

Cardiovascular Biomarkers for the Detection of Cardiovascular Disease

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1. Introduction

Cardiovascular disease (CVD) is the leading cause of death in the Western World. Evidence from post mortem studies demonstrated the presence of atheromatous lesions in coronary arteries in children (Stary 2000). It is often thought of as a disease associated with a modern sedentary lifestyle and a lipid abundant diet. Recently Allam and colleagues (Allam *et al.* 2011) have imaged fifty two ancient Egyptian mummies using multi slice computer tomography and identified atherosclerotic lesions (arterial wall calcification) in 45% of cases with calcification sites located in the aorta, coronary carotid, iliac and femoral arteries as well as in peripheral arteries.

1.1 Cardiovascular epidemiology

The incidence of CVD is higher than for any cancer or other non-CVD condition. CVD caused 29% of global deaths in 2004. It is predicted that by 2030 23 million people will die from a CVD. Data from the USA suggests that CVD was responsible for 34% of deaths in 2006 and over 151,000 Americans who died were <65 years old. The incidence of CVD is declining in the Western World even though rates of lifestyle associated risk factors such as obesity smoking and type II diabetes mellitus are increasing. The decline is in part due to advances in therapeutic and invasive intervention. In creating better outcomes for those with acute cardiac conditions, patients develop heart failure which requires longer term treatment and monitoring and may in fact be a greater health burden than the acute events themselves.

1.2 Cardiovascular pathophysiology

The blood vessels (figure 1) of the cardiovascular system are comprised of three layers, the tunica intima (endothelium), tunica media (concentric smooth muscle) and tunica adventia (longitudinal collagen fibres). The three layers are analogous to the endo-, myo- and epicardium of the heart respectively.

Plaque formation (atherogenesis) is initiated with damage to the endothelium. Cholesterol rich low density lipoprotein (LDL) particles enter the intimal layer via the LDL receptor protein (Brown and Goldstein 1979), a mosaic cell surface protein that recognizes

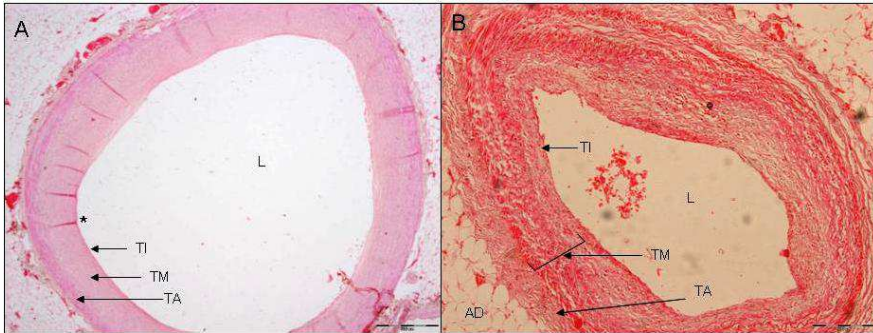


Fig. 1. A) Low powered haematoxylin and eosin (H&E) histological photomicrograph of a normal human artery (x40). B) Medium powered view (x200). AD, adipocytes; L, lumen; *, processing artefact; TI, tunica intima; TM, tunica media; TA, tunica adventitia.

apolipoprotein B100 embedded in the LDL particle. It also recognizes apolipoprotein E found in chylomicrons and very low density lipoprotein remnants, or intermediate density lipoprotein. Macrophage cells accumulate oxidized lipid independently of the LDL receptor by endocytosis. This results in formation of juvenile raised fatty streaks within the endothelium. The macrophage release their lipid content and cytokines into the intima. Cytokines stimulate intimal thickening by smooth muscle cell proliferation, which then secrete collagen, causing fibrosis (figure 2). The lesion appears raised and yellow.

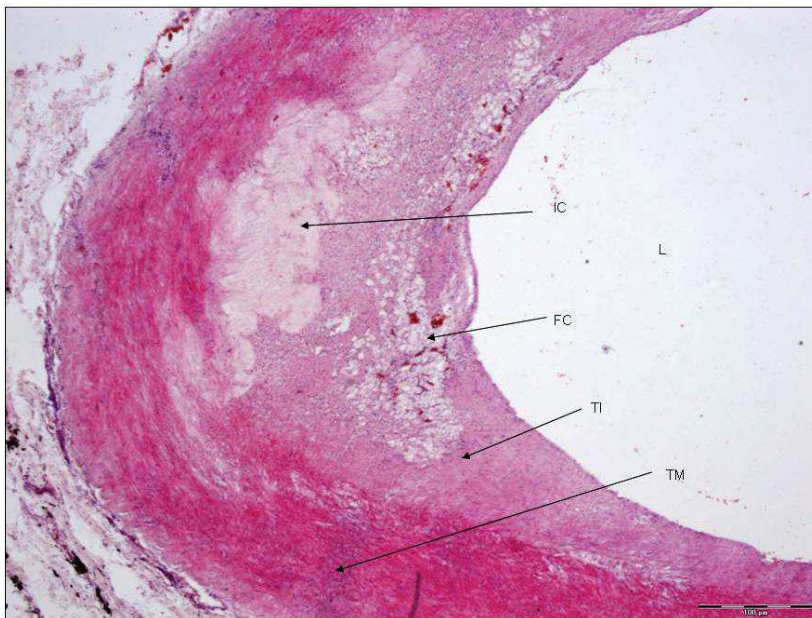


Fig. 2. Medium powered H&E histological micrograph of an intimal lesion (x200). FC, foam cell infiltrate; IC, intimal calcification; L, lumen; TI, tunica intima; TM, tunica media.

As the lesion develops, the medial layer of the vessel wall atrophies and the elastic lamina becomes disrupted. Collagen forms a fibrous cap over the lesion that appears hard and white (known as a fibrolipid plaque). The plaque contains macrophage laden with lipid (foam cells) as well as extracellular or 'free' lipid within the lesion. The endothelium is now in a fragile state. Ulceration of the cap occurs at weak points such as the shoulder region, near the endothelial lining. Rupture to the cap can cause turbulent blood flow in the lumen. The exposed lipid core causes aggregation of platelets and development of a thrombosis. This lesion grows due to further platelet aggregation and is responsible for narrowing of the lumen of the artery resulting in localized ischemia. Distal embolization of a piece of such thrombus may travel downstream and can completely occlude smaller arteries.

