

**ADVANCES IN ROUTINE  
MEASUREMENT OF CARDIAC  
DAMAGE AND CARDIOVASCULAR  
RISK MARKERS**

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Academic Dissertation to be presented with the assent of the Faculty of Medicine, University of Oulu, for public discussion in the Auditorium 8 of Oulu University Hospital, on February 25th, 2005, at 12 noon

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## **Hedberg, Pirjo, Advances in routine measurement of cardiac damage and cardiovascular risk markers**

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### ***Abstract***

The development of commercially available assays from the measurement of enzyme activity to mass concentrations of proteins, especially the assays of cardiac troponin I and T, has been the most important innovation in the field of cardiovascular laboratory diagnostics over the decade. The availability of a simple, rapid test using whole blood to facilitate processing and to reduce the turnaround time could improve the management of patients presenting with chest pain.

The aim of this study was to evaluate the analytical and clinical performance of a new time-resolved fluorometry-based immunology technology using the cardiac marker and high-sensitivity C-reactive protein assays. In addition, the use of high-sensitivity C-reactive protein assay for the investigation of patients with acute atrial fibrillation and the influence of heparin for cardiac marker assays were studied.

The levels of precision attained with pooled serum and plasma samples and control materials were acceptable. The assays were found to be linear within the ranges tested. The correlation coefficient between the Innotracc Aio! 1<sup>st</sup> generation cTnI and Abbott AxSYM cTnI assays was 0.960, and the slope was 0.07. The correlations between the 2<sup>nd</sup> generation Innotracc Aio!, Access AccuTnI and Abbott AxSYM assays were good, but there were biases between the methods. The correlation coefficients between the Innotracc Aio! and Abbott AxSYM CK-MB and myoglobin assays were 0.995 and 0.971, respectively, but the Innotracc Aio! CK-MB assay yielded about 9% higher values than the Abbott assay. The correlations between Innotracc Aio! usCRP and Cobas Integra CRP latex and between Innotracc Aio! usCRP and Hitachi CRP (Latex ) HS were good. Furthermore, the sample material correlation studies showed no significant differences when the Innotracc Aio! System was used. However, the mean Abbott AxSYM CK-MB values and the cTnI values for heparin plasma samples were 17% higher and about 15% lower than for serum samples, respectively. In the investigation of CRP levels in patients with acute atrial fibrillation CRP tended to be higher in the patients with acute FA, and there was a positive correlation between the concentrations of CRP and IL-6.

The results demonstrate the excellent analytical performance of the Innotracc Aio! 2<sup>nd</sup> generation cTnI, myoglobin, CK-MB and usCRP assays, and all the matrices, including serum, plasma and whole blood, are suitable sample matrices to be used with these methods without further standardization.

*Keywords:* acute atrial fibrillation, myocardial infarction, time-resolved fluorometry



*To Kim, Kristian, and Stig*



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Oulu, December, 2004

Pirjo Hedberg

## Abbreviations

AF	atrial fibrillation
ACC/AHA	American Collage of Cardiology/American Heart Association
AMI	acute myocardial infarction
Aio!	all-in-one (AIO)
AP	angina pectoris
CABG	coronary artery bypass grafting
CK	creatine kinase
CK-MB	creatine kinase-MB
CRP	C-reactive protein
cTnI	cardiac troponin-I
cTnT	cardiac troponin-T
CV	coefficient of variation
DL	detection limit
ECG	electrocardiography
EDTA	Ethylenediaminetetra-acetic acid
ESC/ACC	European Society of Cardiology/American College of Cardiology
Hct	hematocrit
FDA	Food and Drug Administration
Hs-CRP	highly sensitive CRP
IFCC	International Federation of Clinical Chemistry and Laboratory Medicine
MI	myocardial infarction
NACB	National Academy of Clinical Biochemistry
NCCLS	The National Committee for Clinical Laboratory Standards
PMI	Perioperative myocardial infarction
PTCA	Percutaneous transluminal coronary angioplasty
TAT	Turnaround time
TnC	troponin-C
RF	rheumatoid factor
UAP	unstable angina pectoris



## List of original articles

The present thesis is based on the following articles, which are referred to in the text by their Roman numerals:

- I Hedberg, P, Melkko, T, Valkama, J & Puukka M (2001) Higher CK-MB Mass values in heparin plasma than in serum measured with the Abbott AxSYM™ System. Clin Chem Lab Med 39(9): 872-874.
- II Hedberg P, Valkama J & Puukka M (2003) Analytical performance of time-resolved fluorometry-based Innotrac Aio!™ cardiac marker immunoassays. Scand J Clin Lab Invest 63: 55-64.
- III Hedberg P, Ylitalo K & Puukka M (2004) Highly sensitive determination of C-reactive protein on the Innotrac Aio! immunoanalyzer. Scand J Clin Lab Invest 64: 677-685.
- IV Hedberg P, Valkama J, Suvanto E, Pikkujämsä S, Ylitalo K, Alasaarela E & Puukka M (2004) Evaluation of Innotrac Aio!™ 2<sup>nd</sup> generation cardiac troponin I assay. Submitted for publication.
- V Ylitalo K, Lindgren K, Hedberg P, Valkama J, Raatikainen P & Huikuri H (2004) Increased interleukin-6 levels in patients with acute atrial fibrillation. Submitted for publication.



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# 1 Introduction

Acute coronary syndromes (ACS) include the conditions of ST-segment elevation myocardial infarction (MI), unstable angina and non-ST-segment elevation MI. ACS is caused by an imbalance between the blood flow/oxygen supply and the oxygen demand in part of the myocardium. These conditions share the pathologic mechanism of atherosclerotic plaque disruption and lead to coronary artery platelet aggregation and thrombosis. The resulting myocardial ischemia or necrosis manifests as elevated concentrations of cardiac markers in the bloodstream. Mass measurement of the MB isoenzyme of creatine kinase (CK, E.C. 2.7.3.2), the different CK isoforms, rapid serum myoglobin measurements and more cardiac-specific markers, such as the troponins, are currently used to facilitate the early diagnosis of acute myocardial infarction (AMI). The timing and concentration of these markers in the circulation depend on the local blood flow, infarct-related artery patency, infarct size and each marker's intracellular location, molecular weight and elimination rate from blood (Hudson *et al.* 2001).

Economic pressure to contain costs and to utilize the limited healthcare resources efficiently has placed an increasing burden on healthcare professionals to triage accurately and efficiently patients with acute chest pain. The advent of thrombolytic therapy and the clear benefit of early thrombolytic intervention underscore the need for rapid diagnosis of AMI and have heightened the interest in rapid methods to diagnose and risk-stratify patients with possible AMI early after arrival at the hospital. The availability of a simple, rapid test using whole blood to facilitate processing and to reduce the turnaround time could improve the management of patients presenting with chest pain (Meinertz & Hamm 1998). The whole blood test could be performed at a variety of testing sites, including emergency departments and point-of-care sites.

Another important challenge for cardiac markers lies in the early risk stratification in patients with unstable angina, where the aim is to identify the patients at high risk for AMI or cardiac death.

Clinical and laboratory studies have shown that inflammation plays a major role in the initiation, progression and destabilization of atheromas. Numerous epidemiologic studies have demonstrated the prognostic utility of C-reactive protein (CRP) in acute coronary

syndromes and its ability to predict future coronary events in apparently healthy men and women (Rifai & Ridker 2001 and refs. therein).

The present study examines the analytical and clinical performance of a dry reagent-based all-in-one immunoassay (AIO) utilizing fluorescent lanthanide chelates and time-resolved fluorometry cardiac marker and ultra-sensitive (usCRP) assays, the effect of interfering factors, such as heparin, rheumatoid factors and hemodialysis, and the role of CRP for the diagnosis of acute atrial fibrillation. These studies were done using the Innotrac Aio! analyzer, which is a fully automated benchtop random-access immunoanalyzer based on this universal all-in-one dry reagent concept.

## **2 Review of the literature**

### ***2.1.1 Clinical diseases***

#### ***2.1.2 Coronary heart disease and acute coronary syndromes***

The clinical presentations of ischemic heart disease include stable angina pectoris, silent ischaemia, unstable angina, myocardial infarction, heart failure and sudden death. Vascular injury and thrombus formation are key events in the origin and progression of atherosclerosis and in the pathogenesis of acute coronary syndromes. Acute coronary syndromes include unstable angina pectoris, myocardial infarction without ST-segment elevation and ST-segment elevation myocardial infarction. Briefly, acute coronary syndromes are due to an acute or subacute primary reduction of myocardial oxygen supply, provoked by disruption of an atherosclerotic plaque associated with thrombosis, vasoconstriction and microembolization, and the active inflammation inside the plaque plays an important role in plaque rupture. Plaques prone to rupture have a large lipid core, low smooth muscle cell density, high macrophage density, thin fibrous cap-disorganized collagen and high tissue factor concentration. The lipid core forms a cellular mass within the collagen matrix of the plaque. After foam cell death, the lipid core may be created by active dissolution of collagen by metalloproteinases. The lipid core of plaques prone to rupture has a high concentration of cholesteryl esters with a high proportion of polyunsaturated fatty acids. Thrombus is induced at the site of plaque rupture or erosion. It may lead to rapid changes in stenosis severity and result in subtotal or total vessel occlusion. It has been shown that thrombi occurring in unstable angina are mainly platelet-rich. Thrombosis at the site of plaque rupture may fragment into small particles, which may migrate downstream and occlude arterioles and capillaries. These platelet emboli may cause small areas of necrosis (minimal myocardial damage, small infarcts) in the absence of occlusion of the epicardial coronary artery. (Bertrand *et al.* 2000, The Finnish Society of Cardiology or Suomen Kardiologinen Seura 2003, Fuster *et al.* 1992, first and second of two parts).

### ***2.1.3 Role of inflammation in the pathogenesis of atherosclerosis***

Accumulating evidence from both basic and applied laboratories indicates that inflammation plays a critical role in several stages of atherogenesis, including acute plaque rupture as well as early initiation of foam cell deposits (Libby 1995). The plaque is typically an advanced atherosclerotic lesion covered by a fibrous cap consisting of smooth muscle cells and connective tissue containing macrophages and T lymphocytes. Under the fibrous cap, the lipid-rich core is filled with macrophages, lipids, calcium and other materials. Unstable plaques often have activated macrophages and leukocytes on their “shoulders”. Certain characteristics may predispose a plaque to rupture. These characteristics include a softer lipid core, the presence of macrophages, fewer smooth muscle cells, a thinner fibrous cap and increased inflammatory activity. (Adams III & Vickie 2001). Cytokines, which cause the *de novo* hepatic production of acute phase reactants, such as C-reactive protein, have been shown to increase in acute coronary syndromes (Liuzzo *et al.* 1994, Pepys & Baltz 1983).

CRP has been shown to activate complement pathway, up-regulate the production of adhesion molecules, increase LDL uptake into macrophages, stimulate nitric oxide production and endothelial nitric oxide synthase expression and increase plasminogen activator inhibitor-1 expression and activity (Devaraj *et al.* 2003, Ledua & Rifai 2003, Pasceri *et al.* 2000, Pasceri *et al.* 2001, Venugopal *et al.* 2002, Verma *et al.* 2002). Recent studies have shown that CRP is of interest in predicting the risk associated with coronary heart disease (Libby & Ridker 1999, Liuzzo *et al.* 1994, Rifai *et al.* 1999).

The American Heart Association (AHA) and the Centers for Disease Control and Prevention (CDC) Scientific Statement have issued recommendations on the use of inflammatory markers in primary prevention and for patients with stable coronary disease or ACS. Highly sensitive CRP assays (hs-CRP or usCRP) are needed for the risk assessment of cardiovascular disease, and such assays are currently available, but may require more standardization because the patients’ results are interpreted using population-based cut-off points. (Pearson *et al.* 2003). According to the CDC/AHA recommendations concerning the currently identified inflammatory markers, hs-CRP is the first choice. Hs-CRP should be measured in metabolically stable patients without known inflammatory or infectious conditions to reduce intraindividual variability, and the assay should be repeated within 2 weeks and the average of two measurements used. If hs-CRP is > 10mg/L, the test should be repeated and the patient examined for sources of infection or inflammation. These recommendations include the laboratory aspect of CRP: hs-CRP low risk < 1mg/L, average risk 1-3 mg/L and high risk > 3mg/L. (Pearson *et al.* 2003).

### ***2.1.4 Atrial fibrillation***

Atrial fibrillation (AF) is the most common sustained cardiac arrhythmia. It is usually classified according to its temporal patterns as paroxysmal, persistent or permanent. AF adversely affects cardiac haemodynamics because of the loss of atrial contraction and the rapidity and irregularity of the ventricular rate. AF causes significant symptoms in

approximately two thirds of patients. AF has been found to associate with a 1.5- to 2-fold increase in mortality and with a 6-fold increase in the risk of stroke. AF is initiated by rapid electrical activity, often arising from arrhythmogenic foci located in the muscular sleeves of pulmonary veins. After a period of continuous AF, electrical remodelling occurs, further facilitating AF maintenance. These changes are initially reversible if sinus rhythm is restored, but may become permanent and be associated with structural changes if fibrillation is allowed to continue. (Markides & Schilling 2003).

AF may persist due to structural changes in the atria that are promoted by inflammation. CRP predicts cardiovascular events and stroke, which are common sequelae of AF. CRP has been found to be elevated in patients with atrial arrhythmias. (Chung *et al.* 2001).

## **2.2 Diagnosis of acute myocardial infarction**

The initial recognition and diagnosis of patients suffering from myocardial infarction include a targeted patient history (associated symptoms, hypertension, sex- and age-related differences in presentation, diabetes mellitus, possibility of aortic dissection, risk of bleeding and clinical cerebrovascular disease), physical examination, electrocardiogram, laboratory examinations (complete blood count with platelet count, international normalized ratio, activated thromboplastin time, electrolytes and magnesium, blood urea nitrogen, creatinine, glucose and serum lipids) and biomarkers of cardiac damage. (Antman *et al.* 2004, The Finnish Society of Cardiology 2003).

## **2.3 Recommendations on the use of biochemical markers of cardiac damage in acute coronary syndromes**

In 1968, Rose & Blackburn introduced the cardiovascular survey methods under the auspices of World Health Organization (WHO) (Fig. 1). Total CK higher than twice the upper reference limit was recommended as an indicator of myocardial necrosis. (Rose & Blackburn 1968). Eleven years later, WHO published guidelines on the criteria for the diagnosis of AMI. In the original criteria for MI, when plans were being made for the MONICA (monitoring cardiovascular disease) project, WHO listed unequivocal changes of serial enzyme measurements as one of the three criteria for diagnosis, the other two being electrocardiographic changes and clinical features, such as chest pain. The ‘gold standard’ in detecting myocardial necrosis has been elevated CK-MB. This measure satisfies the criteria for a diagnosis of MI, as proposed by WHO and later extended for the MONICA study. (Goldman *et al.* 2001, World Health Organization 1979). When the protein markers were introduced, the National Academy of Clinical Biochemistry (NACB) and the International Federation of Clinical Chemistry (IFCC) expanded the recommendations to apply to these protein markers. Both the NACB and the IFCC guidelines recommended the use of two markers: an early marker (reliably increased in blood within 6 h after the onset of symptoms, such as myoglobin) and a definitive marker

(increased in blood after 6-9 h, but with high sensitivity and specificity for myocardial injury, remaining abnormal for several days, such as troponins). The NACB recommended specimen collection at admission, 2-4 h and 6-9 h, and optional determinations at 21-24 h after admission, whereas the IFCC recommended collections at admission and at 4, 8 and 12 h (or next morning) after admission. The NACB and IFCC recommended the use of two decision limits for cardiac troponin. The NACB and IFCC recommended the use of the 97.5<sup>th</sup> percentile of the healthy population, and that cardiac troponin (I or T) should be used as the new biomarker standard for the detection of myocardial damage. The other cut-off sets the lower limit of the reference range for AMI diagnosis. The area between the two cut-offs should not be considered a gray zone. Patients whose troponin I or T falls between these two cut-offs should be labelled as having myocardial injury. According to these recommendations, the assays for cardiac markers should have imprecision (CV) <10% at the AMI decision limits and an assay TAT of about 1 h or less, and institutions unable to consistently deliver cardiac marker TATs of 1 h should implement POC testing devices. Additionally, plasma or anticoagulated whole blood are the specimens of choice for the stat analysis of cardiac markers, but each laboratory must follow the recommendations for acceptable specimen types listed in the manufacturers' package inserts and should use a reference interval specific to the specimen type. In unstable angina, the NACB and IFCC committees unanimously stated that patients with small increases in cardiac troponin should be treated to minimize the risk associated with myocardial injury. (Panteghini *et al.* 1999, Wu *et al.* 1999).

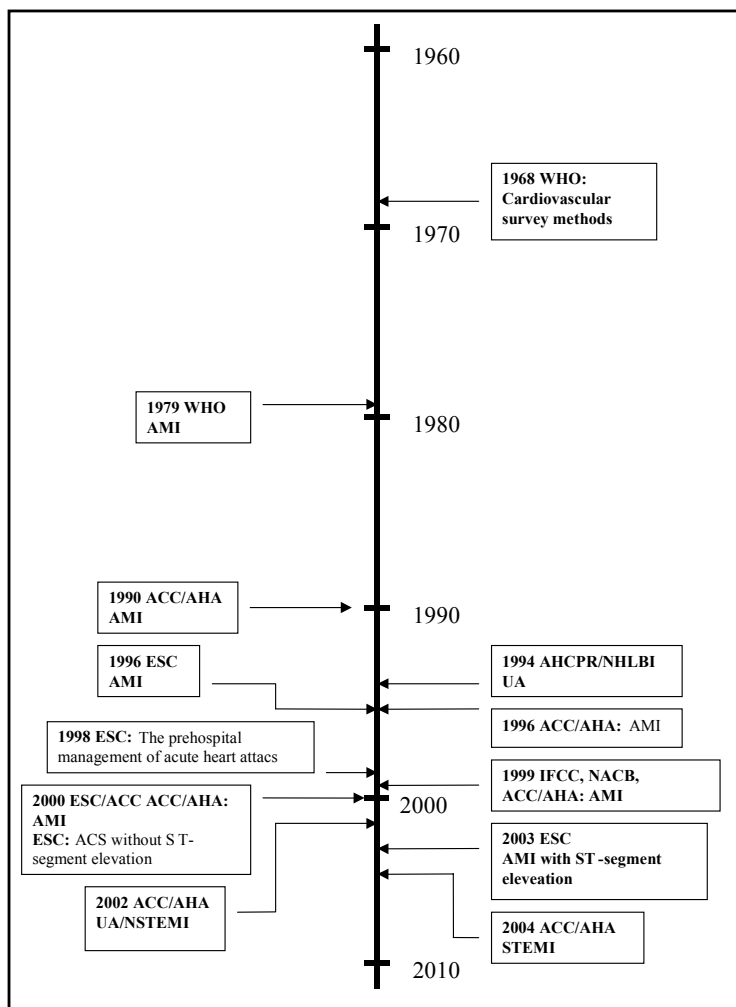
The IFCC Committee on Standardization of Markers of Cardiac Damage (C-SMCD) published proposals for strategies and concepts on the standardization of cardiac marker assays (Dati *et al.* 1999).

Consensus documents recently published by the European Society for Cardiology (ESC) and the American College of Cardiology (ACC) contain specific recommendations on the use of cardiac markers. In response to the issues posed by an alteration in the ability to identify MI, the ESC and ACC convened a consensus conference in July 1999 to reconsider jointly the definition of MI. According to this, myocardial infarction is a general term that should be used with further definition of qualifications, specifically as follows: acute/evolving MI (6 h to 7 days), evolving/healing MI (7 to 28 days) or recently healed MI (29 days or more). Even a small increase of troponin is indicative of myocardial necrosis, and the increases are likely to reflect irreversible rather than reversible injury. The consensus document defines an increased value of cardiac troponin as a measurement that exceeds the 99<sup>th</sup> percentile of a reference control group. The proposed acceptable imprecision of a troponin assay at this level must be  $\leq 10\%$ . Cardiac troponin should be measured from serial blood samples collected at least 6-9 h after the onset of symptoms. If cardiac troponin assays are not available, the best alternative will be CK-MB. For patients in need of early diagnosis, a rapidly appearing biomarker (such as CK-MB isoforms or myoglobin) plus a biomarker that rises later (troponin) are recommended. The new definition outlined in the consensus document will assist in identifying more infarcts than in the past. The manufacturers of cardiac troponin assays must now ensure that the assays are necessarily precise in the cut-off area. (Alpert *et al.* 2000). More recently, Panteghini and colleagues evaluated the imprecision of 17 commercially available cardiac troponin assays, and they found out that no cardiac

troponin assay was able to achieve the 10% CV recommendation at the 99<sup>th</sup> percentile limit (Panteghini *et al.* 2004). Apple with his coworkers demonstrated the 99<sup>th</sup> percentile reference limits for 8 different cardiac troponin and 7 different CK-MB assays. Selective gender and ethnic differences were also demonstrated. Two of the cTnI assays demonstrated a significant ( $p < 0.05$ ) mean difference between males and females, with a 1.2- to 2.5-fold 99<sup>th</sup> percentile for males. Two of the assays also demonstrated a significant ( $p = 0.05$ ) difference between the mean cTnI concentrations in blacks and Caucasians, with a 1.1- to 2.8-fold 99<sup>th</sup> percentile for blacks. For cTnI assays, the 99<sup>th</sup> percentile limits varied by as much as 13-fold between the lowest (0.06  $\mu\text{g/L}$ ) and highest (0.8  $\mu\text{g/L}$ ) concentrations. All of the seven CK-MB assays demonstrated a 1.2- to 2.6-fold 99<sup>th</sup> percentile for males vs females, with mean concentrations significantly higher for males ( $p < 0.0001$ ). For all participants, the 99<sup>th</sup> percentile limits varied by as much as 2.0-fold between the lowest (3.9  $\mu\text{g/L}$ ) and highest (7.9  $\mu\text{g/L}$ ) concentrations. Four CK-MB assays also showed significantly higher (1.2- to 2.7-fold;  $p < 0.02$ ) mean concentrations for blacks vs Caucasians. (Apple *et al.* 2003). The Innotracc Aio! troponin I assay was not involved in either of these studies.

