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## Symposium on ‘Diet and CVD’

### New and emerging risk factors for CVD

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Morphological and immunocytochemical studies have elucidated the complex processes involved in atherogenesis. The notion of plaque instability has emerged from this work and underscored the importance of inflammation in determining clinical complications associated with atherosclerosis, such as acute coronary syndrome. Cells of the immune system have been detected within atherosclerotic lesions and auto-antibodies directed against modified LDL and heat-shock proteins have been identified in the blood of individuals with atherosclerosis. The use of risk ‘engines’, e.g. the Framingham coronary risk score, has facilitated the identification of individuals at high risk, but the constituent classical risk factors used in these algorithms do not adequately differentiate individuals at moderate risk. As age is a major component of the equations used in these algorithms they are not particularly useful in young adults, and their applicability to non-Caucasian populations has been questioned. Biomarkers of early disease and plaque instability have therefore both been sought. Although some of these markers have been shown individually to be associated with a significant hazard ratio, no substantial improvement in discrimination has been demonstrated when they are incorporated into a risk ‘engine’. The latter has generally been assessed by receiver operator characteristic curve analysis, although this approach has been criticised. Other modalities, including imaging and functional assessments of vascular function, are now being developed for clinical use.

#### **CVD: Risk factors: Plaque instability: Biomarkers of early CVD**

The UK prevalence of cardiovascular mortality remains amongst the highest in the world, accounting for approximately one-third of all deaths<sup>(1)</sup>. Atherosclerosis is now recognised as a chronic inflammatory condition, and remains as the major cause of CVD<sup>(2)</sup>. It is characterised by luminal narrowing of arteries and is associated with the deposition of lipid and matrix proteins in the blood-vessel wall<sup>(3)</sup>. A high risk of CVD is associated with abnormalities in lipid metabolism, hypertension and diabetes mellitus<sup>(4)</sup>, associations supported by landmark primary and secondary intervention trials<sup>(5,6)</sup>. These studies have contributed to the development of risk calculators and guidelines for the management of CVD<sup>(4,7)</sup>. The Framingham risk score<sup>(8)</sup> and Prospective Cardiovascular Münster (PROCAM) algorithm<sup>(9)</sup> are perhaps the best known of the risk ‘engines’, and are based on prospective cohort studies from well-characterised Caucasian populations living in the USA and Germany respectively. Deficiencies have

been recognised in extending the data from these cohort studies to individual patients. For example, ethnicity, family history and adiposity are factors that were not originally considered, but for which corrections have been advocated in recent guidelines<sup>(4)</sup>. The risk ‘engines’ also underestimate the absolute risk of patients with pre-existing CVD and diabetes, and it is therefore no longer recommended that risk tables are used to decide whether treatment is necessary in such patients<sup>(4)</sup>.

#### **Classical cardiovascular risk factors**

A strong relationship between baseline mean serum cholesterol levels and year-incidence of CHD was demonstrated in the Seven Countries Study<sup>(10)</sup>. Rates of CHD mortality have fallen in the UK over the past three decades<sup>(1)</sup>. This fall has been attributed to the implementation of intervention strategies for risk-factor