The symptomatic part of the continuum is known as the acute coronary syndrome (ACS) which is due to the rupture/erosion of the plaque. This produces, depending on the plaque size, vascular anatomy and presence of collateral vessels, a mismatch between the supply and demand for oxygen. A net reduction in supply compared to the demand results in ischemia. Tissue hypoxia proceeds resulting in inadequate blood/oxygen perfusion. If blood flow is not re-established, cardiac cell necrosis will occur. Post AMI survival results in remodelling processes in the myocardium and the development of cardiac failure.

2. Cardiovascular biomarkers

Cardiac biomarkers have played an important role in the diagnosis and management of patients with CVD since the 1950's (Gaze and Collinson 2005). The challenge has been the identification of a cardiospecific biomarker. A number of biomarkers are available which can be used for diagnosis and management of patients with CVD (figure 3). Many are not clinically measured due to cost and lack of an evidence base.

The cardiovascular biomarkers essentially fall into three categories. Those that identify patients at risk of atherosclerosis; those associated with plaque destabilisation and those which indicate rupture of the plaque and the detection of necrosis and cardiac insufficiency.

2.1 Plaque formation biomarkers

2.1.1 Cholesterol and lipid fractions

Cholesterol and lipoproteins such as oxidized low density lipoprotein (LDL), small dense LDL and intermediate density lipoprotein are atherogenic. The laboratory repertoire common for the determination of plaque development and progression include the measurement of cholesterol, triglycerides, high density lipoprotein (HDL) and calculation of LDL (Friedewald *et al.* 1972). Increasingly apolipoprotein-AI and lipoprotein B100 are measured. Direct measurement methods of LDL better reveal individuals at risk of CVD but are less often promoted due to the higher cost. A joint consensus statement by the American Diabetes Association and American College of Cardiology stated that direct LDL particle measurement by nuclear magnetic resonance (NMR) is superior to calculated LDL for assessing individual risk of CVD (Brunzell *et al.* 2008). Other lipid components such as small dense LDL and intermediate density lipoprotein which are particularly atherogenic can be measured but this is not common practice. Lipoprotein (a) contains a central core of LDL which is covalently bonded to a polypeptide chain of apolipoprotein a. This peptide shares sequence homology with plasminogen and has been proposed as a mechanism linking plaque rupture and the development of thrombosis.

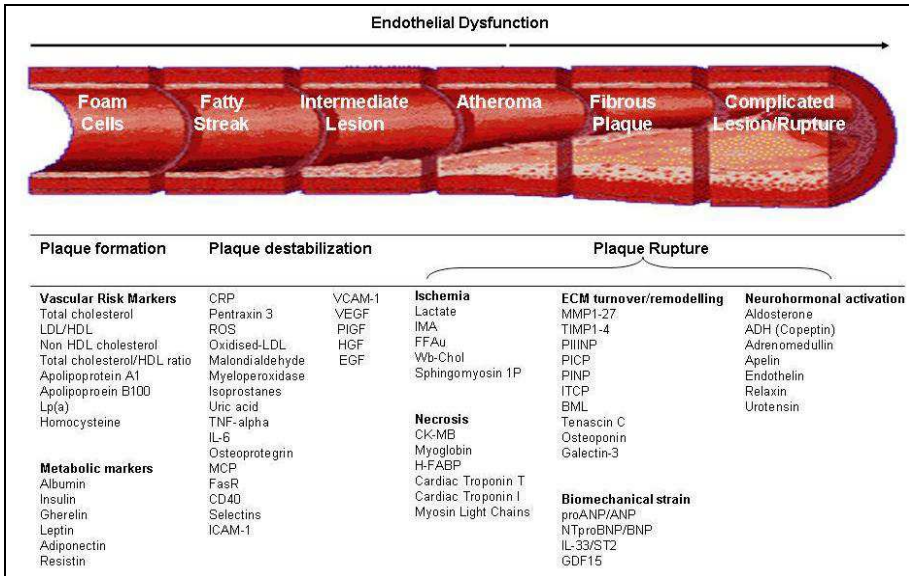


Fig. 3. Cardiovascular biomarkers for the assessment of cardiovascular disease. ADH, antidiuretic hormone; BML, basement membrane laminin; CRP, c-reactive protein; CD-40, cluster of differentiation-40; CK-MB, creatine kinase MB; EGF, epidermal growth factor; FFAu, unbound free fatty acid; GDF-15, growth differentiation factor 15; HDL, high density lipoprotein; HGF, hepatic growth factor; ICAM-1, intracellular adhesion molecule 1; ICTP, type I collagen telopeptide, IL-6, interleukin 6; IL-33, interleukin 33; IMA, ischemia modified albumin; LDL, low density lipoprotein; Lp(a), lipoprotein a; MCP, monocytes chemoattractant factor; MMP1 to 27, matrix metalloproteinase 1 to 27; PIINP, procollagen III aminopropeptide; PICP, procollagen type I carboxy-terminal peptide; PINP, procollagen type I amino-terminal peptide; TIMP1 to 4, tissue inhibitors of matrix metalloproteinase 1 to 4; TNF- α , tumour necrosis factor alpha; VCAM-1, vascular cell adhesion molecule 1; VEGF, vascular endothelial growth factor; VLDL, very low density lipoprotein.

The biggest single cause of plaque initiation and inflammation is modified and oxidized LDL; however plaque development, progression and destabilisation are multifactorial. A number of assays exist for modified and oxidized LDL however they have not made the transition to automated immunoassay analysers. A large number of studies have been performed using oxidized LDL, however when this is translated to routine clinical use in the general chest pain population, the diagnostic and prognostic efficiency of the marker is questionable (Gaze *et al.* 2006b).

2.1.2 Homocysteine

Homocysteine is a sulphur containing amino acid (2-amino-4-sulfanylbutanoic acid, $C_4H_9NO_2S$), closely related to cysteine but with an additional methylene (CH_2) group. Homocysteine is not derived from dietary sources but rather it is biosynthesized from methionine (figure 4). Homocysteine can be recycled back to methionine or converted to cysteine catalysed by cystathionine β -synthase with pyridoxine (vitamin B_6) as a cofactor.