The evolution of ACC/AHA guidelines for the management of patients with acute MI has been reviewed in the publication of ACC/AHA entitled 'Practice Guideline for the Management of Patients with ST-elevation Myocardial Infarction' in 2004 (Antman *et al.* 2004). The first guidelines published by ACC/AHA described the management of patients with acute myocardial infarction (AMI) in 1990. The subsequent documents were the Agency for Healthcare and Quality/National Heart, Lung and Blood Institute (AHCPR/NHLBI) sponsored guideline on the management of unstable angina (UA) in 1994, the revised/updated ACC/AHA guideline on AMI (1996, 1999 and 2000) and the revised/updated ACC/AHA guideline on unstable angina/non-ST-segment myocardial infarction (UA/NSTEMI) in 2002. The present guideline is a revision and deals strictly with the management of patients presenting with ST-elevation myocardial infarction (STEMI) in 2004. (Antman *et al.* 2004)

In addition to the ESC/ACC consensus document, ESC published recommendations for the management of patients with acute coronary syndromes with ST-segment elevation in 1996 and without ST-segment elevation in 2000 (ESC 1996 & Bertrand *et al.* 2000). In 1998, the Task Force Report of 'The Pre-hospital Management of Acute Heart Attacks' was introduced (ESC 1998). In 2003, ESC published recommendations on the management of acute myocardial infarction in patients presenting with ST-segment elevation (ESC 2003).



**Fig. 1. The history of recommendations for the use of cardiac markers and the management of patients with acute coronary syndromes.**

## **2.4 Other markers of cardiac damage and laboratory analyses in risk stratification for myocardial infarction**

Oxidized low-density lipoprotein (LDL) and malondialdehyde-modified LDL have been demonstrated to be a marker of coronary arteriosclerosis and a marker of plaque instability, respectively (Holvoet *et al.* 2001). Other markers of inflammation in the

prediction of cardiovascular disease have traditionally been soluble intercellular adhesion molecule (sICAM-1), total cholesterol, low-density lipoprotein cholesterol, high-density lipoprotein cholesterol and the ratio of total cholesterol to HDL-C (Ridker *et al.* 2000). At the present, the markers of inflammatory process that appear to predict coronary events also include highly sensitive CRP (hs-CRP), serum amyloid A (SAA), fibrinogen and IL-6. Of these markers, hs-CRP may prove to be the most clinically useful because it is easily measured and appears to be the most powerful independent predictor of future vascular events (Libby & Ridker 1999, Rifai *et al.* 1999). Increments in systemic nuclear factor kappa B (NFkB) activation are associated with more rapid onset of clinical cardiac events, implying that its levels may be predictive of events in individual patients (Daniel *et al.* 2001). Additionally, plasma homocysteine has been shown to predict mortality independently of traditional risk factors and CRP in patients with angiographically defined coronary artery disease (Anderson *et al.* 2000).

Increased concentrations of the acute phase proteins, CRP and serum amyloid A, are known to be nonspecific, but may have a role in identifying patients with unstable coronary plaques (Christenson & Azzazy 1998). Patients with abnormal concentrations of acute phase proteins are at an increased risk (Liuzzo *et al.* 1994, Ridker *et al.* 1997). The level of circulating phospholipase A2 (PLA2) has been demonstrated to predict future myocardial infarction (Porela *et al.* 2000).

Circulating pregnancy-associated plasma protein A (PAPP-A) is expressed in unstable but not in stable coronary artery plaques, and circulating levels are elevated in acute coronary syndromes. PAPP-A measurement appeared to be valuable in detecting unstable acute coronary syndrome even in patients without elevated biomarkers of necrosis, such as cardiac troponins or CK-MB, thus potentially identifying high-risk patients whose unstable clinical situation might otherwise remain undiagnosed. (Bayes-Genis *et al.* 2001, Laterza *et al.* 2004, Lund *et al.* 2003, Qin *et al.* 2002). These elevated levels may reflect the new candidate marker of unstable angina and acute myocardial infarction.

Platelet activation is important in the mechanism of thrombus formation and the mechanism of acute coronary syndromes. Indicators of platelet activation, such as platelet function assays or P-selectin, may help to assess a patient's tendency to intracoronary thrombosis. (Christenson & Azzazy 1998). Although not sensitive enough to diagnose MI, insoluble fibrin and cross-linked fibrin degradation are increased in patients at higher risk for complications, and they are thought to indicate increased fibrinolysis before the development of MI (Lee *et al.* 1997). Thrombin-antithrombin III complex (TAT) has been demonstrated to be a sensitive marker of the initial thrombotic process in AMI (Mair *et al.* 1997).

Glycogen phosphorylase-BB (GPBB) release is thought to be linked to the sudden burst of glycogenolysis that occurs in the injured myocardium after acute AMI. Based on its metabolic function, GPBB is an enzyme for early laboratory detection of ischemia. (Mair 1998, Rapitzsch *et al.* 1995). Fatty acid binding protein (FABP) has similarly been proposed as an early plasma marker. The release and plasma kinetics of FABP closely resemble those of myoglobin, but the relatively high concentration of FABP in the heart makes it more specific than myoglobin for the monitoring of myocardial injury. (Glatz & Hermens 2001).

Ischemia-modified albumin has been introduced as a sensitive marker of myocardial ischemia after percutaneous coronary intervention and a useful tool in the risk

stratification of chest pain patients in the emergency department (Sinha *et al.* 2003). Recently, a novel biochemical test has been developed based on the binding of serum albumin and the transition metal cobalt (Albumin Cobalt Binding test, ACB-test).

Measurement of carbonic anhydrase isoenzyme III (CA III) and calculation of the myoglobin to CA III ratio would be helpful for early differential diagnosis of acute myocardial infarction and for differentiation of whether serum myoglobin originates from myocardium or from skeletal muscle (Vuori *et al.* 1996, Väänänen *et al.* 1990). However, this combination of markers has never been commercially available.

Protein S-100 is an acidic and calcium-binding protein. S100<sub>a<sub>0</sub></sub> protein is present at high concentrations in heart muscle, and this protein has been indicated to be a useful marker not only in the diagnosis of AMI, but also in the differential diagnosis of AMI vs angina pectoris. (Usui *et al.* 1990). It is not a heart-specific marker. It increases similarly to myoglobin after AMI, but in contrast to myoglobin, it remains elevated for several days. (Mair 1997).

In recent years, natriuretic hormones, atrial natriuretic peptide (ANP), B-type natriuretic peptide (BNP) and N-terminal proBNP (Nt-proBNP) have been commercially available for clinical assessment of cardiac function, i.e. to identify and manage patients with symptomatic and asymptomatic ventricular dysfunction. Whereas ANP is secreted mainly from atrial cardiomyocytes, BNP and Nt-proBNP are preferentially produced and secreted in the left ventricle. The plasma concentrations of these peptides are increased in diseases characterized by an expanded fluid volume, such as renal failure, primary aldosteronism and congestive heart failure, or by stimulation of peptide production caused by ventricular hypertrophy or strain, thyroid disease, excessive circulating glucocorticoid or hypoxia. The clinical usefulness of cardiac natriuretic peptides in the evaluation of patients with suspected heart failure, in prognostic stratification of patients with congestive heart failure, in detecting LV systolic or diastolic dysfunction and in differential diagnosis of dyspnoea has been confirmed even recently (Panteghini 2004 and refs. therein).

## 2.5 Cardiac markers

### 2.5.1 Development of the biochemical markers of cardiac damage

#### 2.5.1.1 Enzyme markers

The first biochemical markers, aspartate aminotransferase (EC 2.6.1.1., AST, formerly known as serum glutamic oxaloacetic transaminase) and lactate dehydrogenase (EC 1.1.1.27, LD, or LDH), used for the diagnosis of acute myocardial infarction were introduced in 1954 and 1955, respectively (Karmen *et al.* 1954, Wroblewski & Ladue 1955, Wu 1999) (Fig. 2). Karmen and colleagues measured the presence of glutamic oxaloacetic and glutamic pyruvic transaminase activity in human serum, plasma and

whole blood hemolysates by measuring the rate of glutamate formation employing quantitative paper chromatography. Wroblewski and LaDue measured spectrophotometrically the rate of decrease of optical density representing the rate of oxidation of serum diphosphonucleotide activity. AST lacks cardiac specificity, and it currently has no clinical value for AMI diagnosis.

LDH is found in almost every tissue, with high activities in skeletal muscle, liver, heart, kidneys, brain, lungs and erythrocytes. LDH exists as a tetramer composed of two different subunits, M (muscle) and H (heart). It is an important enzyme of glucose metabolism. There are five isoenzymes. In the heart, carbohydrate and fatty acid metabolism is relatively constant, metabolites are completely oxidized, and the LDH-1 isoenzyme is predominant. LDH activity starts to increase at 6 to 12 h after the onset of chest pain, peaks at 1 to 3 d and returns to the baseline 8 to 14 d after the AMI onset. Normally, LDH-1 activity does not exceed 40% of total LDH activity. After myocardial damage, LDH-1 activity exceeds LDH-2 activity, and the LDH-1/LDH-2 ratio is fairly specific for myocardial damage. (Mair 1997). However, LDH-1 is not heart-specific (Adams *et al.* 1993, Lee & Goldman 1986, Mair *et al.* 1994, Mair 1997).

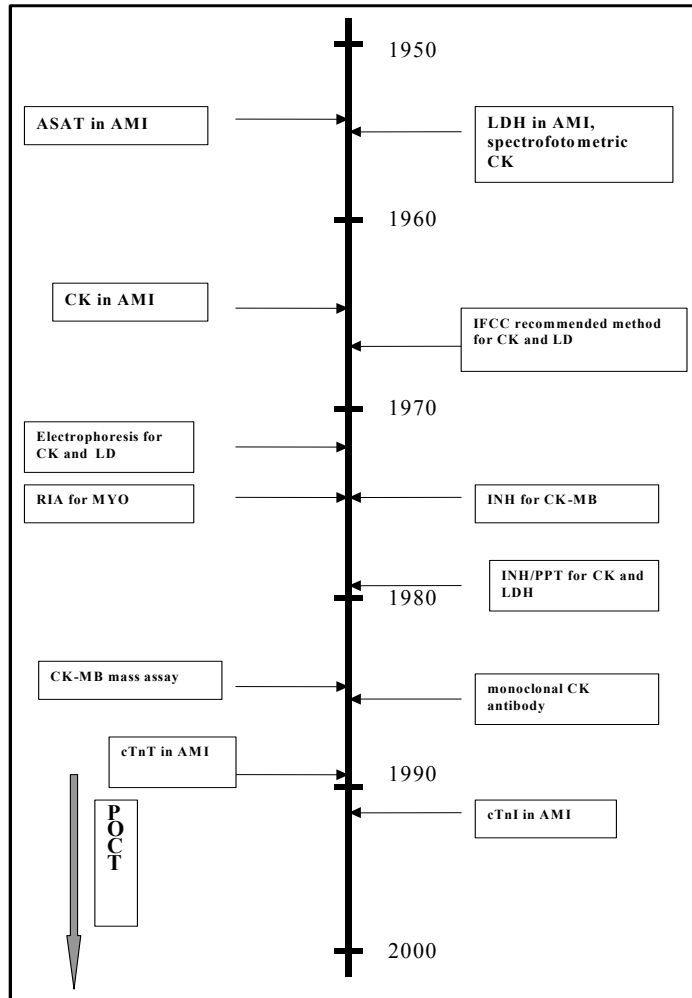
Since 1954, many other enzymes have been studied in serum during infarction: serum malic acid dehydrogenase, serum glutamic pyruvate transaminase, serum alpha-hydroxy butyrate dehydrogenase and serum aldolase have been used. However, the diagnostic value of these enzymes is limited as they are present in significant quantities in both heart and liver tissue. AST was gradually replaced by creatine kinase (CK; EC 2.7.3.2) described in 1965. (Duma & Seigel 1965). Duma and Seigel used a colorimetric technique to measure CK. A sample of 0.1 ml of serum and 15 to 30 minutes' incubation were needed for the colour to develop.

CK is a key enzyme in cellular energetics and in muscle metabolism. It catalyzes the reversible transfer of a phosphate residue and the high-energy binding between adenosine triphosphate (ATP) and creatine, and it functions mainly as a temporary energy buffer and in the transfer of energy from the mitochondria to the cytosol. There are two forms: cytosolic and mitochondrial CK (Mi-CK). Cytosolic CK shows significant binding to myofibrils in heart and skeletal muscle. It is a dimeric molecule composed of either B (brain) subunits or M (muscle) subunits. Three isoenzymes exist: CKBB (CK-1), CKMB (CK-2) and CKMM (CK-3). (Mair 1997). Two mitochondrial subunit isoforms, ubiquitous  $Mi_a$ -CK and sarcomeric  $Mi_b$ -CK, are synthesized in a tissuespecific manner. These subunits combine to form either homodimers or homo-octamers. Mi-CKs from chicken cardiac muscle and brain have been shown to differ in their N-terminal amino acid sequences and to be encoded by multiple mRNAs. (Decking *et al.* 2001 & Schlegel *et al.* 1988). In addition, macromolecular CK isoforms are occasionally seen in plasma (Lee *et al.* 1994). A spectrophotometric method for the determination of creatine phosphokinase was developed in 1955. This method, although no more sensitive than the colorimetric estimation of creatine, made it possible to measure enzymic activities in tissue homogenates and extracts diluted 2 000 to 20 000 times. (Oliver 1955). This method was improved further a few years later. The procedure in which adenosine triphosphate, liberated by the action of the enzyme, was linked to the reduction of nicotinamide adenine dinucleotide phosphate and the formation of reduced nicotinamide adenine dinucleotide phosphate followed spectrophotometrically. This method was more sensitive and less time-consuming than the previous procedures. (Rosalki 1967). This

assay has been recommended by the International Federation of Clinical Chemistry (IFCC) method for the assessment of CK (Horder *et al.* 1991).

To improve the specificity of total enzyme measurements, the assays for CK and LD isoenzymes (combined isoenzyme analysis) were developed using electrophoresis. Electrophoretic analysis of serum enzymes characterized by tissue-specific isoenzyme patterns enhanced the specificity of laboratory diagnosis. The use of nitro blue tetrazolium for the detection of these isoenzymes proved to be somewhat inconsistent and of varying sensitivity for quantification. (Roe *et al.* 1972). Electrophoresis on agar, agarose or cellulose acetate can separate the CK isoenzymes (Moss & Henderson 1996). However, electrophoresis is very labour-intensive and time-consuming. Therefore, immunoinhibition assays were soon developed (Jockers-Wretou & Pfeleiderer 1975). In the technique of immunoinhibition, the inhibiting anti-CK-M sera inhibit both M subunits of CK-3 (MM) and the single M subunit of CK-2 (MB) and thus allow determination of the enzyme activities of the B subunit of CK-2, the B subunits of CK-1, any macro or mitochondrial CKs and adenylate kinase. These methods for measuring the CK isoenzymes require specific antisera against the M and B subunits. These are obtained by preparing monoclonal or polyclonal antibodies. (Moss & Henderson 1996). A modification of this assay was the introduction of precipitation to the method, and this procedure was first developed for CK and LD (Usategui-Gomez *et al.* 1979, Wicks *et al.* 1982). Usategui-Gomez and colleagues used antibodies to LDH-5 (precipitation of LDH-5) as specific and sensitive tools to separate LDH-1 from all other isoenzymes. Both the inhibiting and the precipitating properties of the M-subunit were used to give the desired MB specificity in the determinations by Wicks and colleagues.

In the procedure based on immunoprecipitation, anti-CK-B sera precipitate (after centrifugation) all CK-BB and CK-MB from a serum sample, leaving only the CK-MM activity to be detected. Similarly, anti-CK-M sera precipitate all CK-MM and CK-MB, leaving only CK-BB to be detected. The serum content of CK-MB can thus be measured by calculating the difference between the total serum CK activity and the sum of CK-BB and CK-MM activities. The principle of the method for measuring the LD isoenzymes is similar. (Moss & Henderson 1996).



**Fig. 2. History of the biochemical markers of cardiac damage**

### 2.5.1.2 Protein markers

In contrast to immunoprecipitation and immunoinhibition, which measure isoenzymes by determinations of enzyme activity, immunoassays measure the enzyme mass regardless of whether or not the enzyme molecule is catalytically active. Although mass assays of CK-BB and CK-MM are readily achieved, with less than 2% cross-reactivity, one of the CK-MB units is included in any determination of either the M or the B subunits. Therefore, specific measurements of CK-MB require application of the sandwich

technique, in which two antibodies with affinity for different parts of the CK-MB molecule are used sequentially. The first antibody, which is usually monoclonal, is rendered immobile on a matrix, and the second antibody is conjugated with the label, such as an enzyme or a marker molecule. The sandwich technique ensures that only CK-MB is estimated, because neither CK-MM nor CK-BB reacts with both antibodies. These mass assays also enabled the measurement of protein markers that are not themselves enzymes. (Moss & Henderson 1996).

In radioimmunoassay (RIA,  $^{125}\text{I}$ -myoglobin), myoglobin was the first protein marker investigated for AMI. This method allowed detection of 0.5 ng of myoglobin. All determinations were performed in 12x75 mm glass tubes, and equilibration was reached in 24 h at +4°C. Myoglobin is a low molecular weight (17 kDa) protein present in the cytosol of cardiac and skeletal muscle. Due to these characteristics, myoglobin appears in blood after tissue injury earlier than other biomarkers. (Stone *et al.* 1975). Given the impracticability of stat RIA analysis, however, serum myoglobin was not used in routine practice until the development of automated non-isotopic immunoassays (Wu *et al.* 1999). In 1991, i.e. the immunonephelometric assay of myoglobin, was introduced (Massoubre *et al.* 1991). This assay uses immunolateral in determining serum myoglobin (Behringwerke AG, Warburg, FRG). Rapid immunoturbidometric assays for myoglobin have been introduced since 1988 (Mair *et al.* 1991, Mair *et al.* 1992). These assays used the Behring Turbitimer analyzer to measure myoglobin concentrations in plasma (Turbiquant myoglobin, Behringwerke AG, Marburg, Germany). Quantitative results were available about a minute after the start of the assay.

The first manual CK-MB mass assay was described in 1985, using anti-CK-M and anti-CK-B antibodies. This Tandem-E ImmunoEnzymetric CKMB (Hybritech Inc., San Diego, CA 92121) was a solid-phase, two-site immunoassay. The antibody-antigen binding reaction involved 2-h incubation followed by a 30-min reaction with alkaline phosphatase (EC 3.1.3.1). Absorbance was measured at 405 nm. (Chan *et al.* 1985). In the 1980s, the introduction of automated immunoassays based on monoclonal anti-CK-MB antibodies allowed measurement of the protein (Panteghini 1998). This significantly improved the analytical sensitivity and specificity of the assays. These CK-MB immunoassays helped to minimize analytical interference, which often leads to false-positive results in traditional immunoinhibition assays (Panteghini 2004).

A few years ago, Penttilä *et al.* studied the utility of myoglobin, CK-MB isoforms and CK-MB mass assays in early diagnosis of MI, using cTnT positivity as reference. CK-MB isoforms or myoglobin offered no advantage over Ck-Mb mass as early markers of myocardial infarction. CK-MB isoforms were no more sensitive than CK-MB mass. At 6 h after admission, CK-MB mass and CK-MB isoforms performed better than myoglobin. At that point, i.e. over 6 h after the onset of chest pain, CK-MB isoforms and myoglobin were more sensitive than CK-MB mass. The specificity of myoglobin was lowest at all time points, and CK-MB mass was also more specific than CK-MB isoforms. (Penttilä *et al.* 2002).

Cardiac troponin T (cTnT) and cardiac troponin I (cTnI) as markers for AMI were first demonstrated in 1989 and 1992, respectively (Katus *et al.* 1989, Bodor *et al.* 1992). Katus and colleagues developed an assay in which troponin T was bound to different epitopes by affinity-purified goat anti-cardiac troponin T antibodies immobilized on polyvinyl chloride test tubes as well as horse raddish peroxidase-labelled monoclonal

anti-troponin T antibody in liquid phase. The assay procedure was completed semiautomatically in 90 min. Bodor with colleagues developed a cTnI-specific mAb-based enzyme-linked immunosorbent assay (ELISA) that utilizes two human cTnI-specific mAbs.

The development of commercially available assays for the determination of troponins has been the most important innovation in the field of cardiovascular laboratory diagnostics in the last decade. The increased expression of CK-MB in diseased and damaged skeletal muscle (e.g. muscular dystrophy, after exercise of marathon runners) and in chronic renal diseases supports the growing clinical significance of the replacement of CK-MB by cardiac troponins as the new standard for the detection of myocardial damage in humans and animals. (Apple 1999).

Cardiac troponins are currently regarded as the most specific commercially available biochemical markers of myocardial damage. Due to patent restrictions, assays for cTnT are available from one manufacturer, whereas assays for cTnI are available from multiple manufacturers. There are a considerable number of commercial troponin I immunoassays in the field. A major concern is that there are marked differences in the construction of each assay. However, all currently used immunoassays for troponins, CK-MB and myoglobin are sandwich-type methods based on two monoclonal antibodies or a combination of monoclonal and polyclonal antibodies using the detection scheme of fluorescence, absorbance or chemiluminescence and the label of alkaline phosphatase, acridium ester,  $\beta$ -galactosidase, horseradish peroxidase, europium-chelate or ruthenium complex.

### 2.5.1.3 Point-of-care testing

The rapid turnaround time of biochemical cardiac markers is thought to reduce costs and to improve the outcome of patients with chest pain admitted into emergency rooms. The ideal point-of-care test (POCT) is accurate, precise, rapid (fast turnaround time), easy to sample, easy to operate, with low calibration demands, disposable (one-time use) or easy to maintain and inexpensive and improves clinical decision-making (Valdes *et al.* 2001). POC tests are diagnostic procedures performed at the bedside or near the patient, and they are often performed by non-laboratory personnel with no needs for special skills. As cardiac markers are now the critical element in the diagnosis of non-ST-segment AMI, the target turnaround time (TAT) for reporting the results of cardiac marker assays has become an increasingly important issue. The demand for shorter TATs is also the result of changing practices in the triaging of patients who present in the emergency department with chest pain. TAT has been defined as the time from blood collection to the reporting of the test results (Wu *et al.* 1999).

