has been observed after myocardial ischemia in animals[4,5] and in man.[3,6,7]

In animal models, inhibition of complement activation reduced neutrophil invasion and myocardial cell death.[8-10] More specifically, administration of C1-(esterase)inhibitor (C1-INH), a natural inhibitor of the classical pathway of complement activation, considerably reduced myocardial ischemia/reperfusion injury in the rat,[11] the cat,[12] the pig[13] and the dog.[14]

Thusfar, no studies on the treatment with complement inhibitors in patients with acute myocardial infarction (AMI) have been published, while C1-INH has been used clinically without adverse effects, mainly in patients with hereditary angioedema,[15] but also in patients with sepsis and vascular leak syndrome.[16] Considering the beneficial effects observed in animals, treatment with C1-INH might thus be an option for the benefit of patients with AMI. However, most patients with AMI receive thrombolytic therapy and some plasmin-inhibiting activity of C1-INH has been demonstrated.[17] Therefore it was decided to start C1-INH therapy not earlier than several hours after thrombolytic therapy.

Methods

Study design

The investigation was a prospective, open-label, dose-escalation study. The study protocol, in accordance to the Declaration of Helsinki, was approved by the Review Board of the University Hospital Maastricht. Patients were older than 18 years and had chest pain (> 30 minutes) as well as electrocardiographic evidence for AMI (ST-segment elevation ≥ 0.2 mV in at least 2 contiguous precordial leads and/or ≥ 0.1 mV in at least 2 limb leads).

Patients with known hypersensitivity to plasma products were excluded, as well as patients with recent (< 4 weeks) AMI or surgery, infections, chronic use of acetylsalicic acid, NSAID's or dipyridamole, autoimmune diseases like rheumatoid arthritis or SLE, cardiogenic shock, renal insufficiency (creatinine > 120 µmol/L) or ventricular pacing.

All but four patients received intravenous thrombolytic therapy (see Table 7-1), either with streptokinase (1.5 million IU in 60 min.) or with recombinant tissue-type plasminogen activator (15 mg, followed by 50 mg in 30 min. and 35 mg in 60 min.). In addition, patients received intravenous heparin (5000 IU, followed by 1000 IU/h for 48 h, adjusted to prolongation of the APTT by a factor of 2–3), and acetylsalicylic acid (160 mg intravenous, followed by 80 mg/day orally). Because of possible inhibition of plasmin, C1-INH was not given within 2.5 hours after termination of SK infusion, or within 1 hour after termination of rtPA infusion. In these patients, C1-INH therapy was started 6 hours

after the onset of symptoms. In three patients C1-INH was given 1–2 h after reopening of the infarct-related artery by acute percutaneous transluminal coronary angioplasty (PTCA).

After informed consent, intravenous C1-INH therapy was started in patients with successful coronary reperfusion, as evidenced by both clinical signs of vessel patency and ST-segment normalisation. Three escalating dosage schemes were used (see Table 7-1), starting with a loading dose of 50 U/kg, followed by 1.25 U/kg/h during 48 hours, in the first 6 patients, aiming at reaching a C1-INH plasma concentration of 200% of normal. The following 5 patients received a loading dose of 100 U/kg followed by 1.25 U/kg/h during 48 hours. The remaining 11 patients a loading dose of 100 U/kg followed by 2.0 U/kg/h during 48 hours, aiming at a C1-INH plasma concentration of 300% of normal.

C1-INH preparation and assay

C1-INH (Cetor®, CLB, Amsterdam, The Netherlands) was purified (> 95 %) from human plasma and tested for viral safety according to standard CLB procedures. It was assayed with a functional assay (Berichrom C1 Inhibitor assay, Behringwerke AG, Marburg, Germany), based on a monoclonal antibody recognising human C1-INH, with subsequent binding of biotinylated C1s and HRP-streptavidin.[18] Results were expressed as units (U), with one unit equal to the activity in one mL of pooled donor plasma. The specific activity of C1-INH was 3.8 U/mg, and the detection limit of the assay was 40 U/L.

Blood sample collection

Venous blood samples (5 mL) were obtained on admission, just before and 1, 3, 6, 9, 12, 18, 24, 36, 48, and 72 h after the administration of C1-INH. Samples were taken from indwelling catheters and the first mL of blood was discarded in order to prevent dilution with infused solutions. Plasma was obtained by immediate routine centrifugation and stored at -70°C. In a first sample, used for the assays of C1-INH and C4 activation fragments, clotting was prevented with EDTA. A second serum sample was used for routine chemistry and analysis of the concentrations of troponin T and creatine kinase isoenzyme MB mass ($CK\text{-}MB_{mass}$).

Routine safety parameters

Hemoglobin, hematocrit, leukocyte count, APTT, prothrombin time, creatinine, alanine aminotransferase (ALAT), aspartate aminotransferase (ASAT), γ-glutamyltransferase (γGT), bilirubin, sodium, potasium, magnesium and chloride were determined on admission and 6, 12, 24, 36 and 48 hours after the start of the C1-INH infusion.

Table 7-1. Clinical data of the 22 patients studied.

Patient characteristics												
ID	Sex	Age (y)	Weight (kg)	Previous AMI	Previous UAP	Infarct location	Hospital delay (min.)	Loading dose (U/kg)	Infused dose (U/kg/hr)	Treatment delay (min.)	Type of therapy	Treatment delay (min.)
1	F	74	75	-	-	I, P	180	50	1.25	360	None	-
2	M	55	107	-	-	P, L	60	50	1.25	360	SK	80
3	M	60	85	-	-	I, P, L	90	50	1.25	360	SK	90
4	M	58	105	-	-	I, P	60	50	1.25	360	SK	90
5	F	55	55	-	-	I, P	90	50	1.25*	360	rtPA	95
6	F	38	71	-	-	A	290	50	1.25	390	None	-
7	M	61	88	-	-	A	200	100	1.25	360	None	-
8	F	47	60	-	-	I, P	60	100	1.25	360	SK	60
9	F	70	56	+	+	A	70	100	1.25	360	SK	90
10	M	57	75	-	+	I, P	70	100	1.25	350	SK	75
11	M	40	78	-	+	I, P	55	100	1.25	350	SK	70

I = Inferior; P = Posterior; L = Lateral; A = Anterior

* infusion of C1-INH terminated after 30 hours

SK = Streptokinase; rtPA = recombinant tissue-type plasminogen activator

Table 7-1, (continued): Clinical data of the 22 patients studied.

Patient characteristics												
ID	Sex	Age (y)	Weight (kg)	Previous AMI	Previous UAP	Infarct location	Hospital delay (min.)	Loading dose (U/kg)	Infused dose (U/kg/hr)	Treatment delay (min.)	Type of therapy	Treatment delay (min.)
12	M	51	80	-	+	A	60	100	2.00	360	SK	80
13	M	63	70	-	-	I, P	45	100	2.00	360	SK	60
14	F	83	64	-	-	I, P	210	100	2.00	470	SK	210
15	F	78	73	+	+	A	240	100	2.00	660	rtPA	270
16	M	59	70	-	-	I, P	210	100	2.00	470	SK/PTCA	365**
17	F	74	58	-	-	A	120	100	2.00	360	rtPA/PTCA	300**
18	M	61	100	-	-	I, P	120	100	2.00	260	PTCA	200**
19	M	61	82	-	-	I, P, L	70	100	2.00	360	SK	80
20	M	68	93	-	+	I, L	105	100	2.00	360	SK	105
21	F	58	96	+	-	I, P	105	100	2.00	360	SK	120
22	F	68	87	-	-	A	120	100	2.00	360	rtPA	130

I = Inferior; P = Posterior; L = Lateral; A = Anterior

** Time of reopening coronary artery by PTCA

SK = Streptokinase; rTPA = recombinant tissue-type plasminogen activator; PTCA = percutaneous coronary angiography

Effects of C1-INH on complement activation

Complement activation was estimated by using monoclonal antibodies recognising neo-epitopes on the activation fragments of complement factor C4 (i.e. C4b, C4bi and C4c, together called C4b/c).[19]

Effects of C1-INH on myocardial injury

Troponin T (third-generation) and CK-MB_{mass} concentrations in serum were determined on the Elecsys 2010 from Roche Diagnostics (Mannheim, Germany).

Reperfusion therapy after AMI accelerates and diminishes the release of cardiac marker proteins, and these effects are dependent on treatment delay.[20] To allow detection of additional effects of C1-INH on such protein release, patients should therefore have comparable treatment delays. Because C1-INH therapy was started 6 h after onset of symptoms, but also at a safety interval of several hours after thrombolytic therapy, it was expected that most patients would receive early thrombolytic therapy within a narrow range of treatment delays. Control values were obtained from an earlier study of the diagnostic performance of troponin T and CK-MB_{mass} in patients with AMI who also received thrombolytic therapy.[21] This group was sufficiently large (n = 286) to allow selection of a subgroup of patients who received thrombolytic therapy within the same narrow range of treatment delays as obtained in the present study.

Statistical evaluation

Results were analysed with standard statistical software (BMDP Statistical Software, Los Angeles, USA). Significance of differences was tested according to Kruskal-Wallis.

Results

Safety of C1-INH

The investigation was conducted between March 1998 and June 2000 at the University Hospital Maastricht. Because of the restrictive protocol conditions, only a small number of eligible patients was expected and a randomised study design was not attempted. Twenty-two patients fulfilled the entry criteria. Infarct locations and C1-INH dosages are listed in Table 7-1. Only one serious adverse event (> 350 mL blood loss) occurred in patient 16, who underwent rescue PTCA after SK therapy. This event was not related to C1-INH, as judged by the attending physician. Continuous infusion of C1-INH for 48 hours was completed in all patients but one. The exception was patient 5, in whom the indwelling catheter did not function after 30 hours.