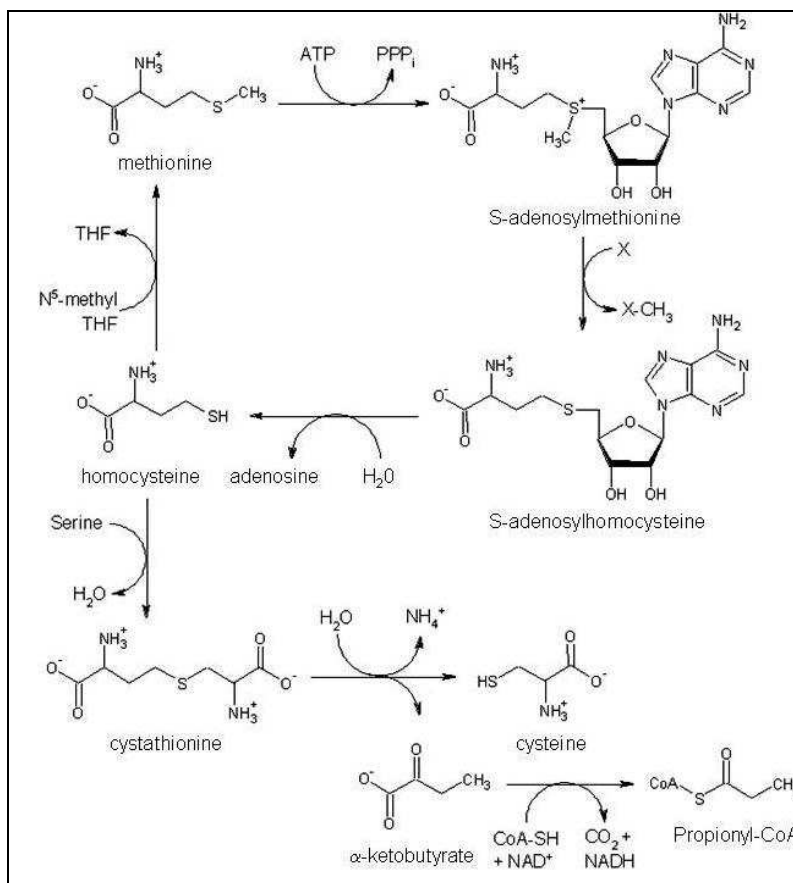


Fig. 4. Metabolism of Homocysteine. Homocysteine is biosynthesised by demethylation of dietary acquired methionine. Methionine receives adenosine triphosphate (ATP) to give S-adenosylmethionine (SAM), catalysed by S-adenosylmethionine synthetase. SAM transfers a methyl group to X (phospholipid, protein, myelin, catecholamine, polysaccharides, creatine, carnitine, DNA, RNA) to form X-CH_3 . The adenosine is hydrolysed to give homocysteine which can be converted back to methionine via tetrahydrofolate (THF) or converted to the amino acid cysteine.

Homocysteine is biosynthesised by demethylation of dietary methionine. Methionine receives adenosine triphosphate (ATP) to give S-adenosylmethionine (SAM), catalysed by S-adenosylmethionine synthetase (EC 2.5.1.6). SAM transfers a methyl group to X (phospholipid, protein, myelin, catecholamine, polysaccharides, creatine, carnitine, DNA, RNA) to form X-CH_3 . The adenosine is hydrolysed to give homocysteine which can be converted back to methionine via tetrahydrofolate (THF) or converted to cysteine. Methionine salvage utilises N^5 -methyl tetrahydrofolate as the methyl donor and vitamin-B12 (cobalamin) associated enzyme tetrahydrofolate methyltransferase (EC 2.1.1.13).

Deficiencies in folic acid (vitamin B₉), pyridoxine (vitamin B₆) or cobalamin (Vitamin B₁₂) can give rise to a high plasma homocysteine. Homocysteinaemia is associated with disruptive damage to the endothelium. *In vitro* studies have demonstrated that homocysteine can induce direct damage to endothelial cells, increase platelet activity, increase collagen synthesis, smooth muscle cell proliferation and has pro-coagulant effects.

The Norwegian Vitamin Trial (NORVIT, clinicalTrials.gov # NCT00266487) was a large study of approximately 5000 post AMI subjects who were treated to reduce plasma homocysteine concentrations (Bonna *et al.* 2006). Patients were randomised to receive one of four daily treatment regimens; 0.8 mg folic acid, 0.4 mg vitamin B₁₂ and 40 mg vitamin B₆; 0.8 mg folic acid and 0.4 mg vitamin B₁₂; 40 mg of vitamin B₆ or finally a placebo. Patients were followed for a median of 40 months for recurrent AMI, stroke and sudden cardiac death. Overall, plasma homocysteine concentrations fell by 27% on patients taking folic acid and vitamin B₁₂. Risk ratio for the primary end-points was 1.08 (95%CI 0.93-1.25). Treatment with vitamin B₆ showed similar results with no benefit with regard the primary end point. Patients who received the polypharmacy of folic acid, vitamin B₁₂ and vitamin B₆ were actually at increased risk of a cardiac event. Conversely in the HOPE-2 study, reduction of homocysteine by polypharmacy reduced the risk of stroke by 25% (Lonn *et al.* 2006). Homocysteine is elevated in patients with chronic kidney disease (CKD). In the renal HOPE-2 study (Mann *et al.* 2008) 307 patients with CKD (eGFR <60 ml/min) at high risk of CVD were randomised to folic acid and B vitamin polypharmacy and a further 312 received placebo for 5 years. The treated group showed a reduction in mean homocysteine from 15.9 $\mu\text{mol/L}$ to 11.9 $\mu\text{mol/L}$ ($p < 0.001$) but did not reduce in the placebo group. Treatment with polypharmacy to reduce plasma homocysteine in CKD patients did not reduce the risk of cardiovascular death, recurrent AMI or stroke.

2.2 Plaque destabilisation biomarkers

Endothelial injury results in chemotactic recruitment of mononuclear cells within the plaque. Interleukin-6 (IL-6) concentrations are increased in patients with established CVD as well as those who have an acute coronary syndrome. Kinetically, IL-6 rises and falls rapidly but produces an acute phase response. This in turn initiates the production of acute phase reactants such as C-reactive protein (CRP) and serum amyloid A protein.

2.2.1 C-reactive protein

C-reactive protein (CRP) is a hepatically derived pentraxin of 23KDa released from the liver during an acute phase response. Physiologically CRP resembles antibody and binds to phosphocholine expressed on the surface of necrotic or dying cells and some bacteria. CRP activates the complement system via the C1Q complex. The primary use of CRP is as a marker of infection, during which concentrations can be 100-fold higher than the limits of normal. CRP rises in response to an increase in plasma IL-6 produced by macrophages. CRP has a long plasma half-life and is now understood to be a mediator as well as a marker of atherothrombotic disease (Ridker 2003). In CVD patients, values are typically in the range 0-3 mg/L. Due to the development of sensitive assays, which can accurately report below 5 mg/L, highly sensitive CRP has been demonstrated to be a strong predictor of future cardiovascular events. Sensitive CRP has demonstrated better predictive power for adverse

cardiac events than low density lipoprotein (LDL) cholesterol (Ridker 2003). A large number of studies have demonstrated that CRP is related to the risk of subsequent cardiac events in both patients with and without pre-existing CVD.