Recently, E. Liikanen in her doctoral thesis described POCT and its quality assurance in the diagnosis and follow-up of heart and cardiovascular diseases in Finnish emergency departments. She also evaluated the POCT carried out particularly by nursing staff with one of the common tests, namely C-reactive protein. This study revealed that a third of the staff in emergency departments had never received additional training in POCT. The most common problems were related to analysis, working arrangements, lack of internal

or external quality assessment and the visual reading of weakly positive tests. The author concluded that, in order to overcome problems and to improve quality, it would be practical to make standard directions that would regulate POCT in emergency rooms and at other POCT sites. Further, a multiprofessional team should administer POCT, and the professionals who use POCT should have adequate basic education, which should be supplemented periodically. (Liikanen 2003).

Since 1994, a visual rapid assay for the bedside testing of cardiac troponin T (Boehringer Mannheim GmbH, Mannheim, Germany) from whole blood specimens has been available (Müller-Bardoff *et al.* 1999). In three developmental modifications, the detection limit of this assay was improved from 0.3 µg/L for the first generation (Collinson *et al.* 1996) to 0.2 µg/L for the second generation (Müller-Bardoff *et al.* 1995, Gerhardt *et al.* 1997) and, in 1997, to 0.1 µg/L (Müller-Bardoff *et al.* 1997). Visual assessment of the available whole blood assay allows a positive or negative statement without definite information regarding the concentration of cTnI in blood. In these tests, blood cells are separated from plasma with glass-fiber fleece. The immunocomplexes formed are concentrated within the reading zone by binding of the biotin-labeled antibody with streptavidine immobilized to the test device. Troponin T bound to the test device serves as a control. (Müller-Bardoff *et al.* 1995). The Cardiac STATus CK-MB/myoglobin device (Spectral Diagnostics, Inc., Toronto, Canada) is a solid-phase chromatographic assay that allows for qualitative detection of CK-MB and myoglobin, utilizing whole blood, serum or plasma (Brogan & Bock 1998).

Although the qualitative assay is sufficient for clinical decision-making, some prognostic information inherent to the concentration of circulating cardiac biomarker is lost. The interindividual variability of visual assessment of the test strip at the detection limit of the assay may cause substantial analytical error. Qualitative POCT are still available for troponin, CK-MB and myoglobin, but the majority of POCT for biomarkers currently on sale are quantitative. An optimized test strip device for cTnT was described in 1999 (Roche Diagnostics, Mannheim, Germany), which can be analyzed by a cardiac reader for quantitative assessment of the test result (Müller-Bardoff *et al.* 1999), and in 2000 the same group developed a quantitative myoglobin assay for this system. The cardiac reader is a camera with a charged coupled device that optically records the reflectance signal from the detection zone of the immunochemical test strips. The signal and control lines are identified by a pattern recognition algorithm. The intensity of the signal line is proportional to the concentration of analytes. (Bardoff-Müller *et al.* 2000). The Biosite Triage® Cardiac Panel (Biosite Diagnostics, San Diego, CA) is a quantitative, multimarker, whole-blood system using a fluorescence technology, where cells are separated from plasma with a filter. The concentration of each analyte, directly proportional to the detected fluorescence, is measured by a Triage meter. (Apple *et al.* 1999). The quantitative cardiac marker tests also include others, such as Stratus CS® (Dade Behring), which is a fluorometric enzyme immunoassay using a radial partition immunoassay technology, and which has been enhanced through the use of a monoclonal capture antibody coupled to Starburst® dendrimers (Altinier *et al.* 2000, Chapelle *et al.* 2000, Heeschen *et al.* 1999, Singh *et al.* 1994). The Alpha Dx System (First Medical, Inc., Mountain View, CA) is a fluorescence immunoassay platform that integrates automated solid-phase sandwich immunoassay capabilities with fluorescence detection in whole blood. The test disc uses three antibodies: a solid-phase antibody, a hapten-labelled

antibody and a fluorescent-labelled anti-hapten (fluorescein) antibody. (Apple *et al.* 2000, Miller *et al.* 1998).

The application of a dry reagent-based all-in-one immunoassay (AIO) utilizing fluorescent lanthanide chelates and time-resolved fluorometry (Lövgren *et al.* 1996) for rapid and quantitative determination of biochemical myocardial infarction markers from whole blood, serum and plasma was recently reported (Pettersson *et al.* 2000).

#### 2.5.1.4 Clinical studies

Several large clinical studies have had a significant impact on the practice of cardiology and the role of biochemical cardiac markers. The TIMI Trial (In Thrombolysis in Myocardial Infarction) was started in 1987. It compared the intravenous tissue plasminogen activator with intravenous streptokinase in 290 patients with evolving acute myocardial infarction. (Chesebro *et al.* 1987). In 1993, The Global Use of Strategies to Open Occluded Coronary Arteries (GUSTO) Trial was begun. This trial was designed to compare aggressive thrombolytic strategies with standard thrombolytic regimens in the treatment of acute myocardial infarction. (Topol *et al.* 1993). There are also clinical trials where troponin T and troponin I have been compared. The GUSTO IIA trial compared cTnI and cTnT for risk stratification in patients with acute coronary syndromes (Ohman *et al.* 1996). The TRIM trial (Thrombin Inhibition on Myocardial ischemia) has also investigated the usefulness of cTnI and T in risk stratification in unstable angina pectoris patients (Holmvang *et al.* 1997, Lüscher *et al.* 1997). There have been several other TIMI/TIMI substudies since 1987, including the TIMI IIB substudy (Morrow *et al.* 2000) and the TIMI 11A (TIMI 11A Investigators 1997) and TIMI 11A substudies (Antman *et al.* 1998). The large TIMI IIB trial addressed the relationship between cTnI and prognosis and the early identification of patients with at an increased risk of death (Antman *et al.* 1996).

There are trials to show that the determination of cTnT has some relevance for the clinical decision-making process in patients with unstable angina. In the Fragmin during Instability in Coronary Artery Disease (FRISC) I trial, cTnT identified patients with unstable coronary artery disease who benefit from long-term antithrombotic protection (Lindahl *et al.* 1997). The c7E3 Fab Antiplatelet Therapy in Unstable Refractory Angina (CAPTURE) trial demonstrated that treatment with the platelet glycoprotein (GP) IIb/IIIa antagonist abciximab reduced the risk of MI in patients with refractory unstable angina during the 18- to 24-hour period preceding the coronary intervention and during the subsequent balloon angioplasty (Hamm *et al.* 1999, The Capture Investigators 1997). Following these initial trials, there have been other FRISC/FRISC substudies (Venge *et al.* 2002, Venge *et al.* 2003).

In the Platelet Receptor Inhibition for Ischemic Syndrome Management (PRISM) trial, patients who were positive for TnI and were treated with tirofiban (a platelet GP IIb/IIIa inhibitor that markedly reduces platelet aggregation) demonstrated a significant decrease in cardiac events compared to patients negative for TnI. No significant difference in outcomes was found for patients without TnI elevations who were treated with tirofiban (The PRISM study investigators 1998). Heeschen and coworkers further investigated

whether cTnT or cTnI could reliably identify high-risk patients who would benefit from tirofiban (Heeschen *et al.* 1999). In the PRISM-PLUS trial, treatment with tirofiban plus heparin significantly reduced the 7-day incidence of death, myocardial infarction or refractory ischemia versus heparin therapy alone (PRISM-PLUS investigators 1998). The RESTORE (Randomized Efficacy Study of Tirofiban for Outcomes and Restenosis) trial addressed the effect of platelet GP IIb/IIIa blockade with tirofiban on adverse cardiac events in patients with unstable angina or acute myocardial infarction undergoing coronary angioplasty (RESTORE investigators 1997).

In the PURSUIT (Platelet Glycoprotein IIb/IIIa in Unstable Angina: Receptor Suppression Using Integrilin Therapy) trial, the inhibition of platelet GP IIb/IIIa with eptifibatid in patients with acute coronary syndromes was investigated. Inhibition of platelet aggregation with eptifibatid reduced the incidence of the composite end point of death or non-fatal myocardial infarction in patients with acute coronary syndromes who did not have persistent ST-segment elevation. (The PURSUIT Trial investigators 1998).

Since the point-of-care assays for cardiac markers have become available, trials investigating the risk stratification of patients and the evaluation of chest pain patients have been reported. The REACTT (Rapid Evaluation by Assay of Cardiac Troponin T) trial (Phase I) evaluated the qualitative Cardiac T device for the early evaluation of chest pain patients (The REACTION Study investigators 1997). The TIMI 11A trial investigated the utility of the qualitative troponin T device for risk stratification in a series of 597 TIMI patients presenting with unstable angina or non-Q-wave myocardial infarction (Antman *et al.* 1998). The Chest Pain Evaluation by Creatine Kinase-MB, Myoglobin, and Troponin I (CHECKMATE) trial showed that multimarker analysis identifies AMI patients earlier and provides better risk stratification for mortality than a local laboratory-based, single-marker approach (Newby *et al.* 2001).

### ***2.5.2 An ideal myocardial marker***

An ideal cardiac marker should have several characteristics. In order to provide absolute cardiac specificity, the marker must be synthesized and released only by the heart. It should have a high concentration in myocardium after myocardial injury. The size and subcellular distribution of proteins and enzymes will dictate how quickly the protein arrives into the general circulation. Early appearance in blood after myocardial injury facilitates the early diagnosis of injury or infarction and is useful for triaging patients in the emergency department for either hospitalization or discharge. A marker for early AMI diagnosis also requires the development of an assay with a short turnaround time to take full diagnostic advantage (e.g. point-of-care testing from whole blood). A marker with long half-life in blood is useful for diagnosing the few patients who present at hospital late, i.e., after normalization of the earlier markers (CK-MB and myoglobin). (Wu 1998).

The ideal cardiac marker assay should have high analytic specificity. Increased cTnI or cTnT in the circulation reflects heart injury, while normal cTnI and cTnT in the presence of increased CK-MB and total CK reflects non-cardiac injury, most likely skeletal muscle injury. (Apple 1999). cTnI is not expressed in skeletal muscle (Bodor *et al.* 1995). cTnT isoforms expressed in skeletal muscle will not cause false-positive increases in blood if

measured by the second- or third-generation cTnT assay published by Roche (Apple 1999). Analytical interference should be minimized, such as heterophilic antibodies, human anti-mouse antibodies, rheumatoid factors and previously described, so far unknown interfering substances (Dasgupta *et al.* 1999, Eriksson *et al.* 2003, Kricka 1999). The assay must be cost-effective, simple to perform, sufficiently precise and accurate, and the assay should be available on automatic analyzers or point-of-care devices or both (Wu 1998). The marker must be highly sensitive; the marker should detect even small damage (e.g., unstable angina, cardiotoxic or infectious myocarditis). It should be able to differentiate between reversible (due to apoptosis) and irreversible (necrotic) damage. In AMI, the marker should allow the monitoring of reperfusion therapy and the estimation of infarct size and prognosis. (Mair 1997).

Although most cardiac markers currently available do not meet the ideal, several meet many of the criteria. A combination of markers may be necessary.

### ***2.5.3 Subcellular distribution and tissue release of cardiac markers***

Cellular injury can be caused by chemical, microbial and physical agents, but the most important cause is myocardial ischemia. Myocardial ischemia is present whenever the coronary arterial flow fails to provide enough oxygen to meet the demands of the myocardium. The extent of ischemia after the occlusion of a coronary artery depends on the presence of preformed collateral anastomoses. The early release of cardiac markers is influenced by a variety of factors, the most important influence being their intracellular compartmentation. (Mair 1997).

Sudden induction of myocardial ischemia by the occlusion of a major branch of a coronary artery in the heart sets into motion a series of events that culminates in the death of markedly ischemic myocytes. These events include the cessation of contraction to reduce the energy demand, ECG changes as a result of localized hyperpolarization and the onset of anaerobic glycolysis. These changes appear rapidly and simultaneously. If ischemia persists, many affected myocytes become irreversibly injured. Ions leak from damaged cardiomyocytes within seconds, metabolites (e.g., lactate, adenosine, inosine) within minutes and macromolecules (e.g., cardiac cytoplasmic enzymes) within hours. (Jennings 1991, Mair 1997, Mair 1999).

Increases in intracellular calcium occur, and this activates phospholipases, endonucleases or the soluble protein calpain. cTnT and I are particularly susceptible to calpain digestion. Lysosomes are stable within the first 3 to 4 h after the onset of ischemia. The accumulation of osmotically active particles arising from ischemic metabolism within cardiomyocytes creates an osmotic load, and water enters the myocyte as a consequence. The reversible, prelethal phase of myocardial ischemia includes edema, condensed and later swollen mitochondria, disappearance of glycogen granules, chromatin condensation and cytoplasmic blebbing. The early irreversible phase is characterized by a very low content of energy-rich phosphates, a depressed adenine nucleotide pool that consists principally of adenosine monophosphate, cessation of anaerobic glycolysis, low pH and low glycogen content, high inosine and hypoxanthine contents and osmolar loads. The intramyocardial changes during the development of

ischemic myocardial necrosis include cell swelling, mitochondrial damage and sarcolemma damage. (Mair 1997, Mair 1999).

The intracellular compartmentation of a molecule is the most important impact of the rapidity with which it is released after myocardial damage. Soluble, cytosolic proteins are released more rapidly than structurally bound proteins after damage of sarcolemma. In the case of cytosolic proteins (such as myoglobin and fatty-acid-binding protein), only membrane leakage is required. Mitochondrial proteins or proteins located in the nucleus (such as CK-MB) are also released at a later stage of the pathological process. The release of bound pool proteins (structural proteins of the contractile apparatus, such as troponin) requires both a leaky plasma membrane and dissociation and/or degradation of the subcellular structure, which is a slower process, and these processes therefore appear last. (Mair 1999). The other important factors that influence the pattern of appearance of a myocardial protein in blood after AMI are: the molecular weight of the proteins (e.g., myoglobin is a small molecule and enters the vascular system earlier than troponins), the concentration gradient between cardiomyocytes, interstitial space and blood, local blood and lymph flow (Mair 1997). Furthermore, cardiac troponins are released into serum as complex forms (Katrukha *et al.* 1997, Lavigne *et al.* 1996, Wu *et al.* 1998). The marker-protein time courses after AMI are also influenced by their rate of disappearance from blood. Proteins are metabolized in organs with high metabolic rates, such as the liver, pancreas and kidneys and the reticuloendothelial system. Smaller molecules, such as myoglobin, pass through the glomerular membranes. (Mair 1997).

#### **2.5.4 CK and CK-MB**

Creatine kinase is a dimer composed of two subunits, each with a molecular weight (MW) of about 40 000. The subunits (B for brain, and M for muscle) are the products of two distinct structural genes. As mentioned above, there are three different subunits. CK-1 (or CK-BB) predominates in brain, prostate, gut, lung, bladder, uterus, placenta and thyroid, whereas CK-3 (CK-MM) predominates in skeletal and cardiac muscle. CK-2 (CK-MB) is present, to varying degrees, in heart muscle (25% - 46% of CK activity) and also in small amounts in skeletal muscle (<5%). Furthermore, CK-MB exists in two and CK-MM in three isoforms. The isoenzyme CK-Mt is located between the inner and outer membranes of mitochondria, and in the heart it constitutes up to 15% of total CK activity. CK activity may also be found in two macromolecular forms: macro CK types 1 and 2. (Moss & Henderson 1996).

Serum CK activity is elevated in all types of skeletal muscle diseases, except in certain neurogenic muscle diseases, such as myasthenia gravis, multiple sclerosis, poliomyelitis and parkinsonism. CK activity is invariably elevated after myocardial infarction and in cardiac trauma following cardiac surgery, angina pectoris, cardiogenic shock, electrical countershock, tachycardia, myocarditis, congestive heart failure and cardiac surgery. Reye's syndrome may involve up to a 70-fold increase in serum CK activity, indicating the severity of the encephalopathy. Serum CK activity demonstrates an inverse relationship with thyroid activity. (Moss & Henderson 1996).

The improved analytical performance of CK-MB mass assays compared with CK-MB activity assays results in higher diagnostic sensitivity of the marker. CK-MB mass concentration, CK-MM and CK-MB isoform ratios, myoglobin, cTnI and cTnT increase significantly earlier than CK and CK-MB activity and are also significantly and markedly more sensitive on admission. (Mair *et al.* 1995). Abnormal levels of CK-MB mass are found within 3 h from the onset of coronary artery occlusion in up to 50% of patients who develop an AMI, and within 6 h after the symptom onset CK-MB mass concentrations are abnormal in approximately 80 to 100% of patients (Mair 1997). Typically, an increased concentration of CK-MB is detectable in blood at 3-10 h after the onset of symptoms, the time to peak elevation is 12-24 h, and the time to normalization is about 3 days (Goldman *et al.* 2001). A fourfold increase in CK-MB mass at 90 min and a twofold increase in the CK-MM<sub>3</sub>/CK-MM<sub>1</sub> (CK-MM isoforms) ratio at 90 min after the initiation of thrombolytic therapy identifies the AMI patients with complete perfusion of the infarct-related coronary artery with acceptable accuracy (Laperche *et al.* 1995).

The BIOMACS study (Lindahl *et al.* 1995) showed that monitoring of a combination of myoglobin and CK-MB or troponin T will allow the confirmation or exclusion of AMI within 3-6 h in almost all patients.

A major reason for between-methods bias is the lack of assay standardization. The American Association for Clinical Chemistry (AACC) nominated a committee in 1992 to identify and validate a standard creatine kinase MB isoenzyme material to improve the comparability of CK-MB mass assays (Christenson *et al.* 1999). The preliminary preparations of standards for CK-MB were made in 1993 (Vaidya *et al.* 1993). Some years ago, a project of the AACC subcommittee on creatine kinase MB (CK-MB) mass assay standardization led to the identification and characterization of a lyophilized CK-MB candidate reference material (in a recombinant form) (Christenson *et al.* 1999). Some manufacturers recently decided to use the AACC CK-MB material (available from Seradyn, Indianapolis, USA) to recalibrate their CK-MB assays, considering it as the highest-level CK-MB reference material currently available. Panteghini and Pagani compared two commercial CK-MB assays using this reference material as a calibrator, and their results showed that the AACC CK-MB standardization material was unable to harmonize the CK-MB results between these two commercial immunoassays. (Panteghini & Pagani 2004).

### 2.5.5 Myoglobin

Myoglobin is an oxygen-binding heme protein of striated muscles that is rapidly released after muscle damage. Myoglobin facilitates oxygen diffusion in striated muscle fibers and also serves as an oxygen reservoir within the muscle fiber. (Mair 1997). It is a low-molecular weight (17 kDa) protein present in the cytosol (5-10% of all cytoplasmic proteins) of cardiac and skeletal muscle (Mair 1997, Panteghini 1999). It is located close to the sarcolemma, the contractile apparatus and the intracellular membranous or fibrillar structures (Mair 1997).

Myoglobin is markedly more sensitive than CK or CK-MB activities during the first hours after the onset of chest pain (Mair *et al.* 1995). It usually starts to rise within 2-4 h

after the chest pain onset and is detectable in all AMI patients between 6 to 10 h from the chest pain onset (de Winter *et al.* 1995, Mair 1992). It peaks mostly within 12-14 h, and the myoglobin entering the vascular space is normally cleared rapidly by renal catabolism, with a half-life of clearance of about 9 minutes for the parent immunoreactive molecule (Klocke *et al.* 1982). The early sensitivities of myoglobin, CK-MB mass, CK isoform ratios, cTnI and cTnT are roughly equivalent, but determination of CK-MB, cTnT or cTnI is preferred in patients admitted later than 10 to 12 h after the onset of chest pain because the myoglobin and CK isoform ratios may already have returned to the baseline by the time the patient has arrived at the hospital. (Mair *et al.* 1995). However, a combination of myoglobin and CK-MB can rapidly diagnose or exclude MI within only 4 hours after the presentation in an emergency department, and the accuracy is not different in patients without diagnostic ECGs. The application of this strategy could potentially lead to a more rapid intervention in patients with MI, while simultaneously allowing early identification of patients at lower risk (Kontos *et al.* 1997).

About fourfold increase in myoglobin at 90 min after the initiation of thrombolytic therapy identifies the AMI patients with complete perfusion of the infarct-related coronary artery with high accuracy. Myoglobin has been considered the best marker to identify AMI patients with and without reperfusion noninvasively. (Laperche *et al.* 1995).

Myoglobin is not cardiac-specific, and patients with renal failure and skeletal muscle damage may have abnormal concentrations in the absence of AMI (Moss & Henderson 1996). Low myoglobin clearance may indicate a high risk for developing renal failure or may be an early marker for kidney dysfunction. Low myoglobin clearance may be useful in indicating the failure of prophylactic treatment to clear myoglobin. (Wu *et al.* 1994).

Myoglobin is an early marker of perioperative myocardial infarction in patients undergoing elective coronary artery bypass grafting (CABG). Patients with and without infarction can be identified as early as 3 h after aortic unclamping, and because of its short half-life in the circulation, myoglobin also allows the investigator to recognize the time of infarction onset more accurately than other markers. (Mair *et al.* 1993, Mair *et al.* 1997).

The prognostic value of serum myoglobin in patients with non-ST-segment elevation acute coronary syndromes have been investigated in two distinct patient populations, namely the TIMI IIB trial and TACTICS-TIMI 18 trial study populations. A serum concentration of myoglobin above the MI detection threshold ( $> 110 \mu\text{g/L}$ ) was associated with increased risk of six-month mortality, independent of the baseline clinical characteristics, the electrocardiographic changes and the elevation in CK-MB and cTnI. Thus, myoglobin may be a useful addition to the panel of cardiac biomarkers for early risk stratification in acute coronary syndromes (ACS). (De Lemos *et al.* 2002).

As with other cardiac markers, such as CK-MB (Christenson *et al.* 1999), the results of different analytical procedures for myoglobin determination have significant biases due to the lack of assay standardization. One goal of the IFCC Committee on the Standardization of Markers of Cardiac Damage (C-SMCD) was to standardize myoglobin assays (Dati *et al.* 1999). Recently, five candidates of secondary reference materials have been evaluated, and one of them, namely lyophilized candidate material (human heart myoglobin in human serum), was selected as the most suitable secondary reference material (Panteghini *et al.* 2004).

## 2.5.6 Troponin I and T

### 2.5.6.1 Structure of troponin and different forms of troponin

Cardiac contractile and regulatory proteins are among the most abundant proteins in cardiomyocytes. These proteins are highly organized in striated muscles, which leads to the typical striation pattern in histological slice images. The basic structure is the sarcomere, which consists of geometrically arranged thick and thin filaments. The thick filaments appear to be the major element in energy transduction and strength development. They are composed of myosin molecules. (Mair 1997). The thin filament of the muscle cell is composed of actin, tropomyosin and troponin. Actin is actually composed of a double-helical formation of G-actin monomers. Tropomyosin is a long thin molecule, which wraps around the length of the double-helical structure. Troponins are located in the thin filament of the myocyte. (Chapelle 1999).