Dose-response of C1-INH

Figure 7-1 presents plasma C1-INH activities for the 3 different dosage schemes. The two loading doses used, of 50 U/kg and 100 U/kg respectively, increased plasma C1-INH activities by the expected amounts of about 100% and 200%. Approximately constant

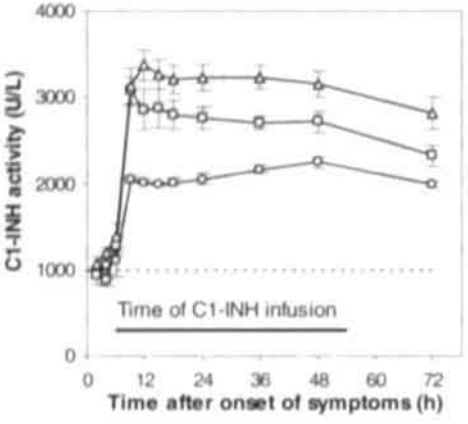


Figure 7-1, C1-INH activities.
Mean plasma C1-INH activities, with SEM, in the 3 dosage groups. Increasing doses (see text) were given to 6, 5 and 11 patients and indicated by (*circles*), (*squares*) and (*triangles*), respectively.

plasma C1-INH activities during the 48 h infusion period indicated that endogenous C1-INH production remained essentially unchanged. Plasma distribution space, calculated from the response to the initial loading dose, was 48.2 ± 1.6 mL/kg (mean ± SEM, n = 22). The average apparent elimination constant, calculated from the C1-INH infusion rate and plasma C1-INH activity after 48 h of infusion, was 0.020 ± 0.001 h^{-1}, corresponding to a half-life time of 35.5 ± 1.8 h.

Effects of C1-INH on inhibition of C4 activation

Figure 7-2 presents dose-dependent effects of C1-INH on activation fragments of

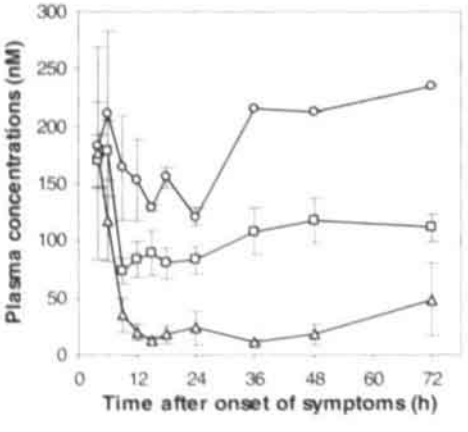


Figure 7-2, Plasma C4b/c concentrations.
Mean plasma C4b/c concentrations, with SEM, in the 3 dosage groups. Increasing doses of C1-INH were given to 6, 5 and 11 patients and are indicated by (*circles*), (*squares*) and (*triangles*), respectively.

complement factor C4 (C4b/c). The dose-dependent reduction of the areas under the C4b/c curves was significant at $p = 0.005$.

Effects of C1-INH on myocardial injury

As explained in the Methods, most patients were expected to have short thrombolytic treatment delays. Indeed, Table 7-1 shows that 13 of the 16 patients receiving successful thrombolytic therapy had treatment delays between 60 and 130 minutes. This

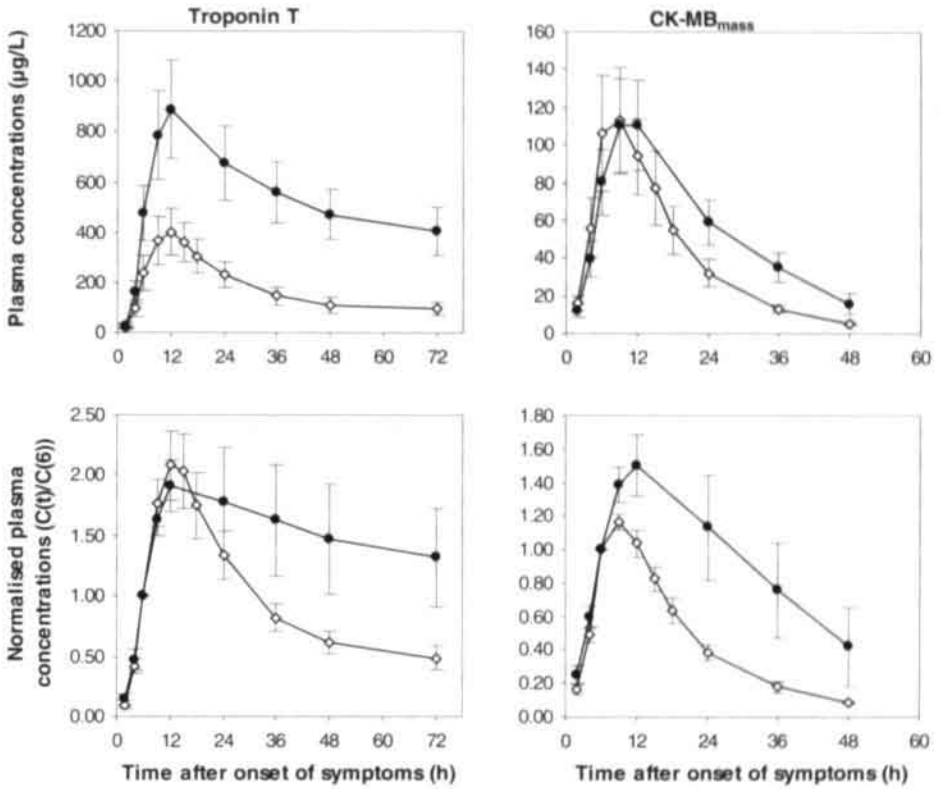


Figure 7-3, Effects of C1-INH on myocardial injury.

Upper panel: Average plasma concentrations, with SEM, of troponin T and CK-MB$_{mass}$ in 13 patients with AMI who were treated with C1-INH (*diamonds*), and in 18 control patients (*circles*). All patients received early thrombolytic therapy, starting 60–130 min. after onset of symptoms. Lower panel: The same data as in the upper panel after normalisation to the 6-hours, pre-treatment, plasma concentration.

homogeneous group of patients was compared to a group of 18 control patients who also received thrombolytic therapy between 1 and 2 h after onset of symptoms. The upper panel of Figure 7-3 shows plasma concentrations of troponin T and CK-MB$_{mass}$ in both groups of patients.

The overall higher troponin T concentrations (also of baseline values) in the control patients shown in the upper panel of Figure 7-3, reflect the use of a second-generation troponin T assay[21], whereas a third-generation test, producing approximately 50% lower values, was used in the present study. Because of the precautions taken to preserve linearity in these assays, such differences in assay conditions can be corrected for by normalisation to the 6-hour, pre-treatment value, as shown in the lower panel of Figure 7-3. Treatment with C1-INH caused earlier return to baseline values and reduction of the areas under the normalised curves by 36% and 57% for troponin T and CK-MB_{mass}, respectively. For CK-MB_{mass} this reduction was significant at $p = 0.001$.

Discussion

Safety of high-dose C1-INH therapy

Clinical experience with intravenous administration of C1-INH has mainly been limited to treatment of angioedema in patients with hereditary C1-INH deficiency.[15] In such patients, therapy has life-saving beneficial effects, but doses are much lower than in the present study, usually 1000 U of C1-INH, repeated once or twice. Such low doses were also given to 3 patients with emergency coronary surgery,[22] without adverse effects.

Much higher doses, comparable to the ones used in the present report, have been given to patients with septic shock, vascular leak syndrome, and to 2 patients with graft failure after lung transplantation.[16,23,24] These studies did not report any C1-INH-related adverse events, but such events could have been difficult to detect in critically ill patients with multi-organ failure. Recently, beneficial effects and absence of side effects have also been reported for 12 neonates who received high preoperative C1-INH doses of 100 U/kg before cardiac surgery.[25] Our study demonstrates that such high initial doses, even when followed by another 100 U/kg in the next two days, caused no adverse events in adults, even although these AMI patients would have allowed detection of relatively mild side effects.

The only report of possible adverse effects of C1-INH was a study of 29 children treated with much higher doses of C1-INH than used in the present study (an initial bolus of 300 U/kg, followed by 750 U/kg over 64 hours). Thrombosis in the vena cava and in the renal vein occurred in 2 of these children and was judged to be possibly related to C1-INH therapy.[16]

Early and late effects of complement activation on myocardial injury

Early reperfusion of ischemic myocardium has been described to induce an acute inflammatory response[26] and to cause complement activation within hours.[7,10,27,28] Production of free radicals by invading neutrophils, and obstruction of microvessels by endothelial dysfunction and/or leukocyte plugging, have been considered as the main mechanisms involved in such injury.[8,11,26] A report on beneficial effects of treatment with

anti-C5 antibodies in patients undergoing coronary artery bypass grafting[29] confirms that complement inhibition may reduce early ischemia-reperfusion injury.
However, much slower complement activation, peaking after days rather than hours, has also been observed after permanent coronary ligation in baboons,[4] in patients with AMI,[1,30] and in myocardial autopsies obtained from patients who died because of AMI.[3] Such late complement activation could explain the beneficial effects noted in the present study for C1-INH given as late as 6 hours after onset of symptoms.
Late activation could also require prolonged availability of C1-INH for lasting beneficial effects. Indeed, it was found in dogs that protective effects of early (< 12 h) treatment with C1-INH had disappeared after 72 hours of permanent coronary occlusion.[14] This finding prompted us to use a continuous 48 hours infusion of C1-INH in the present study, causing elevated plasma C1-INH levels for more than 72 hours (see Figure 7-1).

Inhibition of the contact system as an alternative mechanism for the effects of C1-INH
In addition to inhibiting complement activation, C1-INH may also inhibit contact activation in blood coagulation.[1] Thus, inhibition of factor XIIa-induced intrinsic blood coagulation could be an alternative mechanism for beneficial effects of C1-INH. However, it was shown that low molecular weight dextrans strongly potentiated the cardioprotective effects of C1-INH in dogs.[14] These low molecular forms are unable to activate the contact system[31] or to potentiate C1-INH as an inhibitor of contact system proteases.[32] Furthermore, while immunohistochemical analyses of myocardial tissue samples, taken from patients that died from myocardial infarction, revealed depositions of C-reactive protein and activated complement,[3] similar observations have not been described for the contact system. Hence, we favour the explanation that the beneficial effects of C1-INH were due to its effect on complement rather than on the contact system.