Plasma CRP concentrations of <1, 1 to 3, and >3mg/L correspond to low, moderate, and high-risk groups for future cardiovascular events respectively. Individuals with low LDL (<130 mg/dL; 3.37 mmol/L) cholesterol but high plasma CRP levels (>3mg/L) represent a high-risk group often missed in clinical practice. The addition of CRP to standard cholesterol evaluation may provide an improvement to global risk prediction and monitoring compliance with preventive intervention. Currently CRP is used as a risk predictor for primary prevention intervention. The use of aspirin is associated with a reduction in CRP concentrations and subsequent lower CVD event rate. The use of statin therapy (3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase inhibitors, EC 1.1.1.88) for hypercholesterolaemia is also associated with a reduction in serum CRP; suggesting statins themselves also exert a direct anti-inflammatory effect.

Whilst IL-6 may be a more sensitive marker for CVD prediction, it is less readily available in clinical chemistry laboratories as a routine assay and is harder to measure due to analyte stability and storage conditions.

2.2.2 Lipoprotein-associated phospholipase A2

Lipoprotein-associated phospholipase A2 (Lp-PLA₂) or platelet-activating factor (PAF) acetylhydrolase (EC 3.1.1.47) is a 45kDa intracellular phospholipase which hydrolyzes phospholipids in cellular membranes. It catalyses the degradation of PAF to inactive products by the hydrolysis of an acetyl group at position sn-2, resulting in the production of LYSO-PAF and acetate. It is encoded by the PLA2G gene at loci 6p21.2-p12 and consists of 441 amino acid residues. Attached to LDL, it is responsible for the hydrolysis of oxidised phospholipid in the LDL molecule. Less than 20% of Lp-PLA₂ is associated with HDL. Using immunohistochemical staining with monoclonal antibodies, it has been demonstrated that juvenile plaques show less staining for Lp-PLA₂ than rupture-prone or ruptured plaques (Kolodgie *et al.* 2006). In patients with cardiovascular metabolic syndrome, modification of Lp-PLA₂ through treatment of obesity, hyperlipidaemia and diabetes mellitus (Lp-PLA₂ is independent of insulin resistance), who would otherwise be at intermediate risk of a future cardiac event has shown promise. In a large meta analysis of over 79,000 participants (from 32 prospective studies) Lp-PLA₂ activity and mass demonstrate associations with CVD akin to the risk associated with non-HDL cholesterol or systolic blood pressure (Thompson *et al.* 2010). Lp-PLA₂ is a potential therapeutic target for CVD.

2.2.3 Myeloperoxidase

Myeloperoxidase (MPO) is a 144 KDa (144,000 Dalton) peroxidase enzyme (EC 1.11.1.7) present in abundance in neutrophil granulocytes and stored in azurophilic granules. The gene locus for MPO is 17q23.1. MPO is comprised of two 15 KDa light chains and two variable weighted glycosylated heavy chains which bind haem pigment responsible for the green colour in puss and mucus. The enzyme catalyses the conversion of chloride cosubstrate with hydrogen peroxide to chlorinating oxidants such as hypochlorous acid (hypochlorite).

MPO is a sensitive predictor of AMI. Patients with unstable plaques have higher macrophage and neutrophil counts than patients with stable coronary artery disease. MPO degrades the collagen layer of the atheroma making the lesion susceptible to erosion or rupture. MPO is a risk factor for long term mortality. A study of 885 patients receiving coronary angiography have been studied and followed for 13 years for cardiac mortality. MPO independently predicted coronary artery disease. The addition of CRP measurement improved risk prediction. Patients with elevated MPO or CRP had a 5-fold risk of cardiac mortality compared to a 4 fold risk of cardiac mortality if both markers were elevated (Heslop *et al.* 2010).

2.3 Plaque rupture

The classical view of plaque rupture is an increasingly outwardly growing plaque reducing the luminal space and thus limiting blood flow. It is now known that 80% of plaques that cause an ACS event are actually not flow limiting stenoses. Markers of coagulation are raised in the ACS patient and these include prothrombin fragment 1 and 2 and factor VIIa. Thrombin/antithrombin complex is often elevated and associated with increased plasma cardiac troponin. Inflammatory cells contribute to plaque rupture. Foam cells derived from macrophages congregate in the shoulder region of the vulnerable plaque. At this time, the foam cells express matrix metalloproteinase enzymes (MMPs). These proteolytic enzymes can inactivate zymogens (proMMP) enzymes as well as angiotensin II. A number of MMPs are synthesised and released by macrophage. These include interstitial collagenase (MMP-1), gelatinase enzymes (MMP-2 and MMP-9) and stromelysin (MMP-3). MMP-1 expression is increased by oxidised LDL interferon- gamma (IFN- γ), IL-1 and tumour necrosis factor alpha (TNF- α) (Rajavashisth *et al.* 1999).

2.4 Chest pain

Chest pain admissions are resource intensive and account for a large proportion of emergency care finances. >11 million patients with chest pain present annually to the emergency department in the USA. A final diagnosis of ST segment elevation myocardial infarction (STEMI); conferring the highest risk occurs in only 3% of chest pain presentations (Pope *et al.* 2000). Similar data are given for the UK. 700,000 annual patient attendances in England and Wales are due to chest pain and it is responsible for one quarter of hospital admissions (Goodacre *et al.* 2005). Patients demonstrating STEMI benefit from immediate pharmacological thrombolytic therapy or percutaneous coronary intervention with or without stent placement in order to establish coronary reperfusion. A large proportion of the remaining 97% of chest pain admissions do not have a final diagnosis of ACS (Storrow and Gibler 2000). Biomarkers are especially important in identifying those patients without classical electrocardiographic (ECG) changes who may have a non ST segment elevation myocardial infarction (NSTEMI).