Troponin is a component of the troponin complex, which is responsible for the regulation of muscle contraction. The troponin complex consists of three different molecules: troponin C (TnC) with a molecular weight of 18 kDa and pI 4.1, cTnI with a molecular weight of 23 kDa and pI 9.9, and cTnT with a molecular weight of 34 kDa and pI 5.1. (Katrukha *et al.* 1999). This complex regulates the force and velocity of muscle contraction by modulating the interaction of actin and myosin (Chapelle 1999). The function of TnT is to bind the other troponins to the tropomyosin filament of myocyte, TnI inhibits the actomyosin ATPase, and troponin C binds to calcium ions. The contraction of skeletal muscle is regulated by  $\text{Ca}^{2+}$  binding to troponin C, which results in an internal reorganization of the interactions within the troponin-tropomyosin complex. Troponin T is necessary for  $\text{Ca}^{2+}$ -dependent inhibition and activation of actomyosin. Troponin T consists of an extended  $\text{NH}_2$ -terminal domain that interacts with tropomyosin and a globular  $\text{COOH}$ -terminal domain that interacts with tropomyosin, troponin I and troponin C. The  $\text{NH}_2$ -terminal region of troponin T activates the actomyosin ATPase in the presence of tropomyosin. The interaction of the globular domain of troponin T with the thin filament blocks ATPase activation in the absence of  $\text{Ca}^{2+}$ , and the  $\text{COOH}$ -terminal region of the globular domain anchors the troponin C-troponin I binary complex to troponin T through direct  $\text{Ca}^{2+}$ -independent interaction with the  $\text{NH}_2$ -terminal region of troponin I. This interaction is required for  $\text{Ca}^{2+}$ -dependent activation of actomyosin ATPase activity. (Malnic *et al.* 1998).

Bodor and coworkers, studying a panel of cTnI-specific Mabs, noted that two of the eight Mabs recognised better the complexed than the free form of the antigen (Bodor *et al.* 1992). Katrukha with the study group noticed in their Mab studies that troponin I is not released into the bloodstream of patients with acute myocardial infarction in a free form (< 10% found in free form) but as an I-C complex (Katrukha *et al.* 1997). Morjana and colleagues also concluded that the serum of AMI patients contains cTnI complexes, along with partially degraded cTnI (Morjana *et al.* 1997). In contrast, Lavigne with coworkers suggested that cTnI occurs predominantly as free subunits, with no evidence of the troponin I-T complex (Lavigne *et al.* 1996). Wu *et al.* in 1998, using commercial cTnT and cTnI assays on a collected fraction, found troponin to be released into blood as a

ternary complex of cTnT-I-C, a binary complex of cTnI-C and free cTnT, with no free cTnI within the limits of the analytical methodologies. All nine cTnI assays recognized each of the troponin I forms (complexed and free). In five of these assays, the relative responses for cTnI were nearly equimolar. Moreover, there was a substantial difference in the absolute concentrations of the results between cTnI assays due to the lack of mass standardization and heterogeneity in the cross-reactivities of antibodies to various troponin I forms. (Wu *et al.* 1998).

It has been concluded that cTnI-TnT complex formation is a very important factor influencing the immunochemical detection of cTnI. For precise cTnI immunodetection in the patient's blood, the antibodies used in the assay should be insensitive to cTnI-TnC binary complex formation. To decrease the discrepancy between the existing assays, it is a matter of great importance to calibrate assays against the standard representing the natural form of the antigen, which is a ternary cTnI-cTnT-TnC complex purified from human cardiac tissue. (Katrukha *et al.* 1999).

In contrast to TnC, TnI and TnT exist in cardiac, slow-twitch and fast-twitch skeletal isoforms, which are encoded by different genes. cTnI has 31 additional amino acids on its N-terminal region, which are not present in the skeletal forms. Significant primary sequence heterogeneity (approximately 40% difference) exists in the similar regions of the TnI molecules in addition to the differences in the N-terminal regions. (Chapelle 1999). TnT is an asymmetric molecule with a globular C-terminal domain. The sequences of the four human cTnT isoforms that are differentially expressed in fetal as well as normal and failing adult hearts have been identified by cloning and sequencing full-length cDNAs. (Anderson *et al.* 1995). Pronounced differences exist in amino acid composition between cardiac and skeletal troponin T (Chapelle 1999). cTnT isoforms have also been described in fetal human skeletal muscle, where there is a developmental down-regulation of cTnT and up-regulation of skeletal isoforms of TnT, which leads to the absence of cTnT in non-diseased adult skeletal muscle. Several cTnT isoforms have been shown to be re-expressed during regeneration in adult rat skeletal muscle after injury or denervation and in generating human skeletal muscle from patients with Duchenne muscular dystrophy and polymyositis, in normal (non-generating) skeletal muscle and in muscle from chronic renal disease patients. The mechanism for the expression of cTnT isoforms in skeletal muscle from renal failure patients is likely to be associated with peripheral myopathy associated with renal disease. (Bodor *et al.* 1995, Ricchiuti *et al.* 1998 and refs. therein). The first generation cTnT enzyme immunoassay suffered from a lack of specificity, but this problem was solved by the second-generation cTnT assay, and both monoclonal antibodies developed for this assay seemed to be without cross-reactivity with skeletal muscle isoforms (Katus *et al.* 1992). The Elecsys® Troponin T third generation assay uses recombinant human cardiac troponin T as a standard material, which enables reproducible and reliable standardization of troponin T assays (Hallermayer *et al.* 1999).

A very important factor for cTnI measurements is the stability of the cTnI molecule. cTnI is known to be a very unstable protein sensitive to proteolytic degradation. In *in vitro* experiments with necrotic human cardiac tissue, cTnI was found to be susceptible to proteolysis, and its degradation leads to the appearance of a wide diversity of proteolytic peptides with different stabilities. The N- and C-terminal regions were rapidly cleaved by proteases, whereas the fragment located between the residues 30 and 110 demonstrated

substantially higher stability, possibly because of its protection by TnC. Therefore, the antibodies selected for cTnI sandwich immunoassays should preferentially recognize epitopes in the region resistant to proteolysis (the stable part of the molecule). cTnI can undergo proteolytic degradation not only in the necrotic cardiomyocytes but also in the bloodstream of patients or in collected blood/serum samples. The detected stability of cTnI in serum also depends on the assay used for cTnI measurement. (Katrukha *et al.* 1998).

Labugger with colleagues in 2000 studied troponin I and T modification detected in serum from patients with acute myocardial infarction. A western blot direct serum analysis protocol was developed that allowed detection of intact cTnI and a spectrum of up to 11 modified products in serum from patients with AMI. To determine whether the protein modification detected in the serum occurred before or after release from the myocardium, human recombinant TnI, recombinant TnI<sub>1-192</sub> or recombinant TnT was added to normal serum. This experiment indicated that some TnI modification products existed in the myocardium. In contrast, human recombinant TnT did not degrade in normal serum. Although recombinant TnT showed no proteolytic susceptibility in normal serum, AMI patients possessed only a small amount of intact cTnT, but 1 major and 2 minor degradation products of cTnT. (Labugger *et al.* 2000).

The cTnI molecule contains two serine residues at the positions 22 and 23, both of which can be phosphorylated *in vivo* by protein kinase A. Phosphorylation changes the structure and conformation of the cTnI molecule and can, consequently, affect the interaction of some antibodies with the antigen. A significant part of the protein in the patient's blood is phosphorylated. cTnI phosphorylation has been found to be more abundant in myocardium from control hearts than in preparations from failing hearts, and this difference derives from the phosphorylation of the cardiac-specific cTnI NH<sub>2</sub>-terminal extension. (Bereznikova *et al.* 1999, Katrukha *et al.* 1999, Labugger *et al.* 2000). The expected effect on myofibril function, i.e. the greater calcium sensitivity of failing heart preparations, has been found in the human heart (Bodor *et al.* 1997).

cTnI has two cysteine residues, Cys 79 and Cys 96 (Vallins *et al.* 1990), which can be oxidised or reduced (Ingraham & Hodges 1988), and this oxidation or reduction changes the structure and conformation of the antigen.

The other factors interfering with the antibody-antigen interaction, such as heparin or ethylenediaminetetra-acetic acid (EDTA), will be discussed later in the section concerning analytical interferences.

### 2.5.6.2 Features and kinetics of troponin

Despite their structural localization, cTnT and cTnI concentrations start to increase in serum within the few first hours following the onset of AMI in a rather similar way as CK-MB, but the sequence of appearance of the three markers in blood may be slightly different in different patients (Chapelle 1999). cTnI increases above the normal levels between 3 and 8 h after the onset of AMI symptoms. Peak serum concentrations are reached after 12 to 24 h. Unlike the CK and CK-MB markers, cTnI levels remain elevated and may be detected for 5 to 10 days following AMI, depending on the extent of

the AMI. About 3 to 6 % of cTnI is located in the cytoplasmic fraction of the cell. Although the release mechanism is not completely understood, the cytoplasmic fraction is thought to be responsible for the early release. As much as 94 to 97 % of cTnI is located in the myofibril-bound fraction and is responsible for the lengthy ongoing release of cTnI. The level of cTnI is thought to reflect the extent of myocardial necrosis. (Adams *et al.* 1994, Mair *et al.* 1996).

In AMI, a biphasic pattern of cTnT release with peaks at about 14 h and 3 to 5 days has been demonstrated. These serum concentration changes of cTnT after myocardial damage are most probably explained by the intracellular compartmentation of this protein. (Mair *et al.* 1991). cTnT is released within 4-12 h after myocardial necrosis, with a peak value of 12-48 h from the onset of symptoms, depending on the duration of ischemia and reperfusion. In the human heart muscle, approximately 6 % of the total myocardial cTnT content is present as a soluble, cytoplasmic pool. (Goldmann *et al.* 2001).

Reperfusion of an infarct-related coronary artery influences the release of cardiac markers in AMI patients. Apple and colleagues examined the early appearance kinetics of CK-MB, myoglobin, cTnT and cTnI in successfully reperfused AMI patients at 120 min of therapy. Their study showed significantly greater increases in concentration changes for cTnI and cTnT than for either CK-MB or myoglobin. (Apple *et al.* 1995). A  $\geq 6.8$ -fold cTnT increase at 90 min after the start of thrombolytic therapy has been found to indicate complete perfusion of the infarct-related coronary artery with acceptable accuracy (Laperche *et al.* 1995). A review of several studies demonstrated that early monitoring of myoglobin, cTnI, cTnT and CK-MB mass provides greater than 80% sensitivity and specificity in detecting reperfusion within 90 minutes following the initiation of therapy (Apple 1999).

After successful reperfusion therapy, cTnI and cTnT can be used as indices of the size of the myocardial infarction. The extent of the  $^{99m}\text{Tc}$ -isonitrile defect on late images reflects the final infarct size. It has been demonstrated that the cTnI release in patients with first-time myocardial infarction also significantly correlated with scintigraphic estimates of myocardial scarring. However, the correlation was lower than that with serum CK-MB. (Mair *et al.* 1995). Another study revealed a significant correlation between cTnT and both the peak CK-MB concentrations and the perfusion defect size at gated single-photon emission computed tomography (SPECT) of myocardial perfusion using  $^{99m}\text{Tc}$  sestamibi (Panteghini *et al.* 2002). Additionally, in a mouse model, plasma cTnT closely correlated with the pathological infarct size (Metzler *et al.* 2002).

### **2.5.7 Detection of minor myocardial damage**

The term “minor myocardial damage” was first introduced by Katus *et al.* and Hamm *et al.* (Katus *et al.* 1991, Hamm *et al.* 1992). They emphasized the importance of minor myocardial damage as one form of ischemic myocardial disease. Serum cTnI has been prospectively compared with CK-MB mass measurements in patients with minor ischemic myocardial injury on the basis of minimal increases of total CK activity and

electrocardiographic or echocardiographic findings. In this study, cTnI was found to be more sensitive (100%) than CK-MB mass (82%) for the detection of myocardial injury in these patients. (Apple *et al.* 1997).

Antman and colleagues studied the prognostic value of cTnI concentration in patients with unstable angina or non-Q-wave myocardial infarction. Mortality rate at 42 days was significantly higher in the 573 patients with measurable cTnI levels ( $\geq 0.4 \mu\text{g/L}$ ) than in the 831 patients with very low, undetectable levels ( $< 0.4 \mu\text{g/L}$ ). There were statistically significant increases in mortality with rising levels of cardiac troponin I ( $p < 0.001$ ). Each  $1 \mu\text{g/L}$  increase in the cTnI level was associated with a significant increase ( $p = 0.03$ ) in the risk ratio for death after adjustment for the baseline characteristics independently predictive of mortality. (Antman *et al.* 1996).

If the objective of the cTnT measurement is a diagnosis of minor myocardial injury (to include AMI), cTnT may be more sensitive and specific than CK-MB. The reason may be that the cut-off concentration of CK-MB is to set a few units above the upper reference limit because it contains a significant skeletal muscle component, and for cTnT and cTnI, selecting a cut-off value at the upper limit of the reference range is not problematic because troponins have very low residual baseline concentrations. On the other hand, the currently used troponin assays cannot meet the appropriate CV% at the upper reference limit, and a higher concentration that meets this imprecision goal must be used as a cut-off for AMI. The use of troponins for detecting evidence of minor ischemia has led to new clinical applications; one of the most important of which is the risk stratification of patients with unstable angina (UA). (Chapelle 1999). Moreover, it has been shown that increased serum TnI could be used for screening and risk assessment in congestive heart failure patients. However, the reported levels of cardiac troponin I both in the reference population and in the heart failure patients were far below the levels currently used to diagnose acute myocardial infarction, to document perioperative myocardial injury, or to detect myocardial injury in critically ill patients. (Missov *et al.* 1997).

### ***2.5.8 Risk stratification of patients with acute coronary syndrome and with unstable angina pectoris (UAP)***

cTnI measurements have been used for risk stratification of non-AMI patients admitted with chest pain. By using odds ratios, it was shown that poor outcomes were significantly more frequent in the high-cTnI group than in the low-cTnI group, which is a substantial improvement over CK-MB implications. (Wu *et al.* 1996). In 1992, Hamm with colleagues showed for the first time that cTnT detection in the circulation may be a useful prognostic indicator in patients with unstable angina. The patients with abnormal cTnT concentrations were more likely to develop AMI or to die during hospitalization than the others. (Hamm *et al.* 1992). Although the initial studies were with cTnT, the literature on cTnI is increasing. The results obtained with cTnI are similar. A cumulative meta-analysis (Wu 2001) of the five studies that have compared cTnT with cTnI for risk stratification showed that cTnI has a slightly higher odds ratio than does cTnT, but the difference is not statistically significant, although cTnT has been found to be a more powerful predictor of

death within 30 days (Christenson *et al.* 1998). However, in the last few years, the assays have been developed further, and they are now more sensitive and precise.

Lindahl and colleagues elucidated the prognostic value of cTnT in a large cohort of patients with unstable coronary artery disease, and they found that the maximum cTnT value obtained during the first 24 h provides independent and important prognostic information. They showed that cTnT levels of  $< 0.06 \mu\text{g/L}$ ,  $0.06 - 0.18 \mu\text{g/L}$  and  $\geq 0.18$  helped to divide the patients into low-, intermediate- and high-risk groups, respectively (AMI cut-off was  $0.06\mu\text{g/L}$ ). (Lindahl *et al.* 1996). Galvani *et al.* studied 106 patients with chest pain and discomfort at rest thought to be due to myocardial ischemia during the 48 hours preceding admission and diagnosed as unstable angina. Their results confirmed that the presence of myocardial damage identified by elevations of cTnI is a potent prognostic indicator in patients with unstable angina. Elevations were associated with a 2.5-fold increase in the relative risk of death or non-fatal MI both at 30 days and at 1 year. (Galvani *et al.* 1997).

Ideally, a laboratory should establish its own cut-off concentration for risk stratification purposes for the troponin assay being used. However, due to the need to determine patients' outcomes at 4 weeks to 6 months after the initial presentation, this is not practical.

### ***2.5.9 Perioperative myocardial injury after cardiac and non-cardiac surgery***

It has been reported that the release patterns of cardiac markers after uncomplicated heart surgery depend on the type of surgery and the circumstances during surgery. Off-pump coronary artery bypass grafting (CABG) did not produce a significant increase of total CK, CK-MB activity, CK-MB mass, cTnT, Abbott AxSYM cTnI or Beckman Access cTnI. In contrast, patients undergoing CABG with cardiopulmonary bypass (CPB) showed significant increases in the tested markers, whereas valve replacement surgery produced the highest concentrations of cardiac markers. (Swaanenburg *et al.* 2001). Mair and colleagues in 1991 were the first to report on the use of cTnT for the detection of perioperative MI (PMI) in 21 patients undergoing CABG (Mair *et al.* 1991). Mair and colleagues were also among the first to report in 1994 the use of measurements of cTnI in 28 individuals who underwent CABG (Mair *et al.* 1994).

The levels of cardiac troponins and other markers tend to rise and fall rapidly during the first 24 h after cardiovascular surgery, and the proposed reference values must thus be reported in relation to the time of aortic cross-clamping. (Adams III 2001). cTnI, but not myoglobin and CK, has been found to serve as a reliable marker for the identification of patients with early graft failure following CABG (Thielman *et al.* 2004). In another study, cTnI was shown to be a more specific and more sensitive marker than CK-MB activity for the evaluation of perioperative myocardial ischemia (Bimmel *et al.* 2003). Nageh with colleagues proved cTnI to be the most sensitive marker in detecting myocardial necrosis following percutaneous coronary intervention, when cTnI, cTnT and CK-MB were compared. All these markers provided similarly reliable prognostic information in predicting major cardiac adverse events. (Nageh *et al.* 2003). cTnT has also been found to

predict short- and long-term morbidity and mortality after CABG or valve repair (Kathiresan *et al.* 2004, Lehrke *et al.* 2004).

In recent years, minimal invasive coronary artery bypass grafting (MICABG) has been introduced. With this new technique, surgery is performed on the beating heart, without extracorporeal circulation. (Cattozzo *et al.* 2001). Serum cTnI determination has been found to serve as an adequate diagnostic tool for the diagnosis of PMI in MICABG (Bonatti *et al.* 1998, Cattozzo *et al.* 2001).

Cardiac morbidity and mortality can occur secondary to acute or chronic ventricular dysfunction, acute or pre-existing coronary artery disease or MI. Cardiac injury can occur due to inadequate myocardial protection during aortic cross-clamping, a long period on the heart-lung machine, post-bypass reperfusion injury, a spasm of the internal mammary artery, direct injury during surgical manipulation and postoperative injury or occlusion. (Adams III 2001).

Myocardial markers may appear in the circulation after percutaneous transluminal coronary angioplasty (PTCA). In uncomplicated cases, the concentrations of cTnI and cTnT usually do not increase above the cut-off values, and on the contrary, complications have involved cTnI elevations (Davis 2003, Genser *et al.* 1997). Increases in post-procedural cardiac troponin T and cardiac troponin I are associated with a greater degree of morbidity and mortality. Increases are more common and more pronounced following a longer duration of balloon inflation time, stenting and side-branch occlusion. Elevated cardiac troponins pre-procedure are a poor prognostic indicator. (Davis 2003).

### ***2.5.10 Myocardial damage after radio frequency (RF) catheter ablation***

After radio frequency (RF) catheter ablation therapy, myocardial proteins generally increase in blood as a result of the injury to the myocardium (Chapelle 1999). After this therapy, all myocardial proteins, including cTnI and cTnT, frequently increase in blood (Mair 1997). There is a relationship between the increase of markers and the radiofrequency cumulated power and application time. RF catheter ablation is the curative treatment of choice for many cardiac arrhythmias. The sensitivity of cTnI for the detection of ablation has been shown to be 92% vs 63% for CK-MB mass. CK activity had a sensitivity of 30% in this study. (Dell Rey *et al.* 1998). Carlsson and colleagues investigated the extent of myocardial injury incurred by the creation of continuous RF current induced linear ablation lesions (ablation of atrial fibrillation, right atrial procedure) in comparison to focal RF lesions (AV node reentry tachycardia, WPW tachycardia). The levels of CK, myoglobin, CK-MB mass, CK-MB activity and cTnT before and 2, 4, 8, 24 and 48 hours after ablation were determined. In focal lesions, only cTnT provided a sensitivity of 50% in the detection of myocardial injury, while in linear lesions, cTnT, CK-MB mass and CK-MB activity seemed suitable for the detection of RF current induced myocardial damage with 100% sensitivity. (Carlsson *et al.* 2001).

## 2.6 Analyses of cardiac markers

### 2.6.1 Analytical interferences

#### 2.6.1.1 Heterophilic antibodies

The effects on immunoassays of heterophilic antibodies (HAs) in plasma and serum have been well documented (Boerman *et al.* 1990, Boscato & Stuart 1988, Butler & Cole 2001, Hennig *et al.* 2000, Kricka *et al.* 1990, Kricka 2000). These antibodies may arise upon exposure to a protein deemed foreign by the individual's immune system, thereby triggering an immune response. Such exposures may occur in a variety of situations. Most commonly, the use of monoclonal mouse antibodies for radioimaging of tumours as well as in the therapeutic treatment of some forms of cancer can lead to the generation of a subset of heterophilic antibodies specific for mouse proteins termed 'human anti-mouse antibodies' (HAMA). HAs can also arise due to incidental exposure to foreign proteins occupationally, as in the case of veterinarians, farm workers and kitchen staff, or to the presence of domestic animals in the home. (Yeo *et al.* 2000). The prevalence of HAs in the general population has been estimated to be as high as 40% (Boscato & Stuart 1988). Typically, these antibodies are of the IgG class and recognize epitopes on the Fc portion of the foreign immunoglobulin, although instances where the epitope is located in the Fab region have been reported (Boerman *et al.* 1990, Turpeinen *et al.* 1995).