Limitations of the study
The treatment delay of 6 hours, used to prevent a possible interference of C1-INH with thrombolytic therapy, was a limitation of the present study. Because inhibition of plasmin by C1-INH is weak,[17] future research may show that this precaution is unnecessary. Another limitation was the small number of patients eligible for the study, preventing a randomised study design. Further randomised, placebo-controlled studies, providing information on the effects of C1-INH in relation to infarct location, type of reperfusion therapy and timing of C1-INH administration, are required.

Conclusions
The present study is the first to evaluate the effects of complement inhibition in patients with AMI. Plasma target levels of C1-INH were reached accurately by a combination of an initial bolus dose followed by continuous 48-hours infusion. C1-INH treatment did not cause unwanted side effects and allowed effective inhibition of complement activation. In addition, a reduced release of myocardial marker proteins indicated that limitation of myocardial ischemia/reperfusion injury by complement inhibition, as demonstrated in

several animal species, might also occur in patients with AMI. Thus, complement inhibition may constitute a novel approach for the treatment of AMI.

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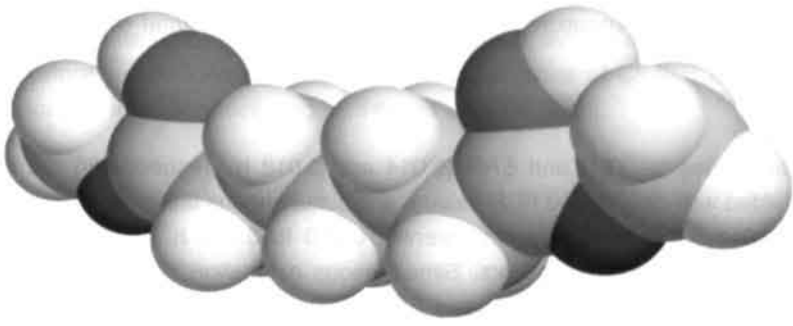
Chapter 8.

Pharmacokinetic aspects of C1-inhibitor



Adapted from Diris JHC, Hermens WTh, Hemker PW, Lagrand WK, Hack CE, Van Dieijen-Visser MP: Pharmacokinetics of C1-inhibitor protein in patients with acute myocardial infarction. *Clin Pharmacol Ther* 2002;72:498–504.

Abstract

Background

C1-inhibitor (C1-INH) purified from pooled human plasma is used for the treatment of patients with hereditary angioedema. Recently, beneficial effects of high-dose C1-INH treatment on myocardial ischemia/reperfusion injury have been reported in various animal models and in man. In the present study we investigated the pharmacokinetic behaviour of C1-INH in patients with acute myocardial infarction to calculate the amount of C1-INH required for optimal efficacy.

Methods

Twenty-two patients received an intravenous loading dose, followed by 48 hours of continuous infusion of C1-INH. Changes in the endogenous production of C1-INH were evaluated in 16 control patients with acute myocardial infarction. A 2-compartment model was used to estimate the Fractional Catabolic Rate constant (FCR), the Transcapillary Escape Rate constant (TER) and the Extravascular Return Rate constant (ERR) of C1-INH. Software designed to analyse and fit measured data to unknown parameters in a system of differential equations was used to fit the experimental data against the 3-parameter model.

Results

With fixed values for TER and ERR (0.014 and 0.018 h^{-1} respectively), 20 of the 22 cases yielded well-determined FCR values, and simultaneous fitting resulted in a median FCR of 0.011 h^{-1} (95% confidence interval, 0.010 to 0.012 h^{-1}) versus 0.025 h^{-1} as reported in healthy control patients. Simultaneous estimation of TER, ERR and FCR demonstrated weakly defined TER and ERR values, whereas the median FCR value remained unchanged. The use of a 2-compartment model resulted in a significantly better fit compared with the 1-compartment model. Physiologic explanations are offered for discrepancies in the literature.

Conclusions

Dose calculation of C1-INH in patients treated with massive doses of C1-INH requires turnover parameters that differ from those found in healthy subjects, possibly because of suppression of continuous C1-INH consumption by target proteases.

Introduction

C1-inhibitor, a protein of approximately 104 kDa, regulates the classical activation pathway of the complement system.[1] Patients with hereditary angioedema (HAE), in whom there is a congenital lack of C1-INH, have attacks of uncontrolled complement activation that may lead to life-threatening laryngeal obstruction or swelling of the gastrointestinal mucosa.[2] Depending on the severity of the edematous attack, treatment of patients with HAE usually consists of one or more bolus injections of C1-INH purified from pooled human plasma.

Other diseases where complement inhibition might have beneficial effects are being explored, and administration of C1-INH has been investigated in patients with sepsis,[1] rheumatoid arthritis,[3] and B cell lymphoproliferative disorders (LPD).[4] Recent studies in various animal models have shown reduction of myocardial ischemia or reperfusion injury after high-dose C1-INH therapy.[5-8] To investigate the effects of C1-INH in patients with acute myocardial infarction, we recently performed a dose escalating pilot study.[9] Patients were given an initial loading dose of C1-INH, followed by continuous infusion during the next 48 hours. Complement inhibition in this study proved to be strongly dose-dependent and necessitated accurate C1-INH dosage control. However, C1-INH was apparently eliminated more slowly than expected from available data of patients with HAE. Therefore we decided to study the pharmacokinetics of C1-INH in patients with acute myocardial infarction receiving high doses of this inhibitor.

Methods

Patients

Twenty-two patients (12 men and 10 women; median age, 60.5 years) with acute myocardial infarction were admitted to the Intensive Coronary Care Unit, University Hospital Maastricht, The Netherlands. After informed consent was obtained for patients to participate in an open-label pilot study, treatment with C1-INH was started 6 hours after the onset of symptoms by means of 3 different dosage schemes as follows: an initial loading dose of 50 U/kg body weight, followed by a continuous infusion of 1.25 U/kg/h; an initial loading dose of 100 U/kg body weight, followed by a continuous infusion of 1.25 U/kg/h; or an initial dose of 100 U/kg body weight, followed by 2.00 U/kg/h during the next 48 hours. Loading doses were infused at a rate of 250 U/min. The administered amounts were chosen in an effort to reach concentrations between 200% and 300% of normal. No presumptions were made about reaching steady state conditions. C1-INH (Cetor®; CLB, Amsterdam, The Netherlands) was purified (> 95%) from human plasma according to good manufacturer's practice guidelines, and was tested for viral safety and clinical efficacy. The lyophilised protein was reconstituted in water before administration.

Blood samples were collected before administration of the loading dose, and at 1, 3, 6, 9, 12, 15, 18, 24, 36, 48, and 72 to 96 hours after the start of loading dose administration.

Clotting was prevented with EDTA, and plasma samples obtained by immediate routine centrifugation were stored at -70°C until further analysis.
Changes in endogenous C1-INH production after acute myocardial infarction were assessed by measuring the course of plasma C1-INH in 16 control patients with acute myocardial infarction who did not receive C1-INH. These patients were admitted to the Department of Cardiology, VU University Medical Center, Amsterdam.[10] Blood samples were collected at admission and 2, 6, 12, 24, 36, 48, 60, and 72 hours thereafter. After centrifugation, plasma was collected and stored in aliquots at -70°C until assays were performed.

Analytical techniques
C1-INH concentrations were determined with the use of a commercially available functional assay based on a chromogenic assay (Berichrom C1 Inhibitor assay; Behringwerke AG, Marburg, Germany). Values were expressed in units per litre, with 1 unit equal to the activity of C1-INH in 1 mL of pooled plasma obtained from healthy donors. The specific activity of C1-INH was 3.8 U/mg protein.

Data analysis
A 2-compartment model, as described in Figure 8-1, was used to estimate individual pharmacokinetic parameters of C1-INH. Time-related changes in plasma and

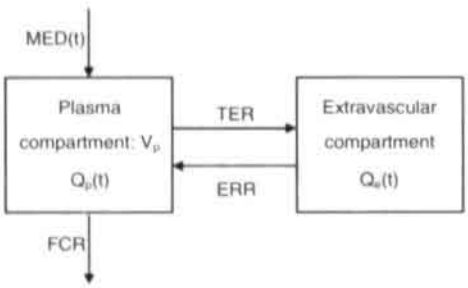


Figure 8-1. Two-compartment model.
$Q_p(t)$ = Amount of C1-inhibitor (C1-INH) in plasma (in units) at time t; $Q_e(t)$ = amount of extravascular C1-INH (in units) at time t; V_p = plasma volume; Med(t) = input function of C1-INH; TER = transcapillary escape rate constant; ERR = extravascular return rate constant; FCR = fractional catabolic rate constant.

extravascular C1-INH pools are given by Equation 8-1 and Equation 8-2 , in which $Q_p(t)$ and $Q_e(t)$ are the plasma- and extravascular pools expressed in Units at time t, MED(t) is the input function of C1-INH, FCR is the fractional catabolic rate constant (per hour) for the elimination of C1-INH from the plasma, TER is the transcapillary escape rate constant (per hour) of C1-INH for extravasation of C1-INH to the extravascular compartment, and ERR is the extravascular return rate constant (per hour) for the return of C1-INH from the extravascular compartment to plasma.

$$\frac{dQ_p(t)}{dt} = MED(t) - (FCR + TER) \cdot Q_p(t) + ERR \cdot Q_e(t) \qquad \textbf{Equation 8-1}$$

$$\frac{dQ_e(t)}{dt} = TER \cdot Q_p(t) - ERR \cdot Q_e(t) \qquad \textbf{Equation 8-2}$$

Equation 8-3, used as an initial condition, presents steady state conditions for the amount of C1-INH in the extravascular compartment at time 0. $Q_p(0)$ is known by measurement of the C1-INH plasma concentration at time 0 (*i.e.*, before C1-INH administration).

$$Q_e(0) = \frac{TER}{ERR} \cdot Q_p(0) \qquad \text{Equation 8-3}$$

All plasma concentrations were converted to total amounts in units by multiplying the concentrations in units per litre by the individual plasma volume in litres. Plasma volume was calculated from the initial increase in C1-INH concentration after administration of the loading dose.