2.5 Biomarkers of cardiac ischemia

The detection of cardiac ischemia prior to myocardial infarction is both a major diagnostic and therapeutic challenge. Detection of cardiac ischaemia prior to necrosis is a potential therapeutic (pharmacological or invasive) target to limit or prevent cellular necrosis. There is currently no gold-standard test for ischemia and currently the only method of detection in

routine clinical use is to observe ST segment changes on the ECG. Historically serum lactate measurements were used however lactate has an extremely low cardiac specificity. Candidate markers including whole blood choline and unbound free fatty acids have been proposed however only ischemia modified albumin (IMA[®]) has taken the translational step from a basic science investigation into an FDA and CE marked commercial assay.

2.5.1 Whole blood choline

Choline is a product of phosphodiesteric cleavage of membrane phospholipids such as phosphatidylcholine and sphingomyelin; catalysed by phospholipase D (EC 3.1.4.4). Physiologically choline provides cell structural integrity, is the precursor for acetylcholine production and a source of methyl groups that participate in the S-adenosylmethionine synthesis pathway.

Whole blood (WBCHO) and plasma choline concentrations increase after stimulation of phospholipase D and the activation of coronary plaque cell surface receptors or ischemia. Phospholipase D activation in coronary plaques causes stimulation of macrophage by oxidised LDL, secretion of MMP and activation of platelets. WBCHO can be measured by high performance liquid chromatography coupled to mass spectrometry (HPLC-MS). In a study of over 300 patients with suspected ACS, WBCHO measured at admission was a significant predictor of cardiac death, cardiac arrest, arrhythmia, heart failure or the need for percutaneous coronary intervention at 30 day follow up (Danne *et al.* 2003). The predictive power was enhanced by the addition of either cTnT or cTnI and served not as a marker of myocardial cell necrosis but identified patients at high risk with unstable angina. WBCHO is therefore a better predictive tool than plasma choline for early risk stratification in patients who are cardiac troponin negative on admission. The current detection methodology using HPLC-MS is not suitable for urgent clinical use.

2.5.2 Unbound free fatty acids

Free fatty acids are produced from the breakdown of triglyceride. The majority of free fatty acids (FFA) circulate bound to albumin with a very small percentage appearing as the unbound free fatty acid (FFAu) form (Richieri and Kleinfeld 1995). The circulating level of FFA is limited to the availability of the albumin binding sites. The mechanism of FFAu release is not fully understood however increased catecholamines following cardiac ischemia may activate FFA release following lipolysis in adipocytes. FFAu are 14-fold higher post PCI, compared to pre procedural concentrations and were higher in those with associated ischemic ST segment changes on the ECG (Kleinfeld *et al.* 1996). An assay using recombinant fatty acid binding protein bound to a fluorescent tag (ADIFAB) (Richieri *et al.* 1999; Richieri *et al.* 1992) has been developed and a second generation assay using a fluorescent molecular probe (ADIFAB2) and a portable reader makes this a potential early marker for the point of care setting. Whilst this marker shows promise in the early phase of ischemia induced ACS, further trials are required to evaluate the diagnosis and prognostic value of FFAu in the chest pain population.

2.5.3 Ischemia modified albumin

A reduction in oxygen supply versus demand causes localized acidosis and the generation of free radicals. Copper and zinc ions, normally bound to proteins in the plasma are released

from protein binding sites to circulate in the free form (Chevion *et al.* 1993; Cobbe and Poole-Wilson 1980; McCord 1985). The N-terminus of albumin binds transition metals such as, Co^{2+} , Ni^{2+} and Cu^{2+} ions (Sadler *et al.* 1994). The N-terminus however, is susceptible to biochemical alteration (Chan *et al.* 1995). Following a period of ischemia, a reduction in the ability of albumin to bind cobalt is apparent. This is the basis of the albumin cobalt-binding test (ACB[®] test) for IMA (figure 5) (Bar-Or *et al.* 2001). The assay measures the ability of albumin to bind a known amount of free cobalt added to the sample. Dithiothreitol (DTT) is added which binds the remaining unbound cobalt. The colorimetric change is read spectrophotometrically.

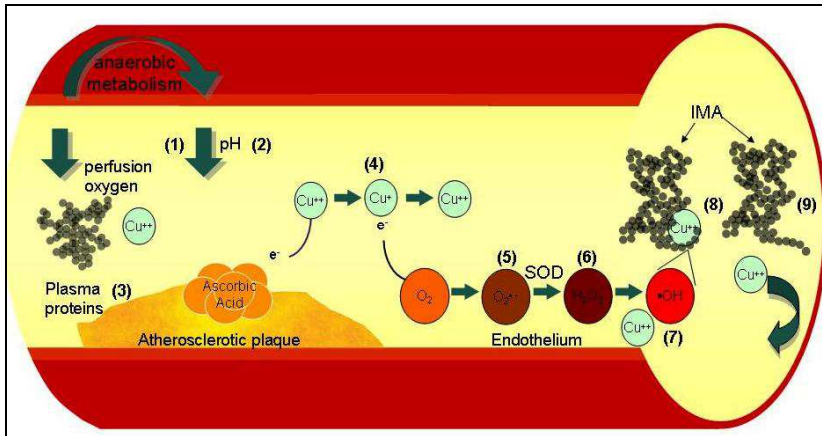


Fig. 5. Mechanism of Ischemia Modified Albumin generation: Reduced blood flow results in tissue hypoxia¹ resulting in a lower pH². Cu^{++} is released from plasma proteins³. In the presence of ascorbic acid, Cu^{++} is converted to Cu^+ . Cu^+ reacts with O_2 to form $\text{O}_2^{\bullet-}$ ⁵. Superoxide dismutase (SOD) dismutates the $\text{O}_2^{\bullet-}$, forming H_2O_2 ⁶. Normally, H_2O_2 is harmlessly degraded to H_2O and O_2 by catalase. However, in the presence of metals such as copper or iron, H_2O_2 undergoes the Fenton reaction, forming OH^\bullet radicals⁷. Free Cu^{++} is scavenged by HSA, where it binds tightly to the N-terminus. OH^\bullet are highly reactive capable of damaging nucleic acids, lipids and proteins, including albumin. One site of damage is the N-terminus, where OH^\bullet alter the amino acids⁸. Altered albumin is incapable of binding Cu^{++} . Bound copper is released from the albumin⁹, where it may be taken up again by the N-terminus of another albumin in a chain reaction which repeats the process of albumin binding and OH^\bullet formation.