Current immunoassays contain non-specific 'blocker' immunoglobulins originating from the same species as the analyte-specific antibodies in an attempt to limit the effect of heterophilic antibodies. Sometimes the addition of blocking agents is not sufficient to neutralize the interfering factor. (Yeo *et al.* 2000). The false-positive cTnI results have been attributed to the HAs using the Abbott AxSYM analyzer (Fitzmaurice *et al.* 1998), but later, in 2000, an 'enhanced' version of the Abbott AxSYM cTnI reagent was designed to greatly reduce or eliminate HA interference, and it has since replaced the original reagents (Yeo *et al.* 2000).

#### 2.6.1.2 Anti-TnI antibodies

Recently, Eriksson and colleagues found in their experiments an interfering factor (IF), which causes negative interference in cTnI immunoassays. It interferes with the binding of antibodies against epitopes in the central part of the cTnI molecule in some assays. Since the recommendation has been to utilize mid-fragment epitopes in cTnI immunoassays, many commercial cTnI assays are likely to be disturbed by this factor. (Eriksson *et al.* 2003). The presence of IF has been demonstrated by gel filtration, where samples with low recovery of cTnI showed that the approximate molecular mass of IF was 50-200 kDa, and that the cTnI elution profiles of samples containing IF shifted towards higher molecular mass compared to samples with less IF. Two-site

immunoassays using combinations of 16 monoclonal and 2 polyclonal cTnI antibodies and 1 monoclonal troponin C antibody were used to measure the analytical recovery of cTnI or cTnI-TnC in serum samples. (Eriksson *et al.* 2004).

### 2.6.1.3 Anticoagulants

cTnI has a very high positive charge (pI 9.87) and thus readily forms complexes with negatively charged molecules, such as heparin. The interaction between cTnI and heparin can interfere with the antibody-antigen interaction. (Katrukha *et al.* 1999). In some assays, different cTnI concentrations have been observed in heparinised plasma samples and serum samples (Gerhardt *et al.* 2000, Stiegler *et al.* 2000). In both studies, the cTnI and cTnT concentrations were lower in heparin plasma than in serum samples. In another study, contrariwise, there were no statistical differences between the serum and heparinized plasma results for myoglobin and cTnI (Pagani *et al.* 2000).

Wu and coworkers examined the release of troponin subunits into the blood of patients after AMI using a gel filtration column, and the serum samples were also examined after incubation with EDTA and heparin. EDTA broke up troponin complexes into individual subunits. When the serum sample was incubated with EDTA, the results showed disappearance of the ternary complex for both cTnT and cTnI. The absolute concentrations of each band were also markedly decreased. When the samples were incubated with heparin, no effect was found on the assays tested. (Wu *et al.* 1998). EDTA can cause discrepancies, especially in assays which use antibodies that differentially recognize free and complexed forms. EDTA can cause partial unfolding of the calcium-dependent troponin complex. (Segura *et al.* 1999).

One source of concern for cardiac marker assays is the frequency of false-positive results due to the presence of fibrin clots in serum. Fibrin strands and clots form when non-anticoagulated blood is centrifuged, and the serum is removed before complete retraction of the clot. Because cardiac patients are routinely given intravenous heparin, *in vitro* clotting times are prolonged. The presence of fibrin strands can produce a false-positive result through incomplete separation of analyte-bound signal antibodies from labeled antibodies free of the analyte. (Wu 2001).

Although the use of plasma instead of serum was initially advocated as a means of shortening the overall turnaround time for reporting a result, each assay needs to be validated for both serum and plasma.

### 2.6.1.4 Rheumatoid factors

Rheumatoid factors are another interfering mechanism. Gamma globulins with measurable anti-gamma globulin activity have long been called rheumatoid factors because of their presence in the serum samples of more than 80% of patients with rheumatoid arthritis. These factors also are found in other diseases, such as hypergammaglobulinemia, and in some elderly persons. Antinuclear antibody is found in some patients with rheumatoid arthritis. The false-positive cTnI results have been

attributed to the presence of rheumatoid factors using the Abbott AxSYM analyzer. This interference could be eliminated by using a polyclonal antiserum against rheumatoid factors (rheumatoid factor blocking agent). This reagent is commercially available. None of the specimens showed detectable cTnT (Elecsys 2100, Roche Diagnostics GmbH, Mannheim, Germany) in this study, and the concentrations of total CK (Roche Diagnostics GmbH, Mannheim, Germany) and CK-MB (Abbott AxSYM) were within normal ranges. (Dasgupta *et al.* 1999).

### 2.6.1.5 Other interferences

There may be many potential sources of interference from the sample. In plasma samples, in addition to the factors mentioned above, complement, polyanions, autoantibodies, drugs and metabolites may cause interference in immunoassays. In serum and whole blood samples, autoantibodies, drugs and metabolites as well as chromogens and enzymes producing active oxygen species, respectively, can interfere. (Wood 1991).

## 2.7 Elevations of troponins without overt ischemic heart disease

Previously, Allan S. Jaffe reviewed and listed quite comprehensively the situations of which there were reports, either published or anecdotally communicated to the author, containing substantial documentation on the nature of the elevation of troponins. Elevations can be due to analytical problem or to cardiac injury of one sort or another. In most cases, there is no proof of cardiac cell death. Some of these elevations have prognostic value. (Jaffe 2001).

In addition to the acute forms of ischemic heart disease, there are many other clinical conditions that may cause necrosis of cardiac tissues: trauma (including contusion, ablation, pacing, implantable cardioverter-defibrillator firings, including atrial defibrillators, cardioversion, endomyocardial biopsy, cardiac surgery), congestive heart failure (acute and chronic), hypertension and hypotension (often with arrhythmias). Patients who undergo major uncardiac surgery and are well may have elevated troponins during the postoperative period. Numerous studies have shown that cTnI and cTnT can be increased in the serum and plasma of renal failure and hemodialysis patients. It is thought that cTnT elevations are more common than elevations of cTnI because cTnI may be removed during dialysis. (Jaffe 2001). However, the second-generation cTnT assays have not yielded positive results in renal failure patients (Ricchiuti *et al.* 1998). Pathological studies have provided evidence of cardiac damage in patients with renal failure (Ooi 2001). In end-stage renal failure, cTnT and cTnI may be increased in serum without any other signs of acute myocardial damage, and dialysis alters the measured troponins (Wayand *et al.* 2000). At any rate, changes in the plasma levels of cardiac troponins are common in hemodialysis patients in the absence of coronary heart disease (Chapelle *et al.* 2002, Fehr *et al.* 2003, Freda *et al.* 2002, Katus *et al.* 1995) and may have prognostic value (Beciani *et al.* 2003, Choy *et al.* 2003, Goicoechea *et al.* 2004).

Primary myocardial disease may cause troponin elevations, such as pericarditis, myocarditis and amyloidosis, sarcoidosis or rhabdomyolysis in the heart (Bachmaier *et al.* 1995, Franz *et al.* 1996, Jaffe 2001, Smith *et al.* 1997). Cardiac troponins are biological markers of left ventricular dysfunction in septic shock (ver Elst *et al.* 2000). Burns have been associated with troponin elevations because of the release of toxic material (Jaffe 2001).

Critically ill patients (especially ones with diabetes) and patients with hypothyroidism, acute neurological disease (including cerebrovascular accidents, subarachnoid bleeds), transplant vasculopathy and vital exhaustion may have elevated troponins (Jaffe 2001). Certain drugs, such as anthracyclines, 5-fluorouracil, sympathomimetic agents, theophylline, cocaine, ethanol and certain animal toxins (scorpion envenomization in children) may be cardiotoxic (Herman & Ferrans 2001). In addition, cTnI or cTnT concentrations may be elevated after extreme exercise, such as a triathlon competition (Cleave *et al.* 2001, Rifai *et al.* 1999, Shave *et al.* 2004, Whyte *et al.* 2000). However, there have also been opposite results of cTnI within the normal range and increased CK concentrations following an ironman distance triathlon (La Gerche *et al.* 2004).

## 2.8 C-reactive protein

CRP is secreted only by hepatocytes, where its synthesis is regulated by cytokines, of which interleukin-6 is the most important. *De novo* hepatic synthesis starts very rapidly after a single stimulus, and the serum concentrations rise above 5 mg/L by about 6 hours and peak around 48 hours. The plasma half-life of CRP is about 19 hours. Human CRP binds with maximum affinity to phosphocholine residues, but it also binds to a variety of other autologous and extrinsic ligands and aggregates or precipitates the cellular, particulate or molecular structures bearing these ligands. Autologous ligands include native and modified plasma lipoproteins, damaged cell membranes, a number of different phospholipids and related compounds, small nuclear ribonucleoprotein particles and apoptotic cells. Extrinsic ligands include many glycan, phospholipid and other constituents of micro-organisms, such as capsular and somatic components of bacteria, fungi and parasites as well as plant products. (Pepys & Hirschfield and refs. therein 2003). The two major biological activities of CRP have been well defined: first, it has significant activation properties, particularly to activate the complement system and to bind to and modulate the function of phagocytic leukocytes. Second, it is able to bind several biological substrates that are widely distributed. (Chenillot *et al.* 2000).

The single copy of the human CRP gene is located on the proximal arm of chromosome 1 (Kilpatrick & Volanakis 1991). It spans approximately 2.5 kbp of DNA and is located 7.7 kbp upstream of a CRP pseudogene. The CRP protein consists of five identical subunits, which aggregate to form a ring-shaped molecule. CRP belongs to a family of pentameric proteins known as pentraxins. The CRP protein was first described in 1930 by Tillet and Francis. The name is derived from the fact that the protein reacted with the somatic C polysaccharide of *Streptococcus pneumoniae*. Increased concentrations of CRP may, by activating the complement system, contribute to inflammation and enhance tissue damage. Failure of the normal or appropriate CRP

response may also have deleterious effects. The main role of CRP is to provide for enhanced clearance of inappropriate materials from the plasma, regardless of whether these are of extrinsic origin, such as micro-organisms and their products, or autologous products of cell damage and death, and it protects against autoimmunity (Bachofen 1991 and refs. therein, Ledue & Rifai 2003, Pepys & Baltz 1983, Pepys & Hirschfield and refs. therein 2003, Szalai 2004). CRP is likely to have important host defence, scavenging and metabolic functions through its capacity for calcium-dependent binding to exogenous and autologous molecules containing phosphocholine (PC) and then activating the classical complement pathway. The X-ray structures of fully calcified CRP, in the presence and absence of bound PC, have revealed that, although the subunit  $\beta$ -sheet jellyroll fold is very similar to that of the homologous pentameric protein serum amyloid P component, each subunit is tipped towards the fivefold axis. PC is bound in a shallow surface pocket on each subunit, interacting with the two protein-bound calcium ions via the phosphate group and with Glu81 via the choline moiety. There is also a hydrophobic pocket adjacent to the ligand. (Thompson *et al.* 1999).

Ledue and Rifai have reviewed the sources of variability in CRP measurement. The variables may be preanalytic or analytic. Preanalytic physiological variations may be due to race, age, sex, season, biological variation, lifestyle (exercise, smoking, obesity, alcohol, anti-inflammatory drugs, hormone therapy), pregnancy and altitude. There are factors in specimen collection that may influence the CRP results, such as fasting, time of collection, specimen type and duration and temperature of storage. Among analytical variables, the laboratory methodology, the detection limit of the method, precision, antigen excess, matrix effects and the reference materials used in the standardization of the method may cause variation in the results. (Ledue & Rifai 2003).

In serum, the concentration of CRP can increase 1000-fold with acute inflammatory events, such as infection, trauma and surgery. Persistently increased CRP may occur in chronic inflammatory disorders, including autoimmune diseases, and malignancies. Mild inflammation and viral infections generally cause CRP concentrations to increase to 10-50 mg/L. Automatic methods with detection limits of 3-5 mg/L are routinely available in the clinical laboratory for this purpose.

### ***2.8.1 Highly sensitive CRP protein methods (hs-CRP)***

There have been several studies where the clinical and analytical performance of high-sensitivity CRP assays have been evaluated (Rifai *et al.* 1999, Roberts *et al.* 2000, Roberts *et al.* 2001, Rothkrantz-Kos *et al.* 2003, Tarkkinen *et al.* 2002, McDade *et al.* 2004). Initially, highly sensitivity methods were based on the ELISA methodology. This methodology is primarily for research and is not ideal for routine use in highly automated clinical laboratories. Traditional CRP methods in the clinical laboratory lack the desired sensitivity and are therefore unsuitable for the purpose of predicting the future risk of coronary events in apparently healthy individuals. (Roberts *et al.* 2001). A latex-enhanced immunoluminonephelometric hs-CRP method, a time-resolved immunofluorometric method and several automated immunoturbidimetric and immunoluminometric methods have been recently developed (Ledue *et al.* 1998, Rifai *et al.* 1999, Roberts *et al.* 2001,

Tarkkinen *et al.* 2002). In many methodological evaluations, the FDA-approved Dade Behring method has been used as the reference method.

The criteria for both accuracy and precision need to be clearly defined. The hs-CRP results will be interpreted in quartiles or quintiles for risk assessment (Rifai & Ridker 2001). Therefore, hs-CRP assays will need to be standardized for concentrations of 0.2–10 mg/L (Roberts *et al.* 2001). Additionally, the imprecision of hs-CRP assay should be <10% at a concentration of 0.2 mg/L (Roberts *et al.* 2000). Thus, ‘high sensitivity’ refers simply to the lower detection limit of the assay procedures compared to conventional CRP methods.

There are differences in the measured concentrations between the methods. Differences in standardization materials or the use of suboptimal value transfer protocols are likely to explain the differences between the methods. (Roberts *et al.* 2001).

### ***2.8.2 Hs-CRP as a prognostic indicator in acute coronary syndromes and as a predictor of future coronary events***

The initial demonstrations of the prognostic value of CRP in cardiovascular disease were derived from patients with acute coronary ischemia or unstable angina, in whom minor elevations of CRP were found to have short-term predictive value (Biasucci 1999, Haverkate *et al.* 1997, Liuzzo *et al.* 1994, Ridker 2001). Recent evidence has demonstrated that increases in CRP concentrations within the reference interval are associated with future coronary events in apparently healthy men and women (Koenig *et al.* 1999, Ridker *et al.* 1997, Ridker *et al.* 1998, Ridker *et al.* 2000). It has been shown that plasma concentrations of CRP, SAA and IL-6 are increased in men with angiographically documented coronary heart disease (CHD). In this study, the concentrations of these markers were increased in patients with CHD but failed to correlate with the severity of coronary disease. The authors concluded that these markers might reflect the diffuse atherosclerotic process in the vascular system rather than the degree of localized obstruction from coronary lesions. (Rifai *et al.* 1999). Koukkunen with colleagues investigated the prognostic value of CRP, fibrinogen, IL-6 and tumour necrosis factor-alpha (TNF-alpha) in patients with UAP, including factor analysis to assess their joint effects. This study comprised 263 consecutive patients (159 men, 104 women) with UAP. Coronary mortality during the median follow-up time of 17 months was 6-fold in the highest tertile for CRP and IL-6 and 3.5-fold in the highest tertile for fibrinogen and TNF-alpha compared to the respective combined lower tertiles. Factor analysis produced two different factors, an inflammation factor (CRP, fibrinogen, IL-6) and an injury factor (cTnT, Ck-MB mass, TNF-alpha), which were independent predictors of the risk of coronary death and major coronary events. (Koukkunen *et al.* 2001).

There have been several studies in which the availability of hs-CRP assay for CRP has enabled the authors to demonstrate a predictive relationship between increased CRP production and future atherothrombotic events, including coronary events, stroke and progression of peripheral arterial disease (Pepys & Hirschfield and refs. therein 2003). In the TIMI 11A study, hs-CRP helped to identify the patients with negative cTnT who were

at an increased risk of mortality (Morrow *et al.* 1998). de Winter and coworkers showed that hs-CRP concentrations  $>5$  mg/L at admission in patients with non-ST-elevation ACS were associated with an increased risk of future cardiac events within 6 months, regardless of cTnI values (de Winter *et al.* 1999). One year later, de Winter and colleagues demonstrated that increased CRP as an early independent risk indicator should be measured as soon as possible after the onset of symptoms, whereas increased cTnI is most reliable at 12 or more hours after the onset of symptoms (de Winter *et al.* 2000). A few years ago, Sabatine and coworkers in a multicentre study hypothesized that simultaneous assessment of cTnI, CRP and B-type natriuretic peptide (BNP) would provide complementary information and enable clinicians to stratify the risk more effectively among patients with ACS. They tested the hypothesis in patients from trial namely the Orbofiban in the Patients with Unstable Coronary Syndromes (OPU)-TIMI 16 trial and then validated their findings in the Treat Angina with Aggrastat and Determine Cost of Therapy with an Invasive or Conservative Strategy (TACTICS)-TIMI 18 trial. They concluded that cTnI, CRP and BNP each provided unique prognostic information in patients with ASC. This multimaker strategy, which categorized patients based on the number of elevated biomarkers at presentation, allowed risk stratification over a broad range of short- and long-term major cardiac events. (Sabatine *et al.* 2002).

The reduction in the risk of the first myocardial infarction associated with the use of aspirin (Ridker *et al.* 1997) or statins (Ridker *et al.* 2001) appears to be connected to the level of CRP.

### **3 Outlines of the present study**

The availability of a simple, rapid test using whole blood to reduce the target turnaround time (time from venipuncture to test result) could improve the management of patients presenting with chest pain. Several studies have shown that high-sensitivity C-reactive protein assays can be used to predict the risk of future atherothrombotic events, including coronary events, stroke and progression of peripheral arterial disease, in apparently healthy subjects. The recently introduced technology of the Innotracc Aio! analyzer makes it possible to measure rapidly both the cardiac markers and the highly sensitive CRP using whole blood, serum or heparin samples, which reduces the TAT of cardiac marker analysis.

The specific aims of the study were:

- To evaluate the analytical performance of time-resolved fluorometry-based Innotracc Aio! CK-MB, myoglobin and 1<sup>st</sup> generations troponin I assays
- To evaluate the analytical and clinical performance of Innotracc Aio! 2<sup>nd</sup> generation troponin I assay
- To validate the use of different sample matrices on the Abbott AxSYM and Innotracc Aio! immunoassay systems
- To evaluate the analytical performance of Innotracc Aio! ultrasensitive C-reactive protein assay
- To study the diagnostic value of CRP in patients with acute atrial fibrillation

## **4 Materials and methods**

For detailed descriptions of the materials and methods, see the original articles I-V.

### **4.1 Samples**

Lithium heparin plasma samples (Terumo Venoject Lithium Heparin, Cat. No. VP 050SHL, Terumo Europe N.V. Leuven, Belgium, with a total heparin concentration of 15.9 IU/ml of whole blood) or serum samples (Terumo Venoject II, Cat. No. VP-050SPZ, Terumo Europe N.V. Leuven, Belgium) for cardiac marker and CRP determinations were randomly collected from routine samples and from apparently healthy persons. For the comparisons of whole blood, serum and plasma samples, an additional lithium-heparin sample, serum sample or K2-EDTA sample (Beckton Dickinson Vacutainer, Cat. no. 388010, Beckton Dickinson Systems, Plymouth, UK) was taken. In order to obtain lithium-heparin plasma, serum or EDTA plasma samples, the whole blood samples were centrifuged at 2000 g for 15 minutes. The supernatants were either analyzed immediately, stored at 4°C or stored long-term at -20°C (maximum about 2 weeks). Before analysis, the frozen samples were thawed at room temperature, mixed and centrifuged to remove any particulate material. All the patients, except the patients whose samples were randomly collected from routine samples, and volunteers granted permission for blood samples to be collected, and the local hospital ethical committee approved the whole study.

### **4.2 Patients**

The patients and the normal reference groups were selected as shown in Table 1.

*Table 1. Study population in the studies I-V.*

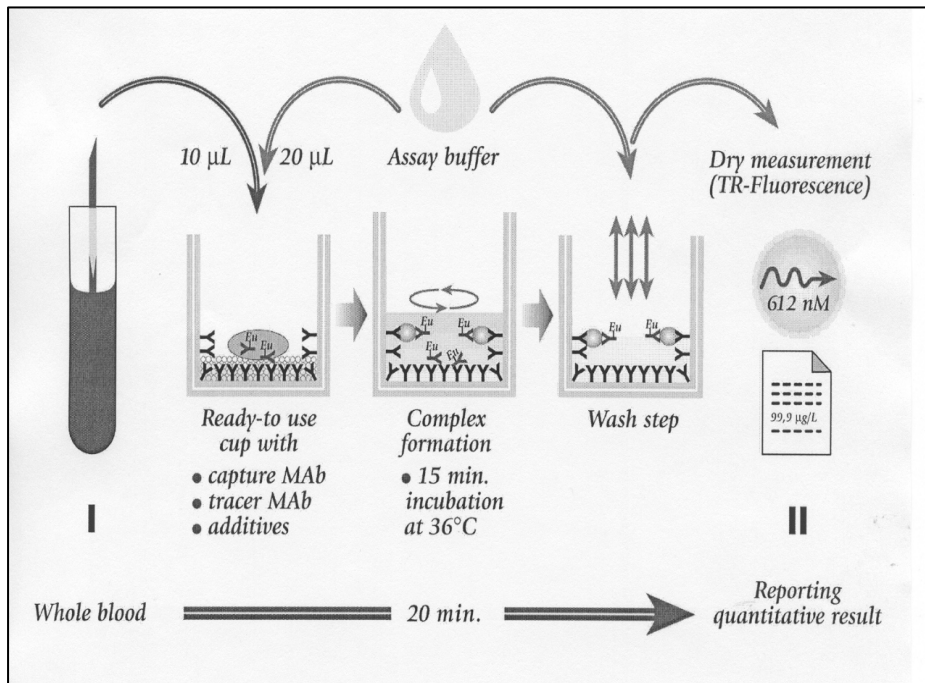
Study	Study population (n= persons approximately)
Study I	
Sample material comparison	Randomly selected patients with chest pain. (n=79)
Study II	
Analytical evaluation.	Randomly selected patients with different cardiac marker concentrations measured by Abbott AxSYM. (n= 280)
Study III	
Analytical evaluation.	Randomly selected patients with different CRP concentrations measured by Cobas Integra. (n= 250)
CRP concentrations at selected percentiles	Apparently healthy persons recruited based an oral health interview and echocardiography. (n= 172)
Study IV	
Method and sample material comparisons	Samples for Innotrac Aio! determinations were randomly collected from routine samples of chest pain patients and according to the Abbott AxSYM cTnI results. (n= 256)
99 <sup>th</sup> percentile reference limit	Apparently healthy persons recruited based an oral health interview and/or echocardiography. (n= 120)
Influence of hemodialysis	Renal failure patients in the Division of Nephrology (Oulu University Hospital). (n=48)
Influence of rheumatoid factor	Patients suffering from Rheumatoid Arthritis in the Division of Rheumatology (Oulu University Hospital). (n=15)
Sample stability	Patients suffering from acute MI (different durations of chest pain) or cardiac surgery (Division of Critical Care, Internal Medicine) (n=10)
cTnI profiles of patients	Patients for this study were selected according to their clinical diagnosis (cardiac surgery, acute MI, etc.) and Abbott AxSYM cTnI results. (n=38)
Study V:	The acute atrial fibrillation group consisted of patients with acute AF admitted into the emergency room of Oulu University Hospital. The paroxysmal AF group consisted of patients with lone paroxysmal AF or paroxysmal AF associated with mild hypertension. For details, see the original manuscript of study V. (n=56)

### 4.3 Analytical methods

Analyses were done using the Innotrac Aio!<sup>TM</sup> analyzer (Innotrac Diagnostics Oy, Turku, Finland), and comparative analyses were undertaken with the Abbott AxSYM® (Abbott

Laboratories, Diagnostic Division, Abbott Park, Illinois, USA), Beckman Access® (Beckman Coulter, Ins., Chaska Minnesota, USA), Hitachi 911™ (Roche Diagnostics GmbH, Mannheim, Germany) and Cobas Integra™ (Roche Diagnostics GmbH, Mannheim, Germany). The Hct of the whole blood samples was determined by Abbott Cell-Dyn® 4000 (Abbott Laboratories, Diagnostic Division, Abbott Park, Illinois, USA). All the analyses were done in Oulu University Hospital Laboratory, except the analyses using the Beckman system, which were done in Kuusamo Hospital Laboratory.