**Abbreviations:** CRP, C-reactive protein; PROCAM, Prospective Cardiovascular Münster.

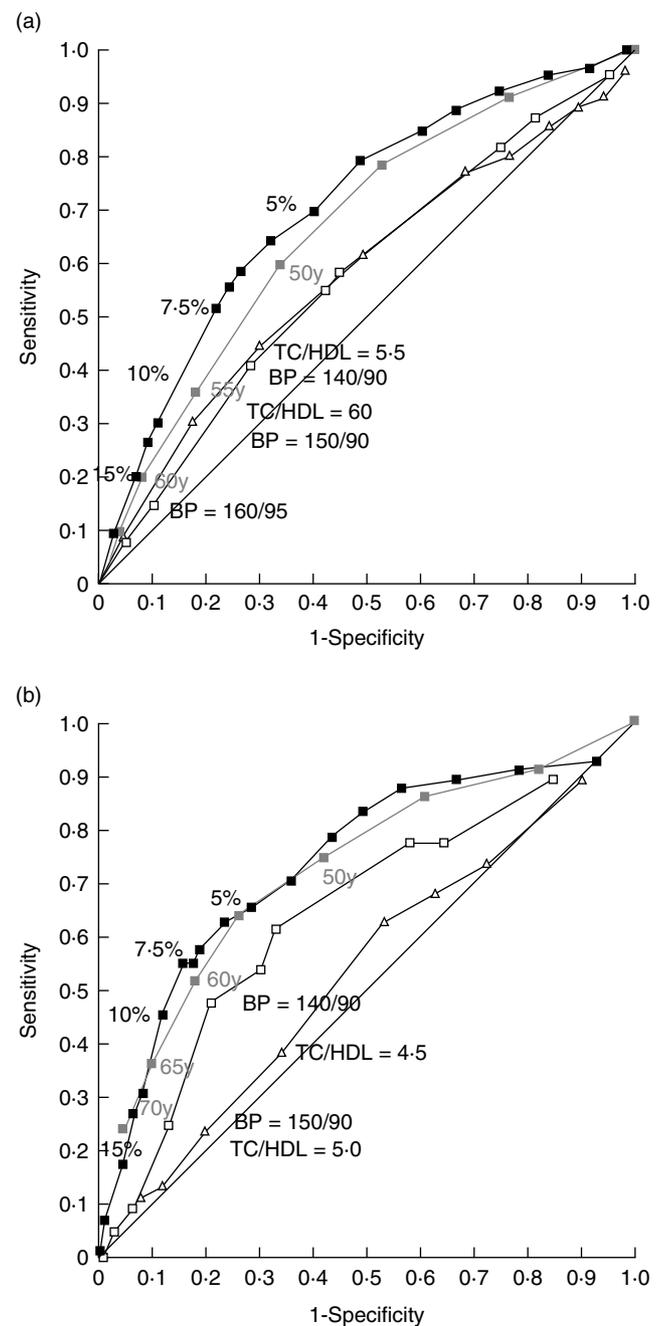
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management including dietary change, statin treatment for hypercholesterolaemia, antihypertensive treatment, reduction in smoking habit and improved management of patients with established acute coronary syndrome<sup>(11)</sup>. Cohort studies such as the Framingham Heart Study<sup>(8)</sup>, the PROCAM Study<sup>(9)</sup> and the Interheart Study<sup>(12,13)</sup> have been key in identifying these and other risk factors, including physical inactivity, the post-menopausal state and psychological factors. The Framingham risk score was derived from data within a middle-class middle-aged Caucasian population and may therefore underestimate lifetime risk and risk in the elderly<sup>(14)</sup>. The applicability of the Framingham data to European populations has been questioned<sup>(15,16)</sup>. The risk scores derived from the PROCAM cohort and the QRESEARCH database in the UK<sup>(16)</sup> have attempted to address this issue.

The importance of adopting a multi-factorial approach in managing coronary risk has become widely accepted<sup>(7)</sup>. Over the recent past several new putative risk factors have been identified, which include plasma homocysteine, lipoprotein (a)<sup>(17)</sup>, soluble adhesion molecules<sup>(18)</sup>, cardiac troponins<sup>(19)</sup>, type B natriuretic peptide<sup>(20)</sup>, C-reactive protein (CRP)<sup>(21)</sup> and coronary Ca score<sup>(22)</sup>. Meticulous histopathological studies<sup>(23)</sup> and imaging studies<sup>(24)</sup> have led to an appreciation of the impact of risk factors early in life on atherogenesis and the notion of plaque instability in patients with advanced disease<sup>(25,26)</sup>.

#### Why the need for more risk markers?

The algorithms described earlier do not discriminate well between individuals who will and will not develop cardiovascular end points. The utility of a test may be evaluated using the c statistic, or area under the receiver operating characteristic curve (see Fig. 1), and although useful in evaluating diagnostic tests, e.g. in Down's screening, it is not the ideal tool for assessing models that predict future risk or stratify individuals into risk categories<sup>(27)</sup>. Individual classical risk factors such as serum lipids (LDL-cholesterol, HDL-cholesterol and TAG), hypertension and smoking only have a modest impact on the c statistic, although they lead to more accurate reclassification of large proportions of patients into higher-risk or lower-risk categories. Even well-validated models of complex disease only achieve values for the c statistic that are well below the theoretical maximum of 1<sup>(27)</sup>. Hence, the use of the c statistic alone for validating the utility of novel CVD risk factors may not be appropriate. The reported values for c using the most-widely-available risk 'engines' is approximately 0.7, and a number of refinements have been suggested, e.g. the inclusion of markers that reflect other aspects of susceptibility such as inflammation and insulin sensitivity<sup>(28,29)</sup>. The impetus for discovering new biomarkers of CVD risk therefore arises for several