Kinetically there is a rapid increase in serum IMA. In an angioplasty model, IMA values decreased at 6 hours post inflation and returned to baseline by 24 hours (Bar-Or *et al.* 2001; Sinha *et al.* 2006). The magnitude of IMA elevation correlates with the number and frequency of balloon inflations (Quiles *et al.* 2003), the presence of collateral vessels (Garrido *et al.* 2004), the need for revascularisation (Dusek *et al.* 2006) and is parallel to the transmural lactate gradient (Sinha *et al.* 2006). The half-life of human albumin is 19-20 days. A truncated modified form would remain in the circulation following production for a number of days. The rapid reduction in IMA following the ischemic event suggests the modification is transient and reversible. IMA alone has a diagnostic sensitivity of 82% with

46% specificity if measured in the first three hours of presentation to the ED (Sinha *et al.* 2004). The combination of a positive ECG, cTnT (>0.05 µg/L) and IMA however demonstrated a 95% sensitivity for diagnosis of ACS. In a study of 538 patients admitted to a chest pain evaluation unit, admission measurement of IMA plus cTnT had 100% sensitivity for prediction of a final diagnosis of AMI (Collinson *et al.* 2006). IMA however lacks specificity for cardiac tissue and elevations can occur in a number of non-ACS conditions including autoimmune diseases, cancers, hepatic cirrhosis, haemorrhagic stroke, renal disease, peripheral vascular disease and polytrauma.

Elevated IMA and cTnT on admission predicts 20% of major adverse cardiac events. IMA appear to work best in conjunction with other tests such as cTn or the ECG. One year follow up of 208 patients presenting to the emergency department with chest pain demonstrated a survival disadvantage in those patients with a serum IMA at admission greater than the median concentration of the population group (Consuegra-Sanchez *et al.* 2008). There were 17 vs 9 (p=0.08) episodes of angina, 21 vs 10 combined endpoints (p=0.03) and 12 vs 4 12 month all-cause mortality (p=0.03) in the IMA positive group compared to the IMA negative group.

Although the assay has been cleared for *in vitro* diagnostic use, two problems remain to be resolved with IMA. First is the mechanism by which IMA is generated and the relationship to the underlying pathophysiological mechanisms during ischemia. The second is the assay format; Very low albumin (Zapico-Muniz *et al.* 2004; Gaze *et al.* 2006a) and the presence of co-existing lactic acidosis affect assay performance. Finally samples need to be analysed within a STAT fashion due to the instability of IMA (Gaze 2009).

2.6 Biomarkers of cardiomyocyte necrosis

2.6.1 Cardiac troponins

Markers of cardiac cell necrosis can be split into those located in the cytoplasm of the cell and those that form the structural apparatus which control muscle contraction. The cytosolic component contains myoglobin, LD, CK, CKMB as well as fatty acid binding protein (FABP). The structural compartment consists of the troponin proteins troponin T and troponin I (figure 6). Troponins have tissue specific isoforms. Cardiac troponin T (cTnT) and cardiac troponin I (cTnI) and are expressed uniquely in myocardial cells, whilst predominantly structural both cardiac troponins (cTn) have a small unbound cytosolic fraction (between 6% and 8% for cTnT and 2.8%-8.3% for cTnI).

The measurement of cardiac troponin is now considered the “Gold Standard” test for myocardial necrosis and the globally accepted ESC/ACCF/AHA/WHF universal definition of AMI has been widely adopted (Thygesen *et al.* 2007). The success of the measurement of troponin is due to the analytical and clinical sensitivity and specificity of the assays and the large evidence base for outcome prediction.

The sensitivity of any biochemical test is dependant on its ability to discriminate between the normal and abnormal state. A test that is elevated in all patients with a condition and is normal in all controls will have a sensitivity of 100%. Sensitivity is influenced by the background level of signal in the normal state and is referred to as noise. This in turn is compared to the change of signal in the abnormal state, known as the signal to noise ratio. It

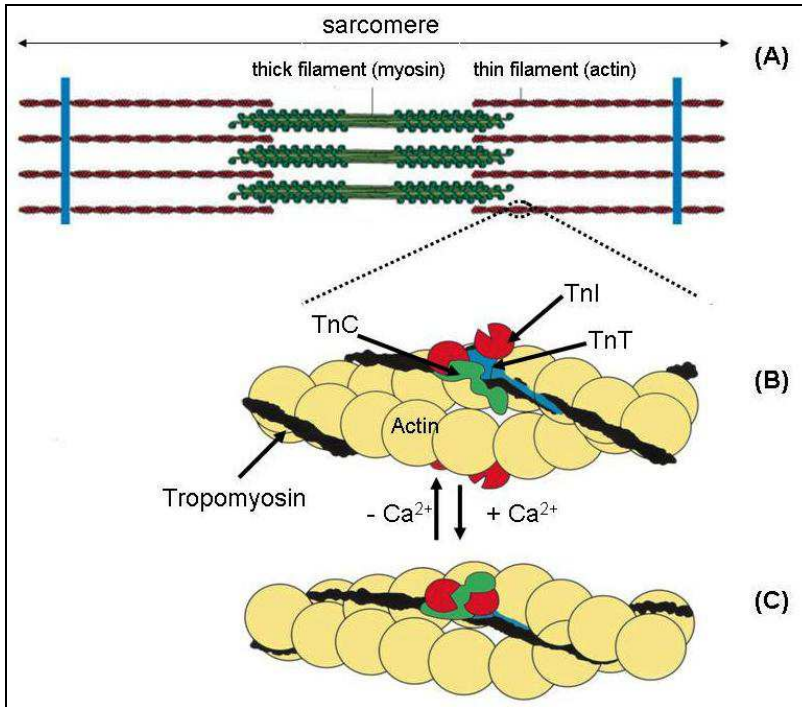


Fig. 6. (A) Anatomy of the sarcomere. (B) Structure of the troponin-tropomyosin complex. Composed of three proteins, troponin T (TnT, 39.7 kDa), which binds tropomyosin, troponin C the Ca²⁺ binding element (TnC, 18 kDa) and troponin I, the Mg²⁺ dependent ATPase inhibitor (TnI, 22.5 kDa), shown in the resting state. (C) During muscle contraction, Ca²⁺ is released from the t-tubules, and bind TnC, leading to a conformational change in the complex

was presumed that the circulating concentration of cTn in apparently healthy people was absolute zero, due to the intracellular location of the proteins. With the advent of more sensitive immunoassay methods the current commercial cTn assays demonstrate a background level in the circulation that appears to be determined by the detection limit of the assay. Although there may be a background level of cTn in the circulation, this is undetectable in the majority of cases by the assays currently commercially available. Elevation occurs only where there is myocardial damage. The combination of low background noise with cardiospecificity means that although in absolute terms the amount of cTn released is less than the amount of CKMB, the ability to detect it is much greater, and the signal to noise ratio is much higher. Using cTn specific assay, it has been demonstrated that elevations of the cardiac isoform do not occur where there is pure skeletal muscle trauma. In a study of 696 apparently healthy individuals aged 18-84 years, of which 45% were male, two commercial cTnI assays have demonstrated a gender difference, with a higher upper limit of normal in males. The other five cTnI assays and the cTnT assay tested did not demonstrate a gender difference. In the same population, CKMB mass was significantly higher in males compared to females (Apple *et al.* 2003).