The Innotracs Aio! analyzer is a fully automated random-access immunoanalyzer based on a universal all-in-one dry-reagent concept (Fig. 3). All analyte-specific reagents are dry-coated on to the bottom of the analyte cups, which are packed into analyte pens (12 cups/pen), and only the addition of the sample and the generic assay buffer is required. To perform the assay, the sample and the buffer are, under continuous shaking, washed and dried, after which the signal of intrinsically fluorescent Europium lanthanide chelate is read from the dry surface. In the usCRP assay, prior to sampling, the Innotracs Aio! analyzer automatically dilutes the sample 1:20. The quantitative results are available within about 20 minutes after the addition of the sample. Calibration of the assays is done whenever a new kit lot is taken into use. Each kit box contains a bar code for reading the factory-defined calibration data, which are used as a lot-specific reference curve. Calibration is performed automatically when the lot changes.



**Fig. 3. Innotracs Aio! assay process**

## 4.4 Imprecision

In study I, within-assay precision was determined using commercial controls (Liquicheck Cardiac Markers levels 1, 2, 3 and LT BioRad Laboratories, Irvine, California, USA) and two human serum pools with different cardiac marker concentrations (own material). The controls and pools were measured single at every 2 h on five separate days. Inter-assay precision was determined by measuring the commercial controls and two human serum pools single twice a day on 21 separate days.

In the evaluation of the 2<sup>nd</sup> generation cTnI assay (IV), three whole-blood and 3 plasma samples with variable cTnI concentrations were used to test within-assay precision (n=20). Commercial controls (Quantimetrix Cardiasure, levels 1, 2 and 3, Quantimetrix Corporation, Redondo Beach, California, USA) and a low serum pool were used to test inter-assay precision. Inter-assay precision was determined by analyzing duplicate control samples twice a day on 10 separate days (n=40).

Patient plasma samples with varying CRP concentrations were used to test within-assay precision (n=20) in study III. Commercial controls (BioRad Liquicheck Control, levels 1, 2, BioRad Laboratories, Irvine, California, USA and Labquality control, myocardial marker CRP low, Labquality Oy, Helsinki, Finland), two lithium-heparin plasma pools and four serum pools were used in the inter-assay precision study. Inter-assay precision was determined by analyzing control samples twice a day on 10 separate days (n=20).

## 4.5 Linearity

The linearity of dilution was investigated by serial dilution of five samples of known cTnI, CK-MB, myoglobin or CRP concentrations. All the samples were diluted with the instrument buffer solution. To evaluate the effect of the matrix used as a diluent in the linearity assessment of study III, samples for linearity were also prepared from two serum pools.

## 4.6 Sample material and comparison of methods

Correlation studies on methods and different sample materials were performed. In study II, comparisons of the Abbott AxSYM and Innotracc Aio! 1<sup>st</sup> generation cTnI, myoglobin and CK-MB were carried out with a total of 136 plasma samples. EDTA whole-blood and lithium-heparin plasma comparisons were carried out with 88 cTnI, 94 CK-MB and 93 myoglobin samples. The lithium-heparin whole blood and plasma comparisons were carried out with 136 patient samples.

Two comparative assessments were included in study IV. The first study of the 2<sup>nd</sup> generation Innotracc Aio! cTnI assay, Beckman Access AccuTnI and Abbott AxSYM cTnI were carried out with a set of 91 lithium-heparin plasma samples. The second study of the Innotracc Aio! 1<sup>st</sup> generation, Innotracc Aio! 2<sup>nd</sup> generation and Abbott AxSYM assays were

carried out with a total of 97 lithium-heparin plasma samples. Comparisons of lithium-heparin whole blood, lithium-heparin plasma and serum were carried out with 38 patient samples using the Innotracs Aio! analyzer.

In study I, 79 paired serum and lithium-heparin plasma samples for Abbott AxSYM CK-MB determinations and 55 serum and lithium-heparin plasma samples for Abbott AxSYM cTnI determinations were used.

Comparative analyses of Innotracs Aio! usCRP and Cobas Integra CRP assays (III) were done with a total of 96 lithium-heparin plasma samples and 76 serum samples. Comparative analyses of Innotracs Aio! usCRP and Hitachi CRP (Latex) HS assays were carried out with a total of 111 serum samples. Comparisons of EDTA whole blood and EDTA plasma were carried out with 29 patient samples. To compare the concordance between Innotracs Aio! usCRP, Cobas Integra, Hitachi 911 and Dade Behring assay (Dade Behring, ref.std, BNII), three commercial controls (Labquality Low CRP, Bio-Rad Immunology control level 1 and 2) and one calibrator (c.f.a.s. proteins, Roche Diagnostics, as a calibrator in Cobas Integra hs-CRP assay and Hitachi CRP latex HS assay) were assayed for CRP. The corresponding Dade BNII values were taken from the package inserts. Each sample was run in duplicate in one analytical run.

#### **4.7 Carry-over**

Carry-over was determined using standard procedures, where triplicate measurements of a sample with a high concentration are followed by triplicate analyses of a sample with a low concentration. Carry-over determinations were done in the studies II and III, and the concentrations were 0.18 and 110 µg/L for cTnI, 43 and 539 µg/L for myoglobin, 2.8 and 352 µg/L for CK-MB and 0.47 and 55 mg/L for CRP.

#### **4.8 Analytical detection limit, limit of quantification, 10% CV and functional sensitivity**

To determine the lowest measurable concentrations of the Innotracs Aio! cardiac markers (II and IV) and usCRP (III) that could be distinguished from zero, 20 replicates of a zero calibrator were analyzed. The detection limits were calculated for each analytical run by determining the mean concentration plus 3SD. Functional sensitivity and the concentration giving a 10% CV of the 2<sup>nd</sup> generation cTnI assay (IV) were determined by analyzing lithium-heparin plasma pools serially diluted with instrument buffer 1-2 times per day in duplicate for 10 days.

To determine the limit of quantification of usCRP assay (III), samples for linearity were also prepared from two serum pools. The low pool was prepared by combining randomly collected samples from blood donors giving a target usCRP concentration in the lowest quartile, and the high pool was prepared using samples from blood donors in the highest quartile (about 7 mg/L). The high pool was diluted with the low pool to the following final percentages out of the high pool: 100%, 75%, 50%, 30%, 20%, 10%, 5%,

2.5% and 0%. The samples were assayed in duplicate in one analytical run. An allowable systematic error limit of 10% was chosen for the linearity assessment, and the limit of quantification was the lowest measured concentration that fulfilled this criterion.

The functional sensitivities of the usCRP, Cobas Integra (Latex) CRP and Hitachi CRP (Latex) HS assays (III) were defined as the CRP concentration corresponding to a 20% CV.

## 4.9 Sample stability

In study II, to evaluate the stability of the samples at room temperature, 8 plasma samples with different cTnI and myoglobin values and four samples for CK-MB were determined immediately after the routine analysis by Abbott AxSYM. All samples were then kept at room temperature and analyzed four times within 8 h.

A more comprehensive sample stability test was used in study IV. The sample stability studies were made with lithium-heparin whole blood, lithium-heparin plasma, EDTA whole blood, EDTA plasma and serum samples. Samples were taken from a total of 10 patients suffering from acute MI or undergoing cardiac surgery. The samples were divided into three tubes. The first measurements were made about 30 min after the venipuncture. The cTnI concentrations recorded from the samples kept at +4°C and +20°C were measured at 0h, 2h, 3h, 5h, 8h and 24h. Additionally, sample stability at +20°C was determined at 0.5h and 2.5h after the first measurement. The stabilities of the serum, lithium-heparin plasma and EDTA plasma samples were also determined at -20°C. These samples were kept frozen for at least 24 hours, and the cTnI concentrations were measured after three freeze-thaw cycles. Any particulate material was removed by centrifugation.

## 4.10 Reference limits

Samples for the 99<sup>th</sup> percentile determination were collected from an apparently healthy reference group (IV). cTnI determinations were carried out using a total of 71 EDTA whole blood, 69 EDTA plasma, 120 lithium-heparin plasma and 72 serum samples.

Samples for the determination of Innotracc Aio! usCRP concentrations at selected percentiles and quartiles were collected from healthy adult volunteers (III). Of these, 91 were female and 81 were men. A total of 111 serum samples from apparently healthy subjects, including 68 women and 43 men, were used for the determination of CRP using Hitachi CRP (Latex) HS assay. In both studies, the participants had an oral health interview and echocardiography or only an oral interview to exclude any current or previous coronary artery disease, chronic disease, acute infection or cardiac-related medical condition or other permanent medication. An oral questionnaire also included a question on smoking habits. No other information regarding non-cardiac illnesses or diseases was obtained.

## 4.11 Clinical studies

In study IV, cTnI concentrations were determined on admission and at different time points after admission to determine the cTnI profiles of patients suffering from acute MI and other cardiac events. In the first experiment the cTnI profiles were compared between Innorac Aio!, Beckman Access and Abbott AxSYM, and in the second experiment comparisons were made between Innorac Aio! and Abbott AxSYM. The total of 7 patients suffering from acute MI, 5 with CABG, 1 with myocarditis and 2 patients without a diagnosis of AMI were recruited into the first study. The second study consisted of the cTnI profiles of 12 patients with AMI, 9 patients with CABG, 1 with bypass surgery and 1 with cardiomyopathy.

To determine the influence of hemodialysis and rheumatoid factors, the total of 48 chronic renal failure patients before and after hemodialysis were examined for cTnI (IV). The time between the methods was 4-5 hours. cTnI concentrations in serum samples were also determined in 15 patients with positive rheumatoid factor (determined by Hitachi 911 Rheumatoid Factor assay) but with no indication of myocardial infarction. cTnI was determined with the Innorac Aio! and Abbott AxSYM analyzers in both studies.

In study V, a total of 32 healthy volunteers, 31 patients with paroxysmal atrial fibrillation and 25 patients with acute atrial fibrillation were recruited. A comparison of the Innorac Aio! usCRP and Cobas Integra CRP assays was done using plasma samples from these volunteers and patients to compare the new method to the commonly used CRP method.

In order to investigate the application of CRP assay in a group of patients with acute atrial fibrillation, plasma concentrations of CRP were measured in 25 patients admitted into an emergency room due to acute atrial fibrillation, 31 patients with previously documented paroxysmal atrial fibrillation and 32 control subjects without any signs or symptoms of cardiac disease (V). Echocardiography was performed on all subjects. Blood samples were collected during normal sinus rhythm. CRP determinations were made using Cobas Integra latex CRP and Innorac Aio! usCRP assays.

## 4.12 Statistic

Deming regression analysis and plots of differences among the evaluated methods were used in the correlation studies (I, II, III, IV). In study V, all data were expressed as mean with standard deviation (SD), unless otherwise indicated. Statistical analyses were performed with the SPSS for Windows software package (SPSS 10.0.1, SPSS Inc., Chicago, Illinois, U.S.A.). The differences between the groups were compared by analysis of variance (ANOVA) followed by Wilcoxon's rank sum test if the data were skewed and with Bonferroni's t-test otherwise. The correlations between normally distributed and skewed data were analyzed using Pearson's correlation coefficient and Spearman's bivariate correlation coefficient, respectively. A p value <0.05 was considered statistically significant.

## 5 Results

For detailed results, see the original articles I-V.

### 5.1 Analytical performance of Innotracc Aio! CK-MB, myoglobin, 1<sup>st</sup> generation cTnI, 2<sup>nd</sup> generation cTnI and usCRP (I, II, III IV)

The analytical detection limits are shown in table 2. Within-assay CVs were 3.2 – 24.9 %, and between-assays precision ranged from 4.0 to 17.2%. Precision at the concentration of 0.04 mg/L using the serum pool was 66.7% for the usCRP assay. The functional sensitivity (CV = 20%) and the concentration giving a CV of 10% were about 0.02 and 0.04 for the 2<sup>nd</sup> generation cTnI assays, respectively, according to the precision results. The 10% CV was determined for the Innotracc Aio! 1<sup>st</sup> generation assay to be at about 0.19 µg/L. Innotracc Aio! usCRP, Cobas Integra latex CRP and Hitachi CRP (Latex) HS had functional sensitivities of 0.10, 0.52 and 0.08, respectively. The 10% CV limits for Innotracc Aio!, Cobas Integra and Hitachi 911 were 0.15, 0.8 and 0.23 mg/L, respectively.

*Table 2. Analytical detection limits (DL) of Innotracc Aio! cardiac markers and usCRP (II, III, IV)*

	1 <sup>st</sup> gen cTnI	2 <sup>nd</sup> gen cTnI	CK-MB	myoglobin	usCRP
DL	0.01 µg/L	0.007 µg/L	0.8 µg/L	0.5 µg/L	0.003 mg/L

In the linearity studies where the patients' plasma pools were serially diluted with instrument buffer, the recoveries with five lithium-heparin plasma samples (0.09 – 62 mg/L) ranged from 76 to 120% for the usCRP assay. The limit of quantification was determined to be 0.03 mg/L.

The linearities within the tested ranges of 0.09 – 76.3µg/L for 1<sup>st</sup> generation cTnI, 0.71– 449.9 µg/L for CK-MB, 0.64 – 1573.3 µg/L for myoglobin and 0.063 –112 µg/L for 2<sup>nd</sup> generation cTnI were within acceptable limits. The recoveries for the different dilutions were 74 – 124.9%.

To compare the Innotracc Aio!, Beckman Access and Abbott AxSYM cardiac marker assays and the Innotracc Aio! usCRP, Cobas Integra latex CRP and Hitachi 911 CRP (Latex) HS assays, Deming linear regression analyses were performed on a single test from each sample over the dynamic range (Table 3). Table 3 also shows the differences (%) between the methods, expressed as a percentage of the average and plotted against the method average. The correlation coefficients between the Innotracc Aio! and Abbott AxSYM 1<sup>st</sup> generation cTnI assays was 0.960, and the slope was 0.07. The correlation coefficients between the Innotracc Aio! and Abbott AxSYM CK-MB and myoglobin assays were 0.995 and 0.971, respectively, but they involved some differences in the measured concentrations. The correlations between the 2<sup>nd</sup> generation Innotracc Aio!, Beckman Access and Abbott AxSYM assays were good, but also involved some differences in the measured concentrations.

Regression results between the Innotracc Aio! usCRP and Cobas Integra CRP yielded the following results: correlation coefficient 0.983 and slope 1.17 (<50 mg/L) and correlation coefficient 0.974 and slope 1.137 (<10 mg/L). For the serum samples, the correlation coefficient was 0.979 and slope 1.106. Regression analysis between Innotracc Aio! usCRP and Hitachi CRP (Latex) HS gave the following results: correlation coefficient was 0.942 and slope 0.879 (<20 mg/L), and correlation coefficient was 0.973 and slope 0.983 (<10 mg/L).

To examine the concordance between the Innotracc Aio!, Roche Cobas Integra, Hitachi 911 and Dade Behring (BNII) methods, the results of three commercial controls and the Roche Cobas Integra c.f.a.s. proteins calibrator were compared (III). The results were as follows: CRP at a low concentration measured with Innotracc Aio! was about 9.4% lower than the value obtained with the Dade Behring method, and good agreement was attained at higher concentrations. The Cobas Integra and Hitachi 911 methods gave about 2.5-14.1 % and 3.0-11 % higher results than Innotracc Aio!, respectively.

Table 4 shows the results of the matrix comparison studies (I, II, III, IV). The slopes were close to 1.0 for all correlations, with the exception of that between CK-MB EDTA whole blood and lithium-heparin plasma (0.83). The largest differences of both sample material comparisons (-16 and -29%) were seen at the very low cTnI concentrations due to the high imprecision of the 1<sup>st</sup> generation cTnI assay in that concentration area (see the figures in study II). Good correlation coefficients were obtained ( $\geq 0.97$ ). Using the Abbott AxSYM analyzer, higher CK-MB mass and lower cTnI values in heparin plasma than in serum were obtained (I). The correlation coefficients and the slopes were 0.995 and 0.987, and 1.08 and 0.82 for the CK-MB and cTnI methods, respectively.

The carry-over results (II) for all cardiac markers were good, ranging within 0.0 – 0.3%.

Table 3. Comparison of methods using Innotracc Aio!, Abbott AxSYM, Beckman Access, Cobas Integra and Hitachi 911(II, III, IV)

Assay	x=reference method y=Innotrac Aio!	Deming regression	Correlation coefficient/n	Mean difference*
1 <sup>st</sup> gen cTnI	x = AxSYM	y= 0.07x-0.07 (y < 60 µg/L)	0.960 / 128	163%
		y=0.07x+0.04 (y < 0.5 µg/L)	0.634 / 56	
CK-MB	x = AxSYM	y= 1.06x-0.02	0.995 / 136	-9%
Myoglobin	x = AxSYM	y= 1.33x-15.8	0.971 / 131	-19%
2 <sup>nd</sup> gen cTnI	x = AxSYM	y=0.06x+0.11 (y < 20 µg/L)	0.996 / 91	155%
		y=0.05x-0.01 (y < 0.5 µg/L)	0.588 / 55	
	x = Access	y=0.45x+0.03 (y < 20 µg/L)	0.947 / 91	78%
		y=0.38x+0.01 (y < 0.5 µg/L)	0.784 / 55	
	y= 1 <sup>st</sup> gen cTnI Aio!	y=0.881x+0.05 (y < 25 µg/L)	0.912 / 97	30%
	x=2 <sup>nd</sup> gen cTnI Aio!	y=0.809x+0.00 (y < 1.0 µg/L)	0.854 / 50	38%
usCRP	x = Cobas Integra	y= 1.17x-0.82 (y < 50 mg/L)	0.983 / 96	21%
		y=1.14x-0.71 (y < 10 mg/L)	0.974 / 41	
	x = Hitachi 911	y=0.88x+0.10 (y < 20 mg/L)	0.942 / 111	24%
		y=0.98x-0.10 (y < 10 mg/L)	0.973 / 108	

\* Percentage of difference (y axis) = [(method A – method B)/average of the two methods]x100

Table 4. Sample matrix correlations (I, II, III, IV)

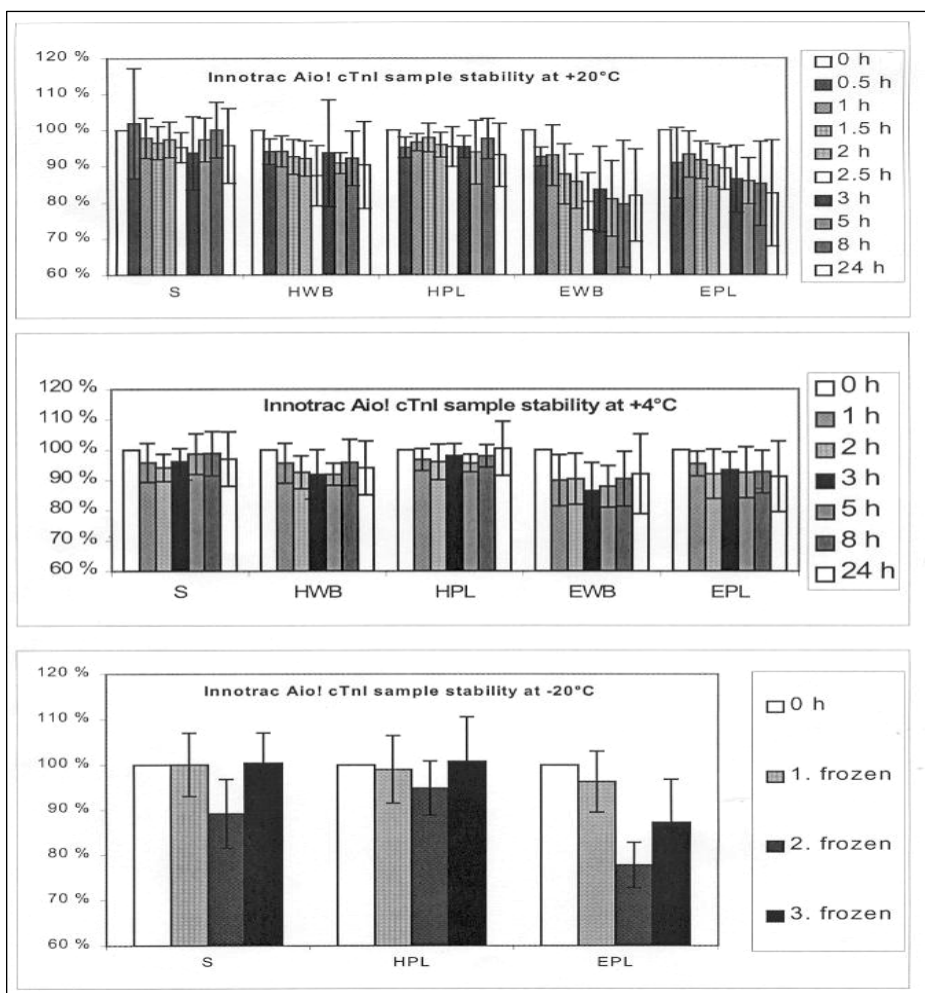
Assay	y, x*	Deming regression	Correlation coefficient / n	Difference %**
1 <sup>st</sup> gen cTnI	y=Li-hep. WB	y=1.02x+0.07	0.973 / 135	-16%
	x=Li-hep. plasma			
	y=EDTA WB	y=1.01x+0.13	0.988 / 87	-29%
	x=Li-hep. plasma			
CK-MB	y=Li-hep. WB	y=1.06x-0.65	0.966 / 136	1.3%
	x=Li-hep. plasma			
	y=EDTA WB	y=0.83x+0.51	0.990 / 97	10%
Myoglobin	x=Li-hep. plasma			
	y=Li-hep. WB	y=1.01x-1.27	0.980 / 129	-1.3%
	x=Li-hep. plasma			
	y=EDTA WB	y=1.04x-7.97	0.970 / 92	4%
	x=Li-hep. plasma			
	y=Li-hep.plasma	Y=0.90x-0.02	0.998 / 38	10%
2 <sup>nd</sup> gen cTnI	x= Li-hep. WB			
	y=serum	y=1.08x-0.01	0.997 / 38	-5%
usCRP	x=Li-hep. plasma			
	y = EDTA plasma	y=0.97x+0.85	0.986 / 29	5%
	x=EDTA WB			
	y=Li-hep.plasma	y=1.08x+3.9	0.995 / 79	17%
CK-MB AxSYM (I)	x=serum			
cTnI AxSYM (I)	y=Li-hep.plasma	y=0.82x+2.0	0.987 / 55	-15%
	x=serum			

\* WB = Whole blood \*\* Percentage of difference (y axis) = [(method A – method B)/average of the two methods]x100

## 5.2 Sample stability (II, IV)

In study II, the stability of the analytes in the lithium-heparin samples at room temperature were studied. The stability values of myoglobin, CK-MB and cTnI were found to decrease by 10%, 10% and 17% in 8 h, respectively.