A software package called *splds*,[11] developed for estimation of unknown parameters in time-dependent dynamical systems,[12] was used to solve the system defined by the differential algebraic Equations 8-1 and 8-2 and the initial condition given by Equation 8-3. It uses the algebra package Maple V (Waterloo Maple Inc, Ontario, Canada) to derive variational equations from the differential equations. These variational equations are then used in the process by combining them with the initial differential equations. The Levenberg-Marquardt method was used to minimise the total sum of squared discrepancies between calculated and measured values of Q_p. A confidence interval (CI) at a user defined level α (*i.e.*, 0.05) is calculated for each estimate. The program also gives a detailed analysis of the reliability and the interdependencies of the parameters. This is used to check whether each parameter independently contributes to a significant decrease in the total sum of squares. Thus we can verify whether the use of a 2-compartment model is to be preferred over a 1-compartment model.

Results

Figure 8-2 shows mean plasma C1-INH concentrations for the 3 patient groups with

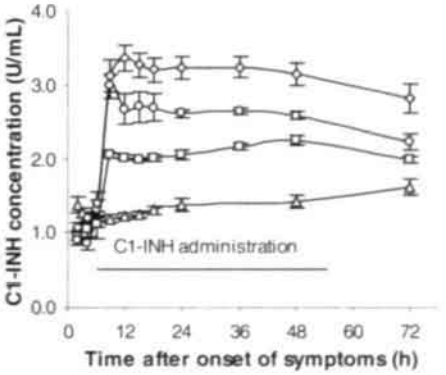


Figure 8-2, C1-INH plasma curves of 3 infusion groups and control patients.
Data presented as mean with standard error. Group 1 (*squares*): Loading dose, 50 U/kg; infusion rate, 1.25 U/kg/h (n = 6). Group 2 (*circles*): Loading dose, 100 U/kg; infusion rate, 1.25 U/kg/h (n = 5). Group 3 (*diamonds*): Loading dose, 100 U/kg; infusion rate, 2.00 U/kg/h (n = 11). Control (*triangles*): No C1-INH administered (n = 16).

different dosage schemes and for the control patients. A clear dose-concentration profile is visible in the 3 groups that received C1-INH therapy. A decrease of C1-INH is visible in the group of control patients in the first 6 hours after the onset of symptoms, and a slow but continuing increase of C1-INH concentration after 6 hours can be noted in the same group.

Figure 8-3 demonstrates the mean observed and fitted C1-INH plasma concentration curves, as well as the calculated extravascular C1-INH pool, for each dosage group.

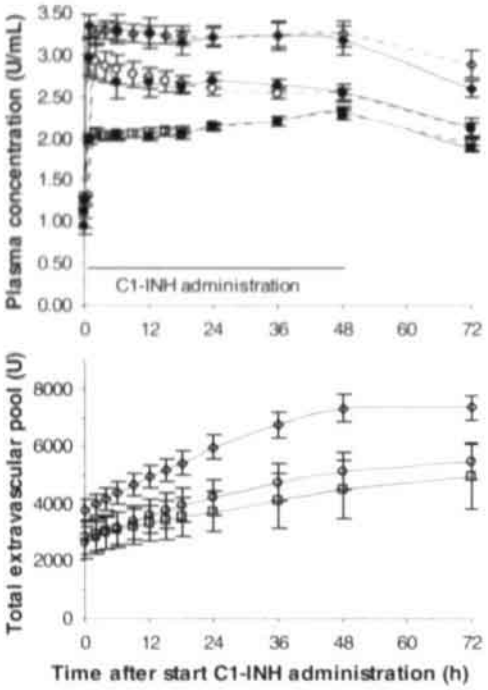


Figure 8-3, Time-course of C1-INH plasma concentration and extravascular content after treatment.

Data presented as mean with standard error (n=22). *Upper panel*, plasma concentrations (in units per millilitre) in group 1 (*squares*), group 2 (*circles*), and group 3 (*diamonds*). *Solid lines*, measured C1-INH plasma concentrations; *dotted lines*, fitted C1-INH plasma concentrations. *Lower panel*, calculated extravascular C1-INH pool (in units) in group 1 (*squares*), group 2 (*circles*), and group 3 (*diamonds*).

After a rapid increase caused by the loading dose, a plateau phase is visible for the duration of infusion. When the infusion was stopped, a gradual decline in the plasma concentration was seen, while the extravascular pool continued to increase, albeit with diminishing speed. The latter indicates that no steady-state conditions were reached.

On the basis of plausible fixed TER and ERR values (0.014 h^{-1} and 0.018 h^{-1}, respectively; see Discussion), the FCR for individual patients was estimated. Results listed in Table 8-1 demonstrate that 20 of the 22 cases had well-determined FCR values, with confidence intervals (CIs) of less than 55%. The 2 cases with CIs greater than 100% both underwent sampling for only a short period of time (< 30 hours) because of difficulties with blood sampling (not drug-related). Simultaneous fitting of the remaining 20 patients with fixed values for TER and ERR yielded the same FCR as the calculated median (0.011 h^{-1}), but this value was better defined (Table 8-1).

Estimation of all 3 parameters resulted in almost the same FCR value: 0.012 h^{-1} (CI, 0.002 to 0.022 h^{-1}). The TER and ERR values were 0.021 h^{-1} (CI, -0.018 to 0.060 h^{-1}) and

Table 8-1. Results 2-compartment model on patients with acute myocardial infarction.

Patient no.	Sex	Age (y)	Body weight (kg)	V_p (mL)	FCR and 95% CI (h^{-1})*
1	F	74	75	2953	0.009 (0.005 to 0.013)
2	M	51	107	5144	0.009 (0.008 to 0.010)
3	M	60	85	4474	0.007 (0.005 to 0.009)
4	M	58	105	4953	0.005 (0.003 to 0.007)
5‡	F	55	55	2500	0.900 (-0.470 to 2.270)
6	F	38	71	3170	0.011 (0.010 to 0.012)
7	M	61	88	3411	0.012 (0.009 to 0.015)
8	F	46	60	2970	0.003 (0.002 to 0.004)
9	F	70	56	2857	0.011 (0.008 to 0.014)
10	M	57	75	5068	0.010 (0.005 to 0.015)
11	M	40	78	3000	0.021 (0.017 to 0.025)
12	M	51	80	4420	0.010 (0.006 to 0.014)
13	M	63	70	3784	0.017 (0.012 to 0.022)
14	F	83	64	2795	0.016 (0.014 to 0.018)
15	F	78	73	3106	0.016 (0.011 to 0.021)
16	M	59	70	3590	0.025 (0.021 to 0.029)
17‡	F	74	58	2929	0.002 (-0.004 to 0.008)
18	M	61	100	6329	0.017 (0.010 to 0.024)
19	M	61	82	4059	0.007 (0.004 to 0.010)
20	M	68	93	4769	0.009 (0.007 to 0.011)
21	F	57	96	3254	0.012 (0.005 to 0.019)
22	F	68	87	3750	0.011 (0.009 to 0.013)
Median (n = 20)		60.5	77	3500	0.011 (0.008 to 0.014)

V_p = Plasma volume; FCR = fractional catabolic rate constant; CI = confidence interval; F = female; M = male.

* Simultaneous fit of 20 patients (fixed transcapillary escape rate constant [0.014 h^{-1}] and extravascular return rate constant [0.018 h^{-1}]): FCR = 0.011 h^{-1} (95% CI, 0.010 to 0.012 h^{-1}). Individual fit of 20 patients (estimation of all 3 parameters): FCR = 0.012 h^{-1} (95% CI, 0.002 to 0.022 h^{-1}).

‡ Sampling period < 30 hours; therefore patient's data were excluded from calculations.

0.019 h^{-1} (CI, -0.055 to 0.093 h^{-1}) respectively, but the large CIs indicate that no precise TER and ERR values could be determined.

Discussion

Recovery of C1-INH

From the plasma volumes and individual body weights listed in Table 8-1, a median plasma volume of 48 mL/kg body weight was calculated. Comparison with the normal plasma value of 41 mL/kg (corrected for sex, weight and age)[13] showed an apparent incomplete recovery (85%) of C1-INH, probably caused by consumption of C1-INH in the acute phase. This finding is supported by the initial decrease in C1-INH in control patients (Figure 8-2).

Validation of model used

On the basis of the independency of the parameters (data not shown), adding a second compartment to the model causes a significant reduction in the total sum of squares. This means that, although the data obtained lack extravascular measurements, the use of a 2-compartment model still results in a better fit of the plasma curves compared with a 1-compartment model.

TER and ERR values

The choice of plausible fixed values for TER (0.014 h^{-1}) and ERR (0.018 h^{-1}) in this study was based on an overview of data on the behaviour of circulating proteins in humans.[14] These data, in most cases obtained from turnover studies of radio-labelled proteins, show an average extravasation of about 1.4 % of the plasma pool per hour for proteins with a molecular weight exceeding 100 kDa. Moreover, it was found that the extravascular pool of such proteins is somewhat smaller than the plasma pool, as also follows from the equilibrium relation E/P = TER/ERR = 0.78, where E/P is the extravascular pool/plasma ratio.

The fact that no precise TER and ERR values for C1-INH could be determined is mainly caused by the lack of extravascular measurements. The small number of observations (2 or 3) made after the end of infusion enlarges the imprecision of TER and ERR because it is primarily this last part of the plasma curve that expresses these values.

Estimation of FCR: Comparison with data from literature

Table 8-2 presents an overview of C1-INH turnover studies in healthy subjects and in patients with various diseases. Apart from this study, all studies used single intravenous bolus injections of C1-INH. With the exception of the study of Kunchak *et al.*,[15] in which total observation time was limited to 24 hours, C1-INH concentrations were measured for at least 72 hours.

In a number of these studies[15-17] the apparent disappearance rate constant (k_d) rather than the true fractional catabolic rate constant for the disappearance of C1-INH from plasma was determined. For instance, Brackertz *et al.*[16] found a biphasic disappearance of injected C1-INH in control subjects and reported a plasma half-life time of 64 h for the final slow disappearance phase from 3 to 8 days after injection. However,

the corresponding apparent disappearance rate constant k_d = (ln2)/64 = 0.011 h^{-1} also incorporates the return of extravascular C1-INH to plasma, which suggests that the FCR would be even lower than 0.011 h^{-1}.