Clinical studies of cTn have concentrated on the diagnostic and prognostic utility of the assays. Initial studies showed that cTnT could be detected in a significant proportion of patients who were classed as having unstable angina using the conventional World Health Organisation (WHO) criteria for myocardial infarction. In these studies, both short term and long term follow up showed that an elevated troponin was associated with an increased risk of cardiac event (Hamm *et al.* 1992).

Determination of cTn is by immunoassay. For both cTnT and cTnI, several generations of assay have existed and have been comprehensively reviewed (Collinson *et al.* 2001a). The measurement technology utilising electrochemiluminescence for cTnT is supplied by one manufacturer (Roche Diagnostics) due to patent restrictions on the antibodies selected for the assay. Measurement of cTnI however is available on a range of immunoassay platforms from different manufacturers. Due to a lack of assay standardisation both for a common calibrator and for antibody selection, comparison between cTnI methods is often problematic. Despite this, a similar series of studies for the different cTnI methods has shown that cTnI elevation predicts adverse cardiac events in patients with unstable angina (Antman *et al.* 1996;Galvani *et al.* 1997;Collinson *et al.* 2001b). The use of cTnT and cTnI determinations results in approximately reclassification of 33% of unstable angina patients into patients who have suffered an AMI.

A number of manufactures have reformulated their assays or introduced high sensitive cTn with the aim of meeting the analytical requirements suggested in the universal definition of AMI. The introduction of such assays provides challenges both in terms of the analytical performance and validation/interpretation procedures for the laboratory and the relevance in the clinical setting. Many of these assays can now demonstrate a normal distribution of detectable cTn concentrations within a reference population (Collinson *et al.* 2009a) (Apple *et al.* 2010;Mingels *et al.* 2009) allowing more robust calculation of the 99th percentile concentration to define normality. These assays also allow the calculation of the biological variability of cardiac troponin (Wu *et al.* 2009).

The introduction of high sensitivity cTn assays will affect the clinical interpretation results. There will be a greater number of positives outside the remit of ACS, but within the ACS population, it may be possible to diagnose AMI earlier. Using the Centaur TnI-Ultra a 99th percentile of 0.039 µg/L (39ng/L) has been obtained from a population of 309 (41% male) apparently healthy individuals highly screened for cardiac risk factors. These included no history of vascular disease, diabetes mellitus, hypertension, or heavy alcohol intake, no cardiac medication, no renal failure (eGFR >60 mL/min/1.73 m²), no significant cardiac disease on echocardiography, with a left ventricular ejection fraction of >50% (Collinson *et al.* 2009b). Within the reference population, cTnI was completely undetectable in 25 subjects and considered negative in 53%. There was no correlation between cTnI and age and there were no significant differences between gender (Collinson *et al.* 2009b). Using the same population, the researchers have defined the 99th percentile of the hs-cTnT assay (Roche Diagnostics) to be 15.5 ng/L and 42 ng/L for the Beckman Coulter enhanced AccuTnI assay. Of note are the differences between the numbers of detectable cTn by assay within the same reference population. Detectable concentrations were achieved in 58% of samples when measured using the hs-cTnT assay, 68% using the TnI-Ultra and 98% using the Access AccuTnI.

The prognostic value of the AccuTnI assay has been demonstrated in the Orbofiban in Patients with Unstable Coronary Syndromes (OPUS)-Thrombolysis in Myocardial Infarction (TIMI) 16 (OPUS-TIMI 16) clinical trial (Morrow *et al.* 2003). A cut point of $\geq 0.04 \mu\text{g/L}$ ($\geq 40 \text{ ng/L}$) was an independent predictor of the 30-day risk of death (odds ratio, OR), 4.1; (95%CI 1.2-13.8), death and AMI (OR, 3.4; 95%CI, 1.8-6.7), and death, MI, or ; 95% confidence interval, 95%CI) need for urgent revascularisation (OR, 2.3; 95%CI, 1.5-3.6) and was also associated with risk of death or development of a further AMI at 10 months.

Using the TnI-Ultra compared to the previous Centaur assay, Melanson and colleagues (Melanson *et al.* 2007) compared the rates of positivity obtained between the two assays over a 24 hour period in 103 patients who presented initially with a negative cTnI but converted to cTnI positive. TnI-Ultra was positive before cTnI in 66(64.1%) of cases demonstrating superior sensitivity. Furthermore, a single admission cTn measurement using hs-cTnI may be a useful rule-out test irrespective of the length of chest pain (Keller *et al.* 2009). Reichlin and colleagues also demonstrated excellent diagnostic performance of sensitive cTn assays at presentation (Reichlin *et al.* 2009).

It has been postulated that sensitive cTn, given the detection in asymptomatic healthy people without a history of cardiovascular disease; may be of value in identifying subjects at long term risk of ACS, i.e. cTn may have a role in primary prevention (Apple 2011). In a large community cohort of adults > 65 years; baseline and delta change in serial hs-cTnT measurements are associated with heart failure and cardiac death (deFilippi *et al.* 2010). Further, the use of N-terminal pro-b-type natriuretic peptide (NTproBNP) nor high sensitive CRP added to the risk assessment. In a second cohort of >3500 subjects aged 30-65 years recruited to the Dallas Heart Study, demonstrable hs-cTnT occurred in 25% of subjects; with higher rates in men compared to women (37% vs 13%) respectively. (de Lemos *et al.* 2010). There was separation in rates of positivity in those aged <40 and >60 years of age. Elevated hs-cTnT was associated with structural heart disease (left ventricular hypertrophy and left ventricular systolic dysfunction) and chronic kidney disease. The overall cardiovascular burden as measured by hs-cTnT in primary care may indicate end organ cardiac damage.