In study IV, the stability of 2<sup>nd</sup> generation cTnI was tested at different temperatures and with different sample matrices (Figure 4). The stability of the cTnI level in the serum, lithium-heparin plasma and lithium-heparin whole blood samples (n=10) was found to decrease < 10% in 24 h at +20° C and at +4°C. The stability of the cTnI level in EDTA whole blood and EDTA plasma decreased by < 10% in up to 1 h and 1.5 h at room temperature, respectively. The stability of cTnI in EDTA whole blood at +4°C decreased in 2 h. Analyte stability decreased at -20°C < 10 % in serum and lithium-heparin plasma, when freeze-thaw cycles were done, but stability in EDTA plasma decreased > 10% after the second freeze-thaw cycle.



**Fig. 4. Innotracc Aio! cTnI sample stability at different temperatures. S = serum; HWB = lithium-heparin whole blood; HPL = lithium-heparin plasma; EWB = EDTA whole blood; EPL = EDTA plasma**

### 5.3 Reference and AMI cut-off limits (I, II, III, IV)

We have established our own normal and diagnostic cut-off limits for AMI patients using specimens from healthy individuals and clinically diagnosed AMI patients for Abbott AxSYM cTnI and CK-MB assays (I). The normal and diagnostic AMI limits for serum were  $\leq 7 \mu\text{g/L}$  and  $\geq 15 \mu\text{g/L}$ , respectively. The normal and diagnostic AMI limits were  $\leq 8 \mu\text{g/L}$  and  $\geq 16 \mu\text{g/L}$ , respectively, for CK-MB and  $\leq 1.0 \mu\text{g/L}$  and  $\geq 2.0 \mu\text{g/L}$  for cTnI

plasma. According to a small group of healthy volunteers, the normal range for 1<sup>st</sup> generation cTnI could be 0.10 µg/L.

The manufacturer recommends a normal range of  $\leq 116$  µg/L for Abbott AxSYM serum myoglobin. Study I showed about 2 % higher results in plasma than in serum specimens around the cut-off limit, and the normal cut-off limit could therefore be 118 µg/L in plasma for Abbott AxSYM myoglobin.

Based on the correlation curves between the Abbott AxSYM and Innorac Aio! assays (II), the corresponding Innorac Aio! concentrations for 1<sup>st</sup> generation cTnI, CK-MB and myoglobin would be about 0.11 µg/L (normal), 0.19 µg/L (AMI), 8.7 µg/L (normal), 18 µg/L (AMI) and 128 µg/L (normal), respectively.

The 99<sup>th</sup> percentiles of the apparently healthy reference group with different sample matrices for 2<sup>nd</sup> generation cTnI were as follow (IV): 0.022 µg/L in EDTA whole blood with mean hematocrit (Hct; 0.37), 0.026 in EDTA whole blood with individual Hct, 0.014 in EDTA plasma, 0.013 in lithium-heparin plasma and 0.013 in serum. The 99<sup>th</sup> percentile limit was chosen to be 0.03 µg/L.

The values for the 25<sup>th</sup>, 50<sup>th</sup>, 75<sup>th</sup>, 80<sup>th</sup>, 90<sup>th</sup>, 95<sup>th</sup>, and 97.5<sup>th</sup> percentiles were determined with the Innorac Aio!, Cobas Integra and Hitachi methods for CRP (Table 5). The Cobas Integra method gave values in the 80<sup>th</sup>, 90<sup>th</sup>, 95<sup>th</sup> and 97.5<sup>th</sup> percentiles comparable to those obtained with the Innorac Aio! method, but higher values at lower concentrations. In the first quartile (0-25<sup>th</sup> percentile), 2<sup>nd</sup> quartile (25-50<sup>th</sup>), 3<sup>rd</sup> quartile (50-75<sup>th</sup>) and 4<sup>th</sup> quartile (75-100<sup>th</sup>), the mean CRP values using Innorac Aio! assay were 0.16 mg/L, 0.52 mg/L, 1.24 mg/L and 4.39 mg/L, respectively. Using the Integra CRP and Hitachi 911 methods, the quartile mean concentrations were 0.42 mg/L, 0.89 mg/L, 1.62 mg/L and 4.63 mg/L, and 0.28 mg/L, 0.56 mg/L, 1.14 mg/L and 3.34 mg/L, respectively. In the health questionnaire of the reference group, two of the volunteers reported being smokers, and their CRP values were both < 2 mg/L (no smokers in study V).

*Table 5. CRP determinations at selected percentiles in adult blood donors using Innorac Aio! usCRP, Cobas Integra Latex CRP and Hitachi CRP (Latex) HS assays (III). The sample type was serum.*

Analyzer	n	25th	50th	75th	80th	90th	95th	97.5th
Aio!	172	0.30	0.74	1.80	2.21	4.19	5.68	6.38
Integra	172	0.63	1.20	2.20	2.45	4.12	5.44	6.56
Hitachi	111	0.40	0.73	1.56	1.80	3.05	5.63	ND

#### **5.4 Methodological comparison studies based on the patients' clinical status (IV)**

As mentioned in the Material and methods, two different comparative analyses were carried out between the methods. The Innorac Aio! 1<sup>st</sup> and 2<sup>nd</sup> generation cTnI, Beckman Access AccuTnI, Abbott AxSYM cTnI methods were also compared with reference to the patients' clinical status. Based on the 99<sup>th</sup> percentile and the 10% CV for these methods, the samples from patients were classified into different categories.

The analytical characteristics of cardiac troponin I, as reported by the manufacturers, are 0.04 for Beckman Access and 0.3 µg/L for Abbott AxSYM as the 99<sup>th</sup> percentiles, while CVs are 10% for Beckman Access 0.06 and 0.8 µg/L for Abbott AxSYM. As described above, the concentrations giving CV of 10% for 1<sup>st</sup> and 2<sup>nd</sup> generation cTnI were 0.19 and 0.04 µg/L, respectively. Based on the 99<sup>th</sup> percentiles as a threshold value of the Beckman Access, Abbott AxSYM and Innorac Aio! 2<sup>nd</sup> generation cTnI assays, 14 samples from patients were classified into different diagnostic categories. When the CV of 10% was used as a threshold value, 29 patients were classified differently. In the second comparative study, discrepancies between the results obtained by using the 99<sup>th</sup> percentile limit and the 10% total CV concentration limit in the Abbott AxSYM, Innorac Aio! 1<sup>st</sup> and 2<sup>nd</sup> generation cTnI assays were seen in 8 samples and 17 samples, respectively. The detailed results are presented in the Tables 6 A-D (previously unpublished data). Previously, Jaffe (Jaffe 2001) published a list of the potential injuries to the heart that could underlie a troponin elevation without causing overt ischemic heart disease. These include congestive heart failure, pulmonary embolism, inflammatory diseases and different types of cardiac surgery, which are also presented in the Tables 6 A-D as the diagnostic groups 3, 4, 5 and 6. Furthermore, the ACC/AHA guidelines for the management of unstable angina recommend monitoring of cardiac troponin in acute coronary syndrome patients to differentiate between unstable angina and non-ST-segment elevation MI (Braunwald *et al.* 2002). This document defines cardiac troponin as an indicator of myocardial necrosis when the maximum concentration of cardiac troponin T or I exceeds the decision limit on at least one occasion during the first 24 h after the index clinical event. Thus, we classified the groups 2, 4 and 6 as true positives with positive troponin I concentrations. The group was classified as positive only when there was evidence of infections, pneumonia or pulmonary embolism.

In the first comparative study (Table 6A), where the 99<sup>th</sup> percentile was used as the MI cut-off limit, 2 of the Beckman Access cTnI, 1 of the Abbott AxSYM and none of the Innorac Aio! results were in concordance with the clinical status in the diagnostic group 1. In the samples 2, 3, 7, 10, 12 and 14, cTnI may be increased because of a preceding coronary procedure, UAP, infection, coronary procedure, bronchitis + pulmonary embolism and pneumonia, respectively. When the cut-off limits of CV 10% were used (Table 6B), the results obtained in 8 Beckman Access cTnI, 2 Abbott AxSYM and 5 Innorac Aio! 2<sup>nd</sup> generation assays were in agreement with the MI diagnosis. In the samples 3 + 4, 5, 9 + 10, 11, 17, 18, 27, 28, 29, the positive cTnI may have been caused by a preceding coronary procedure, UAP, pneumonia, infection, bronchitis, chronic bronchitis, pneumonia, sepsis and pneumonia, respectively. In the second comparative study presented in Table 6C, both MI samples were positive by the Abbott AxSYM and Innorac Aio! 2<sup>nd</sup> generation cTnI assays but not by the Innorac Aio! 1<sup>st</sup> cTnI assay. In the samples 2, 3 and 4, the clinical status of infection and congestive heart failure may have increased the cTnI concentration. Coronary procedure and pneumonia in the samples 5 and 7 + 8, respectively, could have caused the positive cTnI results. Finally, in Table 6D, based on the 10% CV threshold values, 9 of the samples were from MI patients, and 9 were positive in Abbott AxSYM, 9 were positive in Innorac Aio! 2<sup>nd</sup> generation, and none of the samples were positive with the 1<sup>st</sup> generation cTnI assays. The positive cTnI results in the samples 4 and 14 could have been caused by

cardiomyopathy and pneumonia, that in 5 by coronary procedures, those in 7-10 by congestive heart failure and infection and that in 13 by pneumonia.

*Table 6. a). Discrepancies between the results using the 99<sup>th</sup> percentile reference limits yielded by Abbott AxSYM cTnI, Beckman Access cTnI and Innotracc Aio! cTnI 2<sup>nd</sup> generation assays.*

Sample	Abbott AxSYM 0.3 µg/L	Beckman Access 0.04 µg/L	Innotrac Aio! 0.03 µg/L	Diagnosis group*
1.	0.1	0.06	0.02	1, 6
2.	0.2	0.08	0.04	5; Unclear, earlier 4
3.	0.0	0.06	0.01	2
4.	1.0	0.02	0.00	3, alcoholic
5.	4.3	0.02	0.01	5; AIHA
6.	1.0	0.02	0.01	2; prol. AP
7.	0.9	0.13	0.01	3; Inf.ac.resp.astma
8.	0.1	0.03	0.04	3
9.	2.0	0.02	0.01	2; prol AP
10.	0.1	0.04	0.03	4
11.	2.3	0.03	0.01	1
12.	2.2	0.02	0.01	5; collapsus, bronchitis pulmonary embolism
13.	2.2	0.06	0.02	1
14.	0.0	0.10	0.02	5; pneumonia

\* The diagnoses were divided into the following categories: 1. Acute myocardial infarction. 2. Unstable angina pectoris, prolonged angina pectoris. 3. Non-ischemic chest pain, dyspnoea. 4. Coronary procedure: PTCA, CABG. 5. Other: Infection, abdominal pain, pulmonary embolism, collapsus. 6. Congestive heart failure.

Table 6. b) Discrepancies between the results obtained using the 10 % total CV concentration limit yielded by Abbott AxSYM cTnI, Beckman Access cTnI and Innotracc Aio! cTnI 2<sup>nd</sup> generation assays.

Sample	Abbott AxSYM	Beckman Access	Innotrac Aio!	Diagnosis group*
	0.8 µg/L	0.06 µg/L	0.04 µg/L	
1.	0.1	0.06	0.02	1,6
2.	0.3	0.08	0.05	1
3.	0.3	0.10	0.05	5; unclear, earlier 4
4.	0.2	0.08	0.04	5; same patient as 3.
5.	0.0	0.06	0.01	2; UAP
6.	1.0	0.02	0.00	3; alcoholic
7.	1.2	0.01	0.00	3; same patient as 6.
8.	4.3	0.02	0.01	5; AIHA
9.	0.3	0.05	0.06	5; pneumonia suspecta
10.	0.4	0.06	0.09	5; same patient as 9
11.	0.9	0.13	0.01	5; inf.as.resp.asthma
12.	0.5	0.11	0.04	1
13.	0.4	0.09	0.07	5; vertigo
14.	0.1	0.03	0.04	5; dolores thoracis
15.	2.0	0.02	0.01	2
16.	2.3	0.03	0.01	1
17.	2.2	0.02	0.01	5; collapsus, bronchitis
18.	0.3	0.11	0.03	5; collapsus, chronic bronc.
19.	0.7	0.19	0.07	1
20.	2.2	0.06	0.02	1
21.	0.6	0.15	0.05	1
22.	1.0	0.02	0.01	2; prol.AP
23.	0.3	0.08	0.03	1,2; non-Q-AMI susp., prol. AP
24.	0.7	0.14	0.07	1,2; same patient as 23.
25.	0.7	0.12	0.07	5; brain tumour
26.	0.5	0.13	0.06	5; collapsus, chronic bronc.
27.	0.5	0.10	0.03	5; pneumonia
28.	2.6	0.09	0.03	5; septic shock, pneumonia
29.	0.0	0.10	0.02	5; pneumonia

\* Same as above.

Table 6. c). Discrepancies between the results obtained using the 99<sup>th</sup> percentile reference limits yielded by Abbott AxSYM cTnI, Innotracc Aio! cTnI cTnI 1<sup>st</sup> and Innotracc Aio! cTnI 2<sup>nd</sup> generation assays.

Sample	Abbott AxSYM 0.3 µg/L	Innotrac Aio! 1 <sup>st</sup> cTnI 0.10 µg/L	Innotrac Aio! cTnI 2 <sup>nd</sup> 0.03 µg/L	Diagnosis group*
1.	0.8	0.05	0.06	1
2.	1.3	0.02	0.09	5,6; infection
3.	0.6	0.00	0.01	5; same as 2.
4.	1.2	0.00	0.06	5; same as 2.
5.	0.4	0.04	0.03	4
6.	1.2	0.04	0.12	1
7.	2.2	0.04	0.05	5; pneumonia, myxoma, exitus 3 days later
8.	2.7	0.05	0.05	5; same patient as 7

\* Same as above.

Table 6. d) Discrepancies between the results obtained using the 10 % total CV concentration limit yielded by Abbott AxSYM cTnI, Innotracc Aio! cTnI 1<sup>st</sup> and Innotracc Aio! cTnI 2<sup>nd</sup> generation assays.

Sample	Abbott AxSYM 0.8 µg/L	Innotrac Aio! 1 <sup>st</sup> cTnI 0.19 µg/L	Innotrac Aio! 2 <sup>nd</sup> cTnI 0.04 µg/L	Diagnosis group*
1.	1.2	0.15	0.38	1, exitus 2 days later
2.	0.8	0.05	0.06	1
3.	1.0	0.08	0.07	1; same patient as 2.
4.	2.7	0.15	0.14	5; cardiomyopathy; pneumonia
5.	2.7	0.17	0.18	4
6.	1.8	0.06	0.11	1, postoperative
7.	1.3	0.02	0.09	6; unclear diag., infection
8.	2.1	0.10	0.12	6; same patient as 7.
9.	1.2	0.00	0.06	6; same patient as 7.
10.	2.0	0.11	0.15	6; same patient as 7.
11.	1.2	0.04	0.12	1
12.	3.0	0.17	0.31	1
13.	2.2	0.04	0.05	5; pneumonia, myxoma, exitus 2 days later
14.	2.7	0.05	0.05	5; same patient as 4.
15.	1.3	0.14	0.15	1
16.	2.4	0.18	0.21	1
17.	1.7	0.06	0.20	1

\* Same as above.

## 5.5 Influence of hemodialysis or rheumatoid factor (IV)

The details of the two studies presented in this chapter are previously unpublished data. The study group consisted of 48 patients undergoing hemodialysis (22 men and 26 women). The mean age of the patients was 57 years (range: 15 – 89 yrs). Samples were obtained before and after hemodialysis (n=96). 17 patients had coronary artery disease, and 6 of them had had a coronary procedure, 6 patients had had an atrial fibrillation coronary procedure, and 4 patients had congestive heart failure. Almost all of the patients had hypertension and/or left ventricular hypertrophy. 17 of the patients had diabetes mellitus. cTnI was measured from EDTA plasma samples with the Innotrac Aio! and Abbott AxSYM analyzers. The concentration ranges were 0.000 – 0.039  $\mu\text{g/L}$  and 0.0 – 1.1 with the Innotrac Aio! and Abbott AxSYM cTnI assays, respectively. Because the manufacturer has not validated EDTA plasma with the Abbott AxSYM assay, we compared 30 lithium-heparin plasma and EDTA plasma cTnI samples from chest pain patients. The results of these samples were equal throughout the concentration range of 0.6 – 42  $\mu\text{g/L}$ .

In the hemodialysis study, a total of 28 samples were above the analytical detection limit (DL) in the Innotrac Aio! cTnI assay. Nine patients had cTnI plasma levels > DL at both measurements, 3 patient samples were above the 99<sup>th</sup> percentile limit (0.039, 0.040 and 0.035  $\mu\text{g/L}$ ), and only one patient had a plasma level above that cut-off limit at both measurements (0.039 and 0.040  $\mu\text{g/L}$ ). During the hemodialysis session the cTnI values of 21/48 patients (43%) increased, while no change in the cTnI values was seen in 15/48 patients (32%), and the cTnI values of 12/48 patients (25%) decreased. However, the changes were not significant and may be due to the higher precision near the detection limit and to the background signal. Additionally, 2 of the samples were at the cut-off limit.

The minimum detectable concentration in the Abbott AxSYM assay was previously evaluated to be 0.24 $\mu\text{g/L}$ , and as mentioned above, the 99<sup>th</sup> percentile was 0.3  $\mu\text{g/L}$ . Altogether 11 samples were above the analytical detection limit in the Abbott AxSYM assay, while 5 samples were above the cut-off limit (0.9, 0.9, 1.1, 0.5 and 0.6  $\mu\text{g/L}$ ), and two patients had both of these values above the cut-off limit (0.9/0.9 and 0.5/0.6  $\mu\text{g/L}$ ). Additionally, 5 of the samples had concentration values at the cut-off. During the hemodialysis session, 4/48 patients (8%) had an increase in their cTnI values, 7/48 patients (15%) showed no change, and 37/48 patients (77%) had a decrease in their cTnI values. Of the 4 patients with positive Innotrac Aio! cTnI levels ( $\geq 0.03$   $\mu\text{g/L}$ ) in at least one of the two samples, the first patient suffered from atrial fibrillation, congestive heart failure and hypertension, the second patient suffered from coronary artery disease, had had a cardiac procedure and had atrial fibrillation and hypertension, the third patient suffered from coronary artery disease, had had a coronary procedure and had atrial fibrillation and diabetes mellitus, and the last patient suffered from hypertension and left ventricular hypertrophy. All of these patients, except the third, had positive cTnI levels in at least one of the two samples with both methods. Additionally, among the 5 patients with positive Abbott AxSYM cTnI levels in at least one of the two samples, all suffered from hypertension, two suffered from diabetes mellitus, one had had a coronary procedure, one suffered from congestive heart failure, and three suffered from coronary

artery disease. In conclusion, based on these results, the positive cTnI results appear to be due to the coronary artery disease or other potential injuries to the heart and not to the hemodialysis. Additionally, the imprecision of > 10% CV at the 99<sup>th</sup> percentile of the reference group does not permit reliable determination of cTnI at this concentration with these methods.

False-positive troponin I values due to the presence of rheumatoid factor have been described (Dasgupta *et al.* 1999). We studied serum samples from 15 rheumatoid patients (Table 7). Ten of them had had surgery due to the primary disease within the past one to nine days, one surgery patient also suffered from secondary amyloidosis and renal insufficiency, and one had very high C-reactive protein and suffered from recurrent respiratory infections. The rheumatoid factors (RF) were measured using Orion Diagnostica's method (RF-PAIA, Orion Diagnostica, Espoo, Finland) and a Hitachi 911 analyzer. Orion Diagnostica's Rheumatoid Factors assay is based on particle-enhanced immunoturbidimetry. The concentrations varied from 20 to 3630 U/L. A total of 4/15 samples (0.008-0.012 µg/L) had cTnI concentrations recorded with Innotracs Aio! above the detection limit. Among these samples, number 4 was from a patient who had had a carpal joint operation 2 days previously, patient 9 had secondary amyloidosis and renal insufficiency and had undergone arthrodesis of the subtalar joints 6 days previously, and patient 11 had very high CRP and suffered from recurrent respiratory infections. Only one of the samples (number 11) had a cTnI concentration (0.070 µg/L) above the 99<sup>th</sup> percentile with a very high RF concentration of 3630 U/L. The concentrations of cTnI in serum samples were also measured using the Abbott AxSYM analyzer. No measurable cTnI was observed in these samples with this analyzer.