Another situation occurred in the study of Kunchak *et al.*,[15] who reported half-lives of 37.8 to 24.0 hours for the initial disappearance phase of C1-INH in patients with HAE, corresponding to k_d values of 0.018 to 0.029 h^{-1}. These values are indeed higher than the true FCR, because C1-INH is not only catabolised, but also is extravasating during the first 24 hours after a bolus injection.

Overall, Table 8-2 shows that an FCR of 0.025 h^{-1} was obtained in healthy subjects, and higher values are obtained in patients with various diseases causing consumption of C1-INH as a result of complement activation, such as HAE, rheumatoid arthritis and various forms of leukaemia.

Possible explanations for discrepancies in FCR values

A first explanation for the relatively low value for FCR could be a considerable increase in the endogenous production of C1-INH in the days after acute myocardial infarction. Such acute phase reactant-like behaviour of C1-INH could compensate for the extra consumption of C1-INH as a result of complement activation during the first 24 hours after acute myocardial infarction. However, the course of C1-INH plasma levels in the control patients did not support this explanation, given that levels initially decreased rather than increased (Figure 8-2), suggesting increased consumption and not increased synthesis.

Furthermore, increased endogenous production of acute phase reactants usually starts after more than 24 hours and Figure 8-2 indeed shows that C1-INH plasma concentrations in control patients are increasing slightly in the days after acute myocardial infarction. On the basis of data from these patients, it was estimated that this slow increase corresponded with an average increase in C1-INH synthesis of 17 U/h. When this production was added to Equation 8-1, it was found that the FCR changed only marginally to 0.015 h^{-1} (CI, 0.013 to 0.017 h^{-1}) (Table 8-2).

Another explanation for the discrepancy between healthy subjects and patients with acute myocardial infarction could be that continuous consumption of C1-INH caused by complex formation with activated proteases and subsequent rapid removal of complexes from plasma is a main determinant of C1-INH elimination in healthy subjects. If it is assumed that the disappearance of functional C1-INH proceeds via 2 almost equally contributing pathways, (1) inactivation due to complex formation and (2) slow hepatic removal of C1-INH with an FCR value of about 0.011 h^{-1}, it is clear that in studies that used lower doses the combination of the 2 removal pathways could be mistaken for a higher FCR value.

Table 8-2, Data from literature on human C1 inhibitor turnover.

Reference	Subjects	C1-INH preparations	K_d (h^{-1})	FCR (h^{-1})	TER (h^{-1})	ERR (h^{-1})	TER /ERR = E/P	Method
Brackertz *et al.*[16] (1975)	Controls (n = 3)	Iodine label	0.011	–	–	–	–	Exponential fit (k_d from second phase)
	Hereditary angioedema (n = 3)		0.010	–	–	–	–	
Quastel *et al.*[18] (1983)	Controls (n = 9)	Iodine label	–	0.025	–	–	–	Matthews (2-compartment model) or Nosslin (non-compartment model)
	Hereditary angioedema (n = 5)		–	0.035	–	–	–	
Woo *et al.*[3] (1985)	Controls (n = 10)	Iodine label	–	0.025	–	–	–	Matthews (2-compartment model)
	Rheumatoid arthritis (n = 13)		–	0.028	–	–	–	
Melamed *et al.*[4] (1986)	LPD (n = 5)	Iodine label	–	0.053	–	–	1.54	Matthews (2-compartment model)
Waytes *et al.*[17] (1996)	Hereditary angioedema (n = 6)	Concentrate	0.015	–	–	–	–	Half-life estimation over 72 h
Kunschak *et al.*[15] (1998)	Hereditary angioedema (n = 10)	Concentrate	0.018	–	–	–	–	$t_{1/2}$ activity
			0.029	–	–	–	–	$t_{1/2}$ antigen (kd first phase)
This study	Acute myocardial infarction (n = 22)	Concentrate	–	0.011	0.014	0.018	0.78	2-Compartment fit: TER, ERR (fixed)
			–	0.012	0.021	0.019	1.11	TER, ERR (free)
			–	0.015	–	–	–	Corrected for endogenous production

C1-INH = C1 inhibitor; k_d = apparent disappearance rate constant; TER = transcapillary escape rate constant; ERR = extravascular return rate constant; E/P = extravascular pool/plasma pool; LPD = lymphoproliferative disorder; $t_{1/2}$ = half-life.

An obvious difference between the other studies mentioned in Table 8-2 and this study is that we used bulk amounts of C1-INH instead of trace amounts of radiolabeled C1-INH. Patients with hereditary angioedema are usually treated with doses of 1000 U. In contrast, the patients with acute myocardial infarction in the present study received total doses of as much as 20,000 U. At these high doses, disappearance as a result of complex formation will play only a relatively minor role, as compared with hepatic removal of functional C1-INH. This could reveal the observed FCR of 0.011.
A similar low FCR value was found by Quastel *et al.*[18] for a dysfunctional C1-INH protein, Ta. They reported that C1-INH Ta hardly binds to C1. This too supports the idea of the important role of continuous C1-INH disappearance from plasma as a result of complex formation. The defective complex formation of Ta blocked one of the two pathways, thus decreasing the speed of disappearance and revealing the true FCR.

Conclusions

Pharmacokinetic data for C1-INH were estimated in patients with acute myocardial infarction receiving high doses of this inhibitor. The obtained FCR value (0.011 h^{-1}) is lower than that previously found (0.025 h^{-1}). Saturation of the normal steady state kinetics of C1-INH may explain this finding. These data may help to design further clinical trails with this anti-inflammatory drug.

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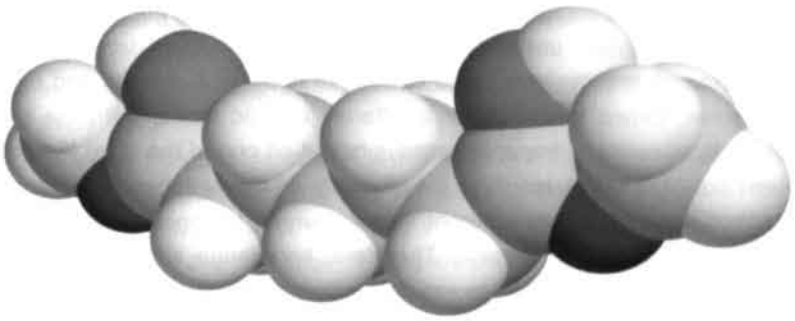
Chapter 9.

General discussion



Discussion

In the Netherlands 47,643 people (= 33.9% of all deaths) died in 2001 from cardiovascular disease.[1] The number of patients that survive a first cardiovascular ischemic event increases every year, but the costs of health care related to treatment of cardiac patients increase accordingly. Much effort is put into the research of mechanisms that cause these cardiovascular diseases and into new ways of better treatment for cardiac patients. In this thesis links between basic research (Chapter 2, 3 and 8), better ways of understanding relations in cardiovascular disease (Chapter 4 and 5) and new clinical practice (Chapter 6 and 7) are explored. Cardiac markers, used as an instrument for the assessment of disease progression and outcome, are also subject of research themselves.

Troponin T as cardiac marker

The development of basic knowledge about troponin T in clinical practice has been highly influenced by the patented rights of the assay. The main advantage was the total absence of standardisation issues between different hospitals. Findings reported in literature could easily be compared because worldwide one and the same assay, giving comparable results, was used. However, the lack of standardisation problems suspended research into the very basic kinetics of troponin T release and breakdown. Other companies, forced to search for a different cardiac marker with the same high cardio-specificity, developed troponin I assays based on one of the other two proteins of the troponin complex. As a result of the diversity in the epitopes recognised by the various antibodies used among different manufacturers, troponin I results were hardly comparable between different assays. Therefore, knowledge about the fragmentation and/or complexation of troponin I has rapidly increased,[2,3] mostly because of the need for explanations for these discrepancies between troponin I assays.[4] This does not mean that no research on troponin T has been conducted at all; various research groups from all over the world have substantially contributed to the knowledge about troponin T and its behaviour after ischemic myocardial events.

The interpretation of troponin T results has changed in the last few years; the initial upper reference limit of 0.10 µg/L for acute myocardial infarction (AMI) has been lowered first to 0.05 µg/L and now even to 0.01 µg/L.[5] This limit is based on the recommended 99th percentile of a healthy population.[6] So, as stated in the discussion section of Chapter 2, a measurable troponin T concentration (*i.e.*, > 0.01 µg/L, third-generation troponin T assay) is indicative for some sort of ongoing myocardial ischemia, even if the person has no symptoms and is otherwise considered to be healthy.

Physiological aspects of troponin T release

Related with the interpretation of troponin T results is the question whether a viable myocyte can release troponin T, explaining the subclinical —but measurable— troponin T concentrations. With the estimation of the loss of cardiac tisse, described in Chapter 3,

we have shown that all troponin T present in the circulation of a healthy subject (0.0002 µg/L[7]) can be fully explained by the natural turnover of cardiomyocytes, resulting in the loss of only 0.006% of cardiac tissue per year (based on an average heart weight of 435 g). This is supported by Hamm *et al.*[8] and recently by the findings of Torbicki *et al.*[9], showing a prognostic value of even the slightest measurable troponin T elevation. These findings indicate that even the minor troponin T elevations are the result of myocardial injury and that viable myocytes will probably not release troponin T. Unfortunately, the lower detection limit of the current third-generation troponin T assay is too high for a more precise determination of these minor elevations from the reference value.

Also important for the diagnosis of very minor ischemic myocardial damage is the reference value of troponin T itself. As described in Chapter 2 it is very likely that the current lower detection limit of the troponin T assay does not allow a precise determination of the reference value. Using an assay with a much lower detection level will probably allow a more reliable detection of minor myocardial damage. Results showing troponin T fragments in serum of patients with severe renal failure while the measured concentration is < 0.01 µg/L (Chapter 3), do support this hypothesis. Perhaps that the fourth-generation of the troponin T assay, which is currently in development[10] and is likely to attain a much lower detection limit, can make this information about early myocardial ischemia more accessible. Maybe that with a better understanding of the reference value and very early stages of myocardial ischemia patients can be treated more effectively.

Another interesting topic that is related to the death of myocytes is the mechanism by which these cell die. Despite numerous publications showing significant amounts of apoptotic cells, the importance of apoptosis in the death of myocytes remains obsure. Also, the specificity for the detection of apoptosis of most used methods (DNA ladder detection and TUNEL staining) is much lower than previously assumed.[11] The minor role of apoptosis seems to be confirmed by the fact that enzymes like HBDH and CK are completely recovered in the plasma after a myocardial infarction.[12] Should apoptotic processes play a significant role, it is to be expected that at least a part of these enzymes should not be recovered due to the proteolytic activity contained within the apoptotic cell. Perhaps that in the very early stages of ischemia, mechanisms are triggered causing the synthesis of apoptotic proteases like caspase-3 (mentioned below), rapidly switching to necrotic processes during sustained ischemia. Troponin T, bound to the myofilaments of the cell, could then still be cleaved by caspase-3 despite necrosis.

Communal *et al.* recently reported of ischemia induced troponin T fragmentation caused by caspase-3[13] which is specifically synthesised by the apoptotic processes. Wang *et al.* found a similar mechanism for the fragmentation of troponin I involving matrix metalloproteinase-2.[14] Another enzyme that is activated upon intracellular Ca^{2+} increase, is neutral Ca^{2+}-activated protease (calpain). Atsma *et al.*[15] showed that calpain was activated in the rat heart and Gorza *et al.*[16] showed that this activation does lead to alterations in the structure of troponin T. Recently, Van der Laarse *et al.* suggested a relation between the troponin alterations and heart failure.[17]

Removal of troponin T from the circulation

Although several questions, like the specificity of cardiac troponin T, have already been answered,[18-20] an important question about the mechanisms involving renal clearance of troponin T has not. The finding that patients with severe renal failure have elevated serum troponin T concentrations has been reported and discussed many times. Several articles have appeared reporting about troponin fragments but either the origin of these samples was cardiac tissue,[21,22] which contains much higher troponin T concentrations, or fragments of troponin I were detected.[23,24] In Chapter 2 and 3, detailed results are shown for the involvement of in vivo troponin T fragmentation as a cause for these unexplained troponin T elevations. In these chapters we describe the in-house development and application of a highly specific immunoprecipitation assay to clearly show that many troponin T fragments are present in the serum of patients with severe renal failure as well as in serum of patients with acute myocardial infarction. Interestingly, AMI patients with a decreased renal function in the days following the infarction show a pattern of troponin T fragmentation similar to patients with severe renal failure. This pattern differs from the troponin T fragmentation pattern present in AMI patients with a normal renal status.

The renal clearance of fragments from larger proteins as the major pathway of removal from the circulation may also be true for other proteins. There are some preliminary indications that this could also be the case for the clearance of the N-terminal fragment of brain natriuretic peptide, a novel cardiac marker for heart failure. With the use of specific anti-NTproBNP antibodies and the same immunoprecipitation this could also be investigated.

Recently achieved knowledge about basic release kinetics does however not alter the usefulness of the troponin T assay in daily clinical practice. One of the applications of the troponin T assay is the infarct size estimation based on the total release after myocardial infarction. Kragten *et al.*[25] found a good correlation between cumulative troponin T and HBDH release. Despite the good correlation, calculation of the infarct size expressed in gram-equivalents of cardiac tissue resulted in only a fraction of recovery based on troponin T release. With the results of Chapter 2 and 3 about troponin T fragmentation, the calculations made previously using troponin T data and a 2-compartment model have been proven to be incorrect. The disappearance rate of troponin T is now estimated much higher than the previously determined 0.11 h^{-1},[25] because of the now known involvement of renal clearance. Determination of the true FCR of troponin T is further complicated by the fragmentation mechanism causing the existence of multiple FCRs, each for a different fragment.

Infarct size quantification

In Chapter 4, a possibly negative influence of the acute phase response on infarct size determination was investigated. As the old cardiac markers (CK, HBDH and LDH) are more and more replaced by the next generation of more specific markers (CK-MB_{mass}

and troponin T as markers for ischemia, and BNP as marker of heart failure), existing methods have to be re-evaluated using the new markers.
Because troponin T, in contrast to the cytosolic enzymes, is bound to myofibrillar structures it was expected that ongoing inflammatory processes could have consequences for the release of troponin T. However, as shown in Chapter 4, activation of the acute phase reaction, expressed by the cumulative CRP-release, causes no complications for the quantitative use of troponin T.
Knowledge about the tissue contents of a cardiac marker is important for the correct determination of infarct size expressed in gram equivalents of healthy myocardium. For years conflicting data have been published whether ischemic myocardial tissue does[26,27] or does not[28,29] contain CK-MB. The cause is now explained in Chapter 5: the role of tissue acidosis seems to have been underestimated, especially in victims of traffic accidents who were thought to have "healthy" hearts at the time of autopsy. Therefore, the results of CK-MB tissue contents obtained from post-mortem samples, in which CK-MB has been inactivated, have erroneously been extrapolated to the in vivo situation.
In Chapter 6, three cardiac markers have been evaluated for a reliable and rapid detection of ischemic myocardial injury after cardiac surgery, the so-called peri-operative myocardial ischemia. Although troponin T is the most sensitive cardiac marker with the best negative predictive value later after surgery, CK-MB_{mass} exceeds these properties in the first hours after the operation.

Complement inhibition and reperfusion injury

Acute myocardial ischemia triggers inflammatory processes via the complement cascade.[30] A significant part of the tissue injury is caused by activation of the complement cascade that promotes invasion of neutrophils and the formation of the cytolytic membrane attack complex. Inhibition of this cascade has been shown to reduce infarct size in various animals.[31-33]
In Chapter 7 the results of the Cetor study are presented: 22 patients with AMI received a complement-inhibiting drug in an effort to prevent myocardial reperfusion injury. Treatment effects were evaluated with the use of the cardiac markers CK-MB_{mass} and troponin T. These markers rise within 3–6 hours to significantly elevated concentrations in the plasma. Because C1-inhibitor was administered after six hours after the onset of symptoms, the 6-hour sample, taken just before the start of administraton, could be used to normalise the data. Normalisation to the 6-hour plasma value reduced the variability of the measured plasma values between patients and corrected for the use of a second-generation troponin T assay in the control group. Despite the small number of included patients, mainly due to the stringent inclusion criteria, a beneficial effect of complement inhibition was found. A double blind randomised study with more included patients should be conducted in order to be able to draw firm conclusions about the quantitative effects.
Vakeva *et al.* showed beneficial effects of anti-C5 therapy on myocardial cell death in a rat model of ischemia/reperfusion injury.[34] In 1999 Fitch *et al.* reported about the

beneficial effects of complement inhibition with a humanised recombinant single-chain anti-C5 antibody in patients undergoing cardiopulmonary bypass.[35] A comparable study (Cardinal trial, unpublished) has been conducted using pexelizumab, an anti-C5 antibody, to reduce complement activation after myocardial infarction, but no significant differences in infarct sizes were found between treated patients and controls. The contradicting results of both studies are difficult to explain.

The differences in outcome between the Cetor study and the Cardinal trail could be explained by the fact that inhibition at the level of C5, as pexelizumab does, allows the activation of C1-C4, including generation of the anaphylatoxins C3a and C4a (see page 6). In contrast, inhibition of C1 completely blocks the classical complement pathway, thus preventing the production of leukotactic activated C3 and C4.

With the results of the Cetor study the pharmacokinetic behaviour of C1-inhibitor was investigated. During and after administration of the drug, the C1-inhibitor plasma concentrations were extensively monitored, providing sufficient data for use in a two-compartment model. The finding that removal of C1-inhibitor from plasma normally occurs not only by slow removal, but also by inactivation via binding to activated C1, shed a new light on previous reports that estimated the disappearance rate of C1-inhibitor to be twice as high. These differences reported in earlier studies are explained in Chapter 8. With this pharmacokinetic *information about C1-INH, target concentrations* of this drug can be reached more accurately in future studies.

Future research

With the visualisation of the troponin T fragments in serum, an important step in gaining complete knowledge of troponin T release has been made. One of the following steps could be the quantification of the fragments and relating these measurements to the results of the third-generation troponin T assay. For this comparison it is preferred that the same antibodies are used, to be certain that exactly the same fragments are measured. The composition of the fragments themselves is also valuable and could be investigated using trypsin cleavage of selected fragments (from the electrophoresis gel) followed by (tandem) mass spectrometry.

Another aspect is the origin of the fragments, *i.e.*, where fragmentation takes place, in the circulation, or already in the myocardial tissue. To find the answers to this question, specific enzymes have to be inhibited with the intention of preventing one specific fragment to appear. One of these enzymes might be caspase-3 which has recently gained attention in the literature as a possible candidate for causing troponin T fragmentation.[13] Another possibility is the measurement of troponin T fragmentation in cardiac tissue itself.

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Summary

The direct consequences of acute myocardial infarction involve many, often complicated, processes. In short, one of the coronary vessels that normally provide the heart of oxygen gets plugged with a blood clot. Without treatment, there is a high probability that part of the heart is too long deprived of oxygen and dies, releasing the content of the muscle cells, including proteins, into the circulation. Some proteins, the co-called cardiac markers, are only present in cells of the heart and can therefore be used by the physician for the diagnosis of myocardial infarction. In this thesis, several chapters describe the release mechanisms of these cardiac markers, and subsequent processes, in more detail.

Should an occluded vessel be opened, either spontaneously or after administration of blood cloth dissolving medication, unfavourable processes will still take place. The presence of dead cells triggers an inflammatory reaction that not only breaks down these dead cells but also attacks viable cells in the surrounding tissue, increasing the damage to the heart even further.

Besides research on cardiac markers, this inflammatory reaction and the so-called reperfusion injury are also investigated. The topics described in this thesis all have the infarcted heart in common and contribute to the explanation of processes that occur during and after myocardial infarction. Goal of this PhD research is two-fold: gaining better understanding of specific fundamental biochemical processes, and providing answers to questions directly related to patients in the hospital.

Chapter 1 provides background information about several (biochemical) processes and techniques that are supposed to be known in the following chapters. The topics discussed are:

- myocardial infarction,
- cardiac markers used in the hospital,
- the complement system,
- mathematical modelling of the time-related release of cardiac markers,
- renal clearance.

In chapters 2 to 4, the cardiac marker troponin T is thoroughly investigated. **Chapter 2** describes the development of an analytical method to isolate, separate and visualise (fragments of) troponin T from blood. Using this method on blood samples of patients with acute myocardial infarction (AMI) has finally proven the long-existing hypothesis about troponin T fragmentation. In the samples taken shortly after the onset of symptoms a pattern of larger troponin T fragments is present that appears to shift to a pattern of smaller ones in the time-course after infarction. By confirming the presence of these troponin T fragments, various (quantitative) aspects about myocardial injury caused by a lack of oxygen are placed in a different light.

In **Chapter 3**, the same method was used on blood samples of dialysis patients in search for an answer to why unexplainable high concentrations of troponin T are often found in the blood of these patients. Troponin T fragments that otherwise would have been rapidly removed by properly functioning kidneys were now found accumulating in

the circulation of the dialysis patients. These findings provide valuable information for a better interpretation of troponin T test-results and give more insight in the normal situation in healthy persons.

The final chapter specifically dealing with troponin T is **Chapter 4**. Animal studies have shown that classic cardiac markers like CK and HBDH are completely released after an infarction and can be totally recovered from the circulation. Nowadays troponin T can also be used for infarct size determination and a possible effect of the before-mentioned inflammatory reaction could have a negative impact on the accuracy of the method. Although a relation between infarct size (based on the enzyme HBDH) and severity of the inflammatory reaction, expressed by the total CRP release, has indeed been found, the influence on troponin T values is the same as on other cardiac markers. This leads to the conclusion that infarct size determination based on troponin T values is not influenced by the inflammatory reaction.

Another protein —the enzyme CK-MB— is subject of investigation in **Chapter 5**. Previous studies suggested an extremely high variation and even total absence of CK-MB in cardiac tissue. If this were to be true, it would have a profound impact on the suitability of CK-MB measurements for infarct size determination. We demonstrated that this absence is merely an artefact caused by the instability of CK-MB. Exposure of cardiac tissue samples to different pH environments has shown that CK-MB is readily degraded at pH values below 6.0. Acidosis of cardiac tissue during the perimortal phase can result in such low pH values and is therefore most likely the cause of the apparent low CK-MB tissue content.

Chapter 6 describes another application for the use of cardiac markers. Patients undergoing coronary artery bypass grafting are at risk for minor myocardial ischemia due to the use of the hart-lung machine. Without infarction, patients can be transferred from the intensive care unit to the (less expensive) medium care unit earlier after the operation. Use of cardiac markers can assist the physician in deciding whether a patient can be transferred to medium care or not. Hence it is important that the cardiac marker has enough sensitivity. By comparing the diagnostic value of three different cardiac markers at various times after the operation, the fastest and most reliable marker for this purpose could be selected. $CK\text{-}MB_{mass}$ is the most sensitive cardiac marker with a very high negative predictive value up to 12 hours after the operation while a significant difference between both patient groups was already present upon arrival at the intensive care unit. Later after the operation troponin T is the most sensitive marker for the presence of a myocardial infarction. Using these results, treatment after cardiac surgery can better be accustomed to the individual patient.

Chapter 7 describes the results of the Cetor study. A complement inhibitor (C1-inhibitor) was administered to 22 patients suffering from myocardial infarction to suppress the inflammatory reaction in the heart. This was the first time high-dose complement inhibition was used in such patients, and despite the fact that it was not a randomised, double-blind study and good controls were missing, results are encouraging.

Because the cardiac markers were already elevated at the time the medication was allowed to be administered (6 hours after the onset of symptoms) is was possible to normalise the differences in the release of the cardiac markers to the 6-hour pre-treament value. Besides a better determination of the effects of the C1-inhibitor treatment, this normalisation process also allowed a comparison between the third-generation troponin T values of the patients and the second-generation troponin T values of the controls. Results indicated both a reduction of complement activation (expressed by C4b/c concentrations) as well as a reduction of infarct size, expressed by total troponin T release.

Based on the detailed patient data obtained from the Cetor study, the pharmacokinetic behaviour of C1-inhibitor is investigated in **Chapter 8**. Several different turnover rates had been published regarding the disappearance of C1-inhibitor in various patient groups. By using a 2-compartment model, C1-inhibitor concentration curves were fitted to the measured C1-inhibitor plasma curves by adjusting the model parameters. With this method the disappearance rate of C1-inhibitor, which turned out to be caused by two equally contributing pathways, could be precisely determined. Results indicate that besides the accepted removal pathway, a pathway of continuous basal complement inhibition *i.e.*, complex formation of C1-inhibitor with activated C1, is present under normal circumstances. This would result in a disappearance rate of only half as fast as previously assumed.

In the final chapter, **Chapter 9**, the main results are reviewed and commentarised and suggestions for related research in the (near) future are given.

Samenvatting

De directe gevolgen van een acuut hartinfarct beslaan vele, vaak ingewikkelde, processen. Kort samengevat wordt één van de bloedvaten die normaal gesproken het hart van zuurstof voorzien afgesloten door een bloedprop. Zonder behandeling is de kans groot dat een gedeelte van het hart te lang van zuurstof verstoken blijft en afsterft. De inhoud van de dode hartspiercellen, waaronder eiwitten, wordt uitgestort en komt in de bloedsomloop terecht. Bepaalde eiwitten, de zogenaamde hartmarkers, komen alleen in hartspiercellen voor en kunnen dus door de arts gebruikt worden om vast te stellen of er sprake is van een infarct. In enkele hoofdstukken van dit proefschrift wordt verder ingegaan op de mechanismen waarop deze hartmarkers vrijkomen en wat er daarna met ze gebeurt.

Mocht het afgesloten bloedvat na verloop van tijd opengaan, hetzij spontaan, hetzij door het toedienen van stolseloplossende medicijnen, dan nog treden er nadelige fenomenen op. Door de aanwezigheid van dode cellen komt in het hart een ontstekingsreactie op gang die niet alleen deze dode cellen opruimt, maar ook nog een deel van de levensvatbare cellen in het omliggende weefsel wat de uiteindelijke schade aan het hart nog groter maakt.

Naast het onderzoek aan de hartmarkers worden ook enkele hoofdstukken gewijd aan onderzoek van deze ontstekingsreactie en de zogenaamde reperfusieschade. De onderwerpen die in dit proefschrift behandeld worden hebben allemaal betrekking op het hartinfarct en dragen bij aan een verklaring van processen die optreden tijdens of na een hartinfarct. Doel van dit promotieonderzoek is tweeledig: dieper inzicht krijgen op bepaalde fundamentele biochemische processen, en vragen beantwoorden die direct verband houden met de patiënten in de kliniek.

Hoofdstuk 1 bevat achtergrond informatie over diverse (biochemische) processen en technieken die in de rest van de hoofdstukken als bekend worden verondersteld. Aan bod komen achtereenvolgens:

- het hartinfarct,
- de verschillende hartmarkers die in de kliniek gebruikt worden,
- het complement systeem,
- het wiskundig modelleren van het tijdsverloop van vrijgekomen hartmarkers,
- de filterfunctie van de nieren.

In de hoofdstukken 2 tot en met 4 wordt de hartmarker troponine T onder de loep genomen. **Hoofdstuk 2** beschrijft de ontwikkeling van een methode om (fragmenten van) troponine T uit bloed te isoleren, om deze vervolgens op grootte te scheiden en zichtbaar te maken. Toepassing van deze methode op bloed van infarctpatiënten heeft de al lang bestaande hypothese betreffende troponine T-fragmentatie eindelijk bewezen. In monsters die kort na het ontstaan van de klachten zijn verzameld is een patroon van grotere fragmenten zichtbaar. In de tijd na het infarct lijkt dit te verschuiven naar een ander patroon met kleinere fragmenten. Met het aantonen van deze troponine T

fragmenten worden verschillende (kwantitatieve) aspecten van hartschade door zuurstofgebrek, in een ander daglicht gezet.
In **Hoofdstuk 3** is dezelfde methode toegepast op bloedmonsters van nierdialyse patiënten om zo een antwoord te krijgen op de vraag waarom er regelmatig onverklaarbaar hoge troponine T concentraties worden aangetroffen in het bloed van deze patiënten. Het blijkt dat fragmenten van troponine T, die normaal gesproken door goed werkende nieren snel gezuiverd worden, zich ophopen in het bloed van dialyse patiënten. Deze resultaten geven waardevolle informatie voor een betere interpretatie van troponine T uitslagen. Tevens wordt op deze manier een beter inzicht verkregen in de normale situatie bij gezonde personen.
Het laatste hoofdstuk waar specifiek naar troponine T gekeken wordt is **Hoofdstuk 4**. Uit dierproeven is gebleken dat klassieke hartmarkers als CK en HBDH na een infarct volledig worden uitgestort en teruggevonden kunnen worden in het bloed. Omdat tegenwoordig troponine T eveneens gebruikt kan worden voor het inschatten van de infarctgrootte, zou een mogelijk effect van de hiervoor beschreven ontstekingsreactie nadelige gevolgen kunnen hebben voor de nauwkeurigheid van deze methode. Hoewel er inderdaad een verband gevonden is tussen de infarctgrootte (op basis van het enzym HBDH) en de ernst van de ontstekingsreactie (op basis van de totale CRP uitstort) geldt dit voor troponine T in dezelfde mate als voor andere hartmarkers. Hieruit kan geconcludeerd worden dat bepaling van de infarctgrootte op basis van de troponine T uitstort niet gestoord wordt door de ontstekingsreactie.
In **Hoofdstuk 5** wordt een andere hartmarker bekeken, namelijk het enzym CK-MB. Eerdere studies rapporteerden voor de weefselinhoud van CK-MB enorm uiteenlopende getallen, tot zelfs complete afwezigheid van het enzym in hartweefsel. Mocht dit laatste inderdaad zo zijn, dan zou dit dramatische gevolgen hebben voor de geschiktheid van CK-MB voor het bepalen van infarctgrootte. Wij hebben kunnen aantonen dat de gemeten afwezigheid slechts een artefact is, veroorzaakt door instabiliteit van CK-MB. Door monsters van hartweefsel bloot te stellen aan verschillende zuurgraden is aangetoond dat CK-MB bij een pH kleiner dan 6.0 snel wordt afgebroken. Verzuring van de hartspier tijdens het sterfproces kan in dergelijke zuurgraden resulteren en is hoogstwaarschijnlijk de oorzaak van de schijnbaar lage CK-MB weefsel inhouden.
Van heel ander aard is het onderzoek omschreven in **Hoofdstuk 6**. Patiënten die een bypass operatie ondergaan lopen kans op een klein infarct ten gevolge van het gebruik van de hart-long machine. Zonder infarct kunnen patiënten na de operatie doorgaans sneller van de intensive care naar de (goedkopere) medium care afdeling. Het gebruik van hartmarkers kan de arts ondersteunen in het maken van de beslissing tot overplaatsing. Het is daarvoor van belang dat de gebruikte marker gevoelig genoeg is. Door de diagnostische waarde van drie hartmarkers met elkaar te vergelijken kon de snelste en meest betrouwbare geselecteerd worden. Tot 12 uur na de operatie is CK-MB_{massa} de beste hartmarker met een zeer hoge negatief voorspellende waarde waarbij er al bij aankomst op de intensive care een significant verschil bestaat tussen de beide patiëntengroepen. Uiteindelijk kan met behulp van troponine T op een later tijdstip het

beste uitsluitsel verkregen worden over een de aanwezigheid van een infarct. Met deze resultaten kan de behandeling na een bypass operatie beter worden afgestemd op de individuele patiënt.

In **Hoofdstuk 7** worden de resultaten van de Cetor studie beschreven. Tweeëntwintig patiënten met een acuut hartinfarct kregen een ontstekingsremmer (C1-inhibitor) toegediend om de ontstekingsreactie in de hartspier te onderdrukken. Dit was de eerste studie waarbij dergelijke patiënten een zo hoge dosis van de remmer kregen toegediend. Hoewel het geen dubbelblind gerandomiseerd onderzoek betrof, en dus een goede controle groep ontbrak, zijn de resultaten bemoedigend. Omdat de hartmarkers al verhoogd waren vóór het medicijn toegediend mocht worden, was het mogelijk om verschillen tussen patiënten in de uitstort van deze markers te normaliseren op de 6-uurs waarde die verkregen werd vóór het toedienen van de ontstekingsremmer. Naast het verkrijgen van een duidelijker beeld van de effecten van behandeling met C1-inhibitor, konden op deze manier ook de patiënten waarden op basis van de 3^{e} generatie troponine T bepaling vergeleken worden met de controle waarden bepaald met de 2^{e} generatie troponine T test. De resultaten laten naast een goede tolerantie voor het medicijn zowel een vermindering zien van de ontstekingsreactie (op basis van C4b/c concentraties) als een reductie van de infarctgrootte op basis van de totale troponine T uitstort.

Hoofdstuk 8 beschrijft de farmacokinetiek van C1-inhibitor die is onderzocht aan de hand van de gedetailleerde patiëntengegevens uit de Cetor studie. In de literatuur zijn namelijk verschillende afbraaksnelheden van diverse patiëntenpopulaties gepubliceerd betreffende de klaring van C1-inhibitor. De op basis van een 2-compartimenten model berekende C1-inhibitor concentratiecurves zijn door het veranderen van de modelparameters aangepast aan de uit de Cetor studie verkregen C1-inhibitor plasmacurves. Op deze manier is de afbraakconstante van C1-inhibitor, welke bleek te zijn samengesteld uit twee aparte afbraakroutes, nauwkeurig bepaald. Naast de bekende afbraakroute lijkt er onder normale omstandigheden nog een continue remming van het complement systeem te zijn waarbij C1-inhibitor samensmelt met geactiveerd C1 en zo uit de circulatie verdwijnt. Hierdoor zou de afbraaksnelheid slechts de helft zijn van wat eerder werd aangenomen.

Het laatste hoofdstuk, **Hoofdstuk 9**, laat de belangrijkste resultaten, voorzien van commentaar nogmaals de revue passeren. Daarnaast worden suggesties aangereikt voor onderzoek in de (nabije) toekomst.

Dankwoord

Vanaf deze plaats wil ik allereerst mijn dank richten aan mijn beide promotores. *Marja*, ondanks je bij tijd en wijle zeer drukke werkzaamheden heb je altijd tijd voor me vrij kunnen maken. Het is fantastisch om op een dergelijk aangename manier begeleid te worden. *Wim*, wanneer voor mijn gevoel het onderzoek weer eens stagneerde heb ik maar een wandeling naar de universiteit hoeven te maken om voldoende ideeën op te doen waar een aantal promovendi de komende jaren mee bezig gehouden kunnen worden. Jullie beider gedegen kennis van het wetenschappelijk onderzoek, aangevuld met mijn enthousiasme, heeft naast dit proefschrift het nodige plezier opgeleverd om alles-overspannende bruggen en slechte tekeningen.
Chris, samen met Marja en Wim heb je het nodige geduld moeten opbrengen wanneer ik met wéér een nieuwe optie kwam aan zetten om de immunoprecipitatie-methode nu eindelijk werkend te krijgen. Ondanks onze sterk verschillende drinkgewoontes heb ik onze samenwerking als bijzonder prettig en leerzaam ervaren.

In de vier jaar dat ik nu op het lab bivakkeer heb ik kennis mogen maken met de nodige kamergenoten. *Appie*, dank voor de wijze waarop je me in korte tijd wegwijs hebt gemaakt, niet alleen in het Cetor project maar ook in het ziekenhuis zelf. *Kim*, het halve jaar van eenzaamheid werd afgesloten met jouw komst. De tijd die we samen op de kamer hebben doorgebracht, jij ploeterend aan de hsCRP bepaling en ik aan het uitwerken van de Cetor studie, heeft aan de basis gestaan van het feit dat je nu een van mijn paranimfen bent. *Snježana*, met jouw komst was de bezetting van de kamer compleet. Ik kijk met plezier uit naar een van onze volgende discussies over het waarom van bepaalde onlogische Nederlandse grammaticale constructies. *Robert*, relatief kort bij ons op de kamer gezeten maar toch lang genoeg om mij een beter beeld te geven van de apothekerswereld. *Jaap*, als voorganger in de opleiding tot klinisch chemicus pas het afgelopen jaar bij ons op de kamer gekomen, maar al voldoende lang aanwezig om een belangrijke bijdrage te leveren aan de uitstekende sfeer op de kamer. Ik weet zeker dat samen met jou en Snježana de komende opleidingstijd dezelfde prettige samenwerking met zich meebrengt. *Etienne*, ondanks het feit dat je vaste werkplek niet bij ons op de kamer is, ben je er toch dusdanig vaak dat je hier genoemd wordt. Als mijn opvolger in het troponine onderzoek aan jou de taak om te beginnen met het uitpluizen van de losse eindjes van mijn onderzoek.

Wetenschappelijk onderzoek is onmogelijk zonder lotgenoten die met een andere bril kunnen kijken naar de praktische problemen waar je tegenaan loopt. Daarom ook dank aan mijn mede *CARIM AIO's* op de universiteit, in het bijzonder *Debby* en *Roy*. De discussies met jullie hebben voorkomen dat ik op een geïsoleerd research eilandje terecht ben gekomen. Veel succes met de afronding van jullie eigen promotieonderzoek.

Alle medewerkers van klinische chemie, inclusief diegenen die inmiddels het lab verlaten hebben, hartelijk dank voor het feit dat ik zonder problemen mijn experimenten heb kunnen uitvoeren in een gezellige en behulpzame omgeving. Ik hoop dat jullie na het lezen van dit proefschrift een iets duidelijker beeld krijgen waarmee ik de afgelopen 4 jaar bezig ben geweest.

Het leven buiten kantooruren is misschien wel even belangrijk geweest om tot de resultaten te komen die de basis vormen voor dit proefschrift. Zonder de nodige afleiding is het onmogelijk om 's ochtends met een frisse blik naar de resultaten van de vorige dag of vorige week te kijken. Daarom ook dank aan mijn *vrienden*, sommigen ver weg in het Brabantse land, anderen dichtbij in het Limburgse, maar allemaal even belangrijk voor de broodnodige ontspanning en de leuke tijd buiten de muren van het ziekenhuis.

Mijn ouders, voor jullie onvoorwaardelijke liefde en de mogelijkheid die jullie me hebben geboden om de studie van mijn keuze te kunnen volgen. Zonder goede thuisbasis en opvoeding zou ik niet daar terecht zijn gekomen waar ik nu ben.

Joyce, een speciale plaats voor jou. Met jouw steun en liefde heb ik de afgelopen jaren glansrijk doorstaan en zie ik de toekomst vol vertrouwen tegemoet.

List of publications

Papers

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1. Diris JHC, Van Dieijen-Visser MP, Kleine AH, De Zwaan C, Hack CE, Tissing M, Strengers PWF; Glatz JFC and Hermens WTh. Beneficial effects of C1-inhibitor treatment in patients with acute myocardial infarction. Ned Tijdschr Klin Chem 2001;26(2):88.
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Curriculum Vitae

Ik ben geboren op 26 juni 1976 te Helmond. Na het behalen van mijn Vwo-diploma aan het Carolus Borromeus College te Helmond in 1994 ben ik aansluitend Scheikundige Technologie gaan studeren aan de Technische Universiteit Eindhoven. Het afsluitend onderzoek aan de vakgroep Instrumentele Analyse, uitgevoerd onder leiding van prof. dr. ir. H.L. Vader is op 22 december 1999 afgerond met het behalen van mijn ingenieurs diploma. Ik ben op 1 januari van het nieuwe millennium begonnen als Assistent in Opleiding aan de Universiteit Maastricht bij het Cardiovascular Research Institute Maastricht (CARIM). Begeleid door prof. dr. M.P. van Dieijen-Visser en prof. dr. W.Th. Hermens heb ik vanuit het klinisch chemisch laboratorium van het naastgelegen Academisch Ziekenhuis Maastricht, 4 jaar lang onderzoek verricht waarvan het resultaat nu voor u ligt. Met het voltooien van dit proefschrift is de weg vrij voor mijn opleiding tot klinisch chemicus waarmee ik de komende jaren zoet zal zijn.

Jart Diris.