2.7 Heart failure

Survival rates following AMI are increasing, primarily due to a combination of early diagnosis and a rapid therapeutic or invasive intervention to open occlusions and to salvage myocardial tissue. Patients with some degree of damaged myocardium however further develop cardiac failure. Cardiac failure is a pathophysiological state in which an abnormality of cardiac function is responsible for the failure of the heart to pump blood at a rate commensurate with the requirements of the metabolising tissues. Cardiac failure affects 0.4-2% of the population and the age adjusted mortality due to cardiac failure is increasing (Remme and Swedberg 2001). There is a 50% survival rate four years after diagnosis (Cowie and Zaphiriou 2002). Currently, the clinical diagnostic methodologies available are inadequate due to poor sensitivity and specificity. There is heavy reliance on the identification of heart failure from symptoms, ECG changes and/or chest x-ray diagnosis. The 'gold standard' for diagnosis of cardiac failure is currently cardiac imaging technology including echocardiography or magnetic resonance imaging; however these are often expensive and not always routinely available. A blood borne marker of cardiac dysfunction is therefore highly desirable.

B type natriuretic peptide (BNP) is a hormone secreted predominantly by ventricular wall myocytes in response to increased ventricular stretch. It is not stored following production, but is under constant renewal by transcription and translation of mRNA. A prohormone proBNP is produced within the myocyte. This prohormone is cleaved by the enzyme corin to give the active portion of the molecule, B type natriuretic peptide (BNP) and the N terminal portion of the hormone, NTproBNP. Both are available as routine biomarker assay tests on a range of immunoassay platforms. The half-life of the active BNP is short (20 minutes) compared to that of the NTproBNP (120 minutes).

Measurements of NTproBNP and BNP are used in the diagnosis of suspected acute or chronic cardiac failure. It has been demonstrated that the measurement of BNP/NTproBNP allows exclusion with a high certainty of left ventricular systolic dysfunction (LVSD) in patients presenting to primary care with symptoms suggestive of heart failure, mainly shortness of breath (McDonagh *et al.* 2008;Cowie *et al.* 1997;Zaphiriou *et al.* 2005). It has been suggested, therefore, that BNP/NTproBNP measurement should be used as the “gold standard” test for the detection of ventricular dysfunction. In a prospective randomised trial of diagnosis based on BNP with conventional clinical diagnosis in 452 patients, the diagnostic pathway including BNP produced reduction in hospital stay; stay in intensive care and lower cost but with equivalent mortality (Mueller *et al.* 2004).

The role of BNP/NTproBNP in patients presenting with ACS however remains more uncertain. Studies have shown that measurement of NTproBNP/BNP on admission in both selected and unselected suspected ACS patients can be used to predict outcome. A study of 2525 patients enrolled in a clinical trial (OPUS-TIMI 16) with a mixed population of ST segment elevation MI (STEMI), non ST segment elevation MI (NSTEMI) and unstable angina pectoris (UAP) showed that quartiles of BNP concentrations were predictive of survival. There was a 9% mortality rate in the highest quartile compared with less than 1% in the lowest quartile at 10 months follow up. The population was dichotomised into those with BNP above or below the cut point of 80pg/L. An elevated BNP ≥ 80 pg/mL showed a significantly greater risk of death, heart failure or MI both at 30 days and at 10 months (de Lemos *et al.* 2001). In a study of 666 patients admitted with AMI, BNP and NTproBNP were measured 24-96 hours from symptom onset. Subsequent death or heart failure was predicted by BNP/NTproBNP levels even when ejection fraction exceeded 40% (Richards *et al.* 2003). BNP/NTproBNP measurements have been combined with other biomarkers including CRP and cTn (Sabatine *et al.* 2002) or cTn alone (Jernberg *et al.* 2002) Measurements of BNP/NTproBNP provide additive information under these circumstances with the worst prognosis seen when the greatest number of biomarkers are elevated.

3. Conclusion

There are a number of cardiovascular biomarkers that aid the clinician in the identification of the risk of developing cardiovascular disease. Many are specialised tests and are not offered in the routine clinical chemistry laboratory. A large evidence base exists for those that are commonly measured as markers of the acute coronary syndrome. Biomarkers should be used in an algorithm along with the clinical signs and the results of other diagnostic modalities such as the ECG, stress test or echocardiography.

Any novel cardiac biomarker should prove clinically useful and cost effective with the ultimate aim of altering patient management and reduce mortality. The cardiac troponins are the most successful of the candidate markers and have influenced an alteration to the definition of acute myocardial infarction. The B type natriuretic peptide has a large evidence base for the diagnosis and more importantly the monitoring of heart failure. A reliable marker of cardiac ischemia remains to be demonstrated and is the focus of current research studies.

4. References

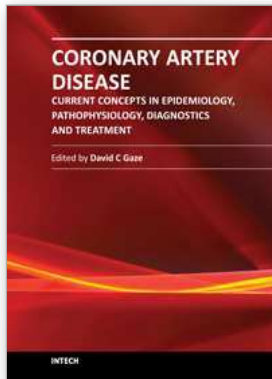
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Coronary Artery Disease - Current Concepts in Epidemiology, Pathophysiology, Diagnostics and Treatment

Edited by Dr. David Gaze

ISBN 978-953-51-0262-5

Hard cover, 272 pages

Publisher InTech

Published online 16, March, 2012

Published in print edition March, 2012

Cardiovascular disease is ranked as the leading cause of death world wide, responsible for 17.1 million deaths globally each year. Such numbers are often difficult to comprehend. Heart disease kills one person every 34 seconds in the USA alone. Although the leading killer, the incidence of cardiovascular disease has declined in recent years due to a better understanding of the pathology, implementation of lipid lowering therapy new drug regimens including low molecular weight heparin and antiplatelet drugs such as glycoprotein IIb/IIIa receptor inhibitors and acute surgical intervention. The disease burden has a great financial impact on global healthcare systems and major economic consequences for world economies. This text aims to deliver the current understanding of coronary artery disease and is split into three main sections: 1. Epidemiology and pathophysiology of coronary artery disease 2. Coronary artery disease diagnostics and 3. Treatment regimens for coronary artery disease

How to reference

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David C. Gaze (2012). Cardiovascular Biomarkers for the Detection of Cardiovascular Disease, Coronary Artery Disease - Current Concepts in Epidemiology, Pathophysiology, Diagnostics and Treatment, Dr. David Gaze (Ed.), ISBN: 978-953-51-0262-5, InTech, Available from: <http://www.intechopen.com/books/coronary-artery-disease-current-concepts-in-epidemiology-pathophysiology-diagnostics-and-treatment/cardiovascular-biomarkers-for-the-detection-of-coronary-artery-disease>

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