*Table 7. Rheumatoid factors (RF) and Innotracs Aio! 2<sup>nd</sup> generation cTnI concentrations of 15 patients suffering from rheumatoid arthritis.*

Sample	RF (kU/L)	Innotrac Aio! cTnI 2 <sup>nd</sup> generation (µg/L)
1.	117	0.000
2.	32	0.011
3.	21	0.006
4.	590	0.010
5.	20	0.002
6.	22	0.005
7.	56	0.000
8.	72	0.005
9.	112	0.012
10.	26	0.001
11.	3630	0.070
12.	1060	0.006
13.	508	0.005
14.	33	0.007
15.	69	0.008

## 5.6 cTnI profiles in acute myocardial infarction and other cardiac events

Figure 5 shows the typical AMI patient's profile using the Innotracs Aio! 2<sup>nd</sup> generation assay in comparison with the commercial assays. There were acute MI patients with patient profiles, CABG patient profiles, UAP patient profiles and one subject with a non-MI patient profile in the Innotracs Aio!, Beckman Access and Abbott AxSYM cTnI assays among all the profiles determined (n=38). The profiles were similar, and the levels in all determinations were much higher than the cut-off level used. In the 2/3 comparisons of all the three methods, the 2<sup>nd</sup> generation Innotracs Aio! and Beckman Access assays detected the released cTnI and the myocardial damage earlier than the Abbott AxSYM assay. On the contrary, in one patient profile, Abbott AxSYM detected MI earlier than the other assays. All of the CABG profiles were similar in the assays, with the exception of one patient, who also suffered from UAP. The Beckman Access assay seemed to identify UAP.

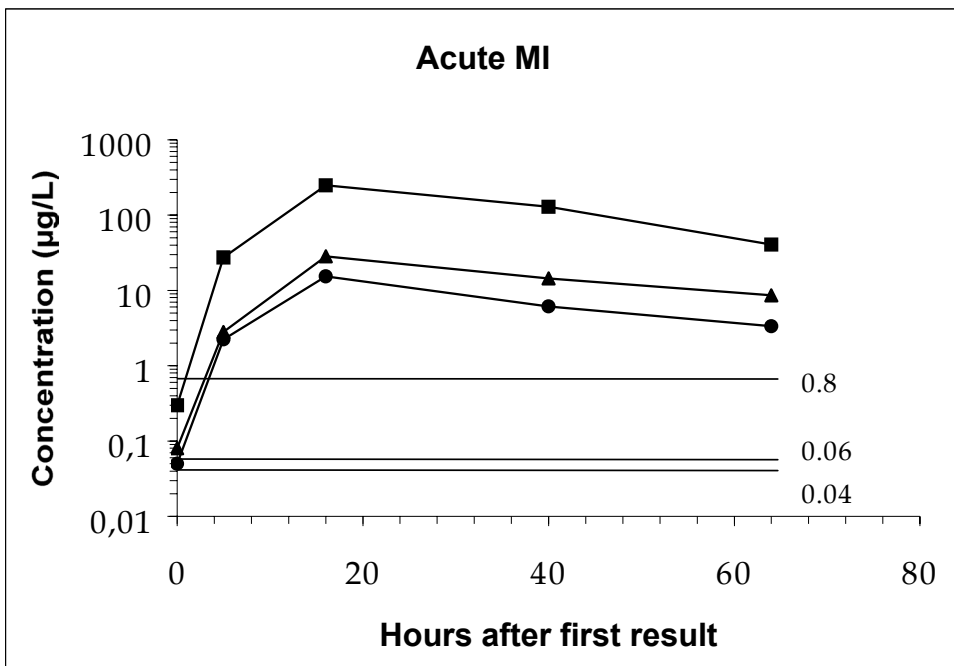


Fig. 5. A typical cTnI profile of a patient with acute MI. The Innotracs Aio! 2<sup>nd</sup> generation assay is indicated as circles, Abbott AxSYM as boxes and Beckman Access as triangles. The 10% CVs of the methods are marked as horizontal lines

## 5.7 Use of CRP assay in the investigation of patients with acute atrial fibrillation (V)

Plasma concentrations of CRP and IL-6 were measured in 25 acute atrial fibrillation (AF) patients, 31 patients with paroxysmal AF and 32 healthy control subjects. CRP concentrations were measured using Cobas Integra (V) and Innotracc Aio! (unpublished data). The analytical detection limits of the Cobas Integra and Innotracc Aio! methods are 0.085 mg/L (according to the reagents insert) and 0.003 mg/L, respectively. The IL-6 values of the patients with acute AF were significantly higher than those of the controls or the patients with paroxysmal AF (Table 8). CRP values using Cobas Integra and Innotracc Aio! tended to be higher in the patients with acute AF: 2.69+/- 3.23 vs. 1.82+/- 2.34 and 1.44+/-0.87,  $p=0.126$ , and 2.61+/-4.09 vs. 1.59+/-2.48 vs. 0.86+/-0.77, respectively. There was a positive correlation between the concentrations of IL-6 and CRP. The imprecision of the Innotracc Aio! usCRP method has been shown to be better at  $< 3$  mg/L than the imprecision of the Cobas Integra method. Therefore, the differences between the control and other groups are more reliable compared with Cobas Integra.

*Table 8. IL-6 and CRP values of the patients with acute AF, paroxysmal AF and controls*

Analyte	AF	Paroxysmal AF	Healthy controls
IL-6 pg/ml	3.34+/-2.65	1.47+/-1.29	2.30+/-2.24
CRP Cobas Integra mg/L	2.69+/-3.23	1.82+/-2.34	1.44+/-0.87
usCRP Innotracc Aio! mg/L	2.61+/-4.09	1.59+/-2.48	0.86+/-0.77

## 6 Discussion

### 6.1 Changing the criteria in the evaluation of chest pain patients challenge the cardiac marker assays (I, II, III, V)

The introduction of the 99<sup>th</sup> percentile reference limit for the diagnosis of myocardial infarction by the European Society of Cardiology/American College of Cardiology (ESC/ACC) Consensus Committee was largely driven by the demonstration that even the lowest detectable amounts of cardiac troponins in blood are associated with an increased cardiac risk, and that the definition of myocardial infarction should be refined as any amount of myocardial necrosis. (Albert *et al.* 2000). Patients with any detectable troponins benefit from early coronary or pharmacological intervention (Lindahl *et al.* 2001, Morrow *et al.* 2001, Venge *et al.* 2002). The consensus committee also proposed that the imprecision of the assay should be <10% at the 99<sup>th</sup> percentile limit. Analytical imprecision is not uniform among troponin assays (Pagani *et al.* 2003, Panteghini *et al.* 2004). A failure to reach this goal could increase the risk of clinically misleading results. The demand for very precise troponin assays presents a major challenge. A recent study showed that only cardiac troponin concentrations that meet the goal of 10% CV as a MI cut-off provide a misclassification rate <1% in a population of patients with suspected acute coronary syndrome (Sheehan *et al.* 2002). The current commercially available troponin assays do not meet this criterion. Nevertheless, there has been substantial improvement in the precision and sensitivity offered by the newer assays. Panteghini and colleagues compared the commercially available assays using the 99<sup>th</sup> percentile reference limit defined by the manufacturer and the CV of 10% determined in the study. Innorac Aio! was not included in this study. No cardiac troponin assay was able to achieve the 10% CV recommendation at the 99<sup>th</sup> percentile. There were marked differences in the results between the different assays measuring cTnI. The analytical responses to each cTnI serum pool varied over 20-fold for the participating cTnI assays, underscoring the need for standardization. Concentrations corresponding to 10% CV imprecision and the 99<sup>th</sup> percentile reference limit for Innorac Aio! cTnI (0.04 µg/L) were nearest to the Beckman Access assay (0.06 µg/L), the Liaison assay (0.03 µg/L;

Byk-Sangtec Diagnostica) and the Beckman Access assay (0.04 µg/L), respectively. Innotracc Aio! has the lowest 10% CV concentration limit of the cTnI assays on the market. Among the tested methods, the 10%/99<sup>th</sup> ratios were 4.1, 1.5 and 2.2 for Abbott AxSYM, Beckman Access and Liaison, respectively. The ratios of the 17 assays involved in the study were  $\leq 9.0$ . (Panteghini *et al.* 2004). In our study of Innotracc Aio! 2<sup>nd</sup> generation cTnI assay (IV), the ratio was 1.3, providing the good imprecision of the assay. On the other hand, it has been suggested that, from a practical point of view, for troponin assays currently unable to meet the 10% CV at the 99<sup>th</sup> percentile value, a predetermined higher concentration that meets this imprecision goal should be used as the cut-off for AMI until the goal of 10% CV can be achieved at the 99<sup>th</sup> percentile (Panteghini *et al.* 2004). Additionally, differences in the 99<sup>th</sup> percentile values between males vs females and blacks vs caucasians have been demonstrated, giving more challenge to the use of the 99<sup>th</sup> percentile as a cut-off limit (Apple *et al.* 2003).

The biological variations for myoglobin (Panteghini & Pagani 1997) and CK-MB (Ross & Fraser 1998) have been estimated. The overall variation of the Innotracc Aio! myoglobin assay was <9.5%. This variation may be clinically unacceptable. CK-MB assay showed a CV% within the target value, defined also on the basis of biological variability (9.2% for CK-MB). However, according to our experience in the Laboratory of Oulu University Hospital, variation may be different in quality controls from different manufacturers (unpublished data).

## **6.2 Differences in results between matrices and assay designs (I, II, IV)**

The complex molecular nature of cTnI complicates measurement by immunoassays, causing the commercial assays to detect cTnI differently. The differences between the results can be explained by the use of different antibodies in the assays, the lack of an international reference material for cTnI standardization and the effect of interfering factors.

It is clear that the problems of cardiac marker standardization will not be quickly solved. A number of projects are under way under the auspices of the IFCC, AACC and other organizations. The subcommittee for cTnI standardization of AACC working in cooperation with the National Institute for Standard and Technology (NIST) and IFCC C-SMCD recently identified a candidate reference material that is a complex of troponin C, I and T purified from human cardiac tissue (Christenson *et al.* 2001). The standardization of CK-MB and myoglobin seems to be simpler. For a single polypeptide chain protein with a molecular mass of about 17 kDa (myoglobin), purification from cardiac tissue can be easily used to produce primary immunogens and calibrators, and for a heterodimeric protein, such as CK-MB, the alternative use of recombinant DNA technology is possible (Christenson *et al.* 1999, Wu *et al.* 1989)

Previously, Eriksson with colleagues evaluated the presence of interfering factors in cTnI assays by measuring the recovery of cTnI added to samples from volunteers and patients with acute coronary syndromes. The factor may cause inhibition of cTnI immunoreactivity ranging from mild to very severe, depending on the amount of

interfering factor in the sample. Eriksson *et al* proposed as a solution to the problem a multi-antibody assay, in which the antibody combination is chosen from the terminal parts with a mid-fragment part, because the most interference-free antibodies are those against epitopes in the terminal parts of the molecule, which are not present in the fragmented cTnI molecules. (Eriksson *et al.* 2003). According to a personal interview of the manufacturer, the Innotracs Aio! 2<sup>nd</sup> generation assay was developed using this information. This may explain the earlier detection of released cTnI and AMI compared to the xSYM assay. The Beckman Access assay seemed to detect cTnI very similarly to the Innotracs Aio! assay, showing that the antibodies may have been chosen from the same regions. On the other hand, Abbott in a Euromedlab congress (2003) recently introduced a new 2<sup>nd</sup> generation cTnI assay using Abbott AxSYM, which is now commercially available (Qiu *et al.* 2003).

Plasma has been recommended as the specimen of choice by both the cardiology and the laboratory medicine communities, to keep the turnaround time short (Panteghini *et al.* 2001). However, there may be significant differences between the serum and plasma concentrations of troponins when different analytical systems are used (Gerhardt *et al.* 2000, Stiegler *et al.* 2000). According to our matrix comparison data (I, IV), there were different CK-MB and cTnI values in heparin plasma compared to serum measured with the Abbott AxSYM system, while the Innotracs Aio! system yielded equal values. On the contrary, it has been published that the concentrations of cTnI in some cases using different commercial methods and cTnT using Elecsys (Roche Diagnostics) were lower in heparin plasma than in serum samples (Gerhardt *et al.* 2000, Stiegler *et al.* 2000). In conclusion, the matrix differences may be due to the analyte measured and the method used. Additionally, titration of sera with heparin concentrations below and above tube concentrations cause increasing losses of troponin with increasing heparin concentrations (Gerhardt *et al.* 2000). We used lithium-heparin tubes with a low total heparin concentration of 15.9 IU/ml of whole blood in our experiments, which value is in routine use in our laboratory. However, the diagnostic and reference limits must be revised and defined whenever the laboratory undertakes to use plasma samples instead of serum.

Changes in the plasma levels of cardiac troponins are common in hemodialysis patients in the absence of coronary heart disease and may have prognostic value (Chapelle *et al.* 2002, Fehr *et al.* 2003, Freda *et al.* 2002, Katus *et al.* 1995, Beciani *et al.* 2003, Choy *et al.* 2003, Goicoechea *et al.* 2004). However, there was no remarkable influence of hemodialysis on cTnI release in our experiment. The changes between the cTnI results before and after hemodialysis were not significant and may be due to the higher precision near the detection limit, to the CV of 10% at the 99<sup>th</sup> percentile limit and to the background signal of Innotracs Aio! method.

As mentioned earlier, heterophilic antibodies and rheumatoid factors may cause false-positive results, and these interferences can be eliminated by using the polyclonal antisera against them. In our study IV of rheumatoid factors, one slightly increased cTnI result was found with the very high concentration of RF, even though blocking agents have been introduced to the reagents. This patient had infections, and this may have caused the elevation of cTnI.

There are many preanalytic and analytic variables that can affect the CRP results. Additionally, the differences in the standardization materials or the suboptimal value transfer protocols are likely to explain the differences between the methods. (Ledue &

Rifai 2003). According to the manufacturers, the Cobas Integra CRP latex, Hitachi CRP (Latex) HS and Dade Behring BNII assays have been standardized against CRP 470, whereas the Innotracs Aio! usCRP assay has been standardized against the WHO 1<sup>st</sup> IS 85/506 reference material.

### 6.3 Requirements of highly sensitive CRP assays (III, V)

It is now believed that CRP is not simply a marker for atherosclerosis, but fundamentally a marker for unstable atherosclerotic plaque, and that atherosclerosis probably involves inflammatory components at the onset of the disease. The inflammatory process continues its destructive mission throughout the evolution of heart disease: plaque development, activation of matrix proteases that cause degradation of the collagen matrix and make the plaque more vulnerable and ultimately oxidative vascular damage (Sainato 1997, see the observations of Tracy R, Ridker 2001). With regard to primary prevention, the most important data relating hs-CRP and vascular disease in large-scale epidemiological studies have linked minor elevations in CRP in apparently healthy individuals to future risks of MI, stroke, congenital heart disease, death and peripheral vascular occlusion (Ridker 2001, Ridker 1998). CRP has also been found to be elevated in patients with either persistent or paroxysmal atrial fibrillation and associated with an increased risk for new AF episodes (Aviles *et al.* 2003, Chung *et al.* 2001).

Highly sensitive CRP assays are required for the prediction of atherosclerotic risk in apparently healthy adults. Therefore, the assay has to provide both good accuracy and good precision. It has been suggested that, for sensitive CRP, the within-laboratory total imprecision should be <10% across the linear range of the assay (Roberts *et al.* 2000), and the assay must be able to reliably measure hs-CRP at least at the lowest cut-off point (1 mg/L). However, the assays used in population-based studies and clinical research should be able to measure much lower hs-CRP concentrations, such as 0.15 mg/L. (Ledue & Rifai 2003). In study III, Innotracs Aio!, Cobas Integra latex CRP and Hitachi CRP (Latex) HS had functional sensitivities (20% CV) of 0.10, 0.52 and 0.08 mg/L, respectively. The 10% CV limits for Innotracs Aio!, Cobas Integra and Hitachi 911 were 0.15, 0.8 and 0.23, respectively. This indicates that Innotracs Aio! met these criteria. Additionally, the imprecision for Innotracs Aio! seemed to increase at the high and very low concentrations, indicating that the main use of this assay can lie in estimating the stratification of atherosclerotic risk or other risk stratifications in apparently healthy individuals.

CDC/AHA redefined the hs-CRP risk categories as low < 1 mg/L, average 1-3 mg/L and high > 3 mg/L. In study III, the healthy population could be divided as follows: Integra serum samples; 73, 72 and 27 volunteers were divided into categories of low risk, average risk and high risk, and Hitachi 911 serum samples (unfortunately, the total sample size was small): 66 were categorized as low risk, 31 as average risk and 12 as high risk, and Innotracs Aio! serum samples: 100, 47 and 25 volunteers were classified into the categories of low risk, average risk and high risk, respectively.

## 6.4 Point-of-care testing (POCT) (II, III, IV)

Rapid diagnosis is clinically relevant and may have a direct impact on patient management. POCT may have a direct influence on the length of stay and the admission and discharge policies. There have been very few studies concerning POCT in Finland, although the method is widely used. Internationally, POC studies rarely concern cost issues or the influence of hospital stay. They usually emphasize the evaluation of POCT and compare the results with those obtained at the central laboratory. Assessing the cost-benefit ratio on a per test basis cannot be done effectively in a generalized manner, because each hospital must evaluate this with respect to its own unique circumstances.

In a prospective randomized controlled trial, POCT was compared to laboratory testing in a district general hospital as part of the UK Health Care Technology assessment program. A total of 263 patients were enrolled in this study. The target turnaround time was improved from 72 minutes to 20 minutes. Rapid diagnosis is effective and cost-effective for patient management, but only within the context of data-driven decision-making protocols. (Collinson *et al.* 1999). A prospective randomized control trial of low-risk patients (ROMIO study) showed cost reduction comparing the costs in emergency department rule out to conventional hospital stay (Gomez *et al.* 1996).

Laboratory costs can be divided into fixed and variable costs. Fixed costs include equipment, depreciation, rent, most labour and utilities. These costs tend not to vary for central laboratories equally much as for alternate-site testing venues. Variable costs, which change with each incremental unit, are lower for most central or core laboratories compared to alternate-site venues. Examples of variable costs include supplies, incremental labour and reagents. The fixed expenses for quality control, maintenance and record keeping account for a smaller part of overall costs in alternate-site venues. There are a number of cost analyses of core vs alternate-site testing strategies in the literature. These studies demonstrate the difficulty of describing the actual costs of the different components of core laboratories and alternate-site testing venues. (Brogan & Bock 1998 and ref. therein).

Faster TAT can be achieved with plasma or whole blood. Because of the easy handling, a whole blood test may be performed at near-patient testing sites, including emergency departments, point-of-care sites and even ambulances, for applications including risk stratification and assessment of cardiac injury (Roth *et al.* 1999). The results of these studies showed that a time-resolved immunological technology using the Innotracs Aio! system is highly suitable for use even outside the central laboratory, because it is quick and easy to use and maintain, the reagents remain stable for a long time, and whole blood samples can be used.

If electrocardiography (ECG) is diagnostic of AMI, patients are immediately treated before the results of biomarker testing are available. In approximately 50% of the cases, ECG is non-diagnostic, and an objective alternative (biochemical markers) is useful in aiding clinicians in their decision-making about patient treatment and disposition. (Hamm *et al.* 1997). Rapid TAT for the testing of biomarkers can reduce any delay in treatment and allow for a triage and discharge decision to be made. Additionally, it is recommended that the results for STAT cardiac markers be available in 1 hour or less, and that POCT should be implemented when this target cannot be consistently met. (Wu *et al.* 1999).

Kilgore and colleagues focused on a very direct outcome of POCT turnaround time. They provide a direct, quantitative comparison of the three primary strategies for reducing turnaround time: central laboratory stat testing, satellite laboratory testing and POCT. They measured traditional analytical TATs and ‘therapeutic TAT’, which is the time between the decision to test and the initiation of a therapeutic intervention. They showed that therapeutic TAT was 1-2 min shorter for bedside testing compared with the satellite laboratory and 9-14 min shorter in the satellite laboratory compared with centralized testing. This not unexpected because central laboratory testing requires several additional steps in specimen handling and transport that are not required for satellite laboratory or bedside testing. Kilgore *et al.* also evaluated the service outcome of staff satisfaction and found that the satellite laboratory produced the greatest overall satisfaction, offering timely, accurate answers while minimizing labour for the medical staff. (Kilgore *et al.* 1998). Time, temperature and duration of storage during transport to the central laboratory may affect the quality of the sample. Transfer to the laboratory is usually done by a courier or a pneumatic tube delivery system. State-of-the-art systems of the latter type ensure gentle transfer, thereby avoiding hemolysis. Rapid transport and short storage times can improve the reliability of the laboratory results. The most important causes for alterations in specimen quality are: metabolism of blood cells, evaporation/sublimation, chemical reactions, microbiological decomposition, osmotic processes, effect of light and gas diffusion.

## 6.5 Conclusions

Cardiac markers have recently received a lot of attention with respect to their utility in point-of-care testing strategies. Several point-of-care devices have been introduced by different manufacturers. In the present studies, one of these was evaluated. The analytical performance of Innotracs Aio! 1<sup>st</sup> and 2<sup>nd</sup> generation cTnI, CK-MB, myoglobin and usCRP was investigated. Different sample matrix comparison studies were also carried out. The ability to work with whole blood samples saves time. According to the results of this study, it is possible to analyze cardiac markers and usCRP serum, plasma and whole blood samples by using the same system and by using the same sample for all analytes. The results demonstrate the very good analytical performance of Innotracs Aio! assays. Therefore, this system is particularly suitable for use in emergency rooms, coronary care units, satellite laboratories and central laboratories.

A clinical evaluation of 2<sup>nd</sup> generation of Innotracs Aio! cTnI assay and the utility of Cobas Integra latex CRP and more sensitive Innotracs Aio! usCRP assays in the investigation of patients suffering from acute atrial fibrillation was also performed. Additional studies will be needed to study these markers in other clinical conditions, including invasive procedures for coronary artery diseases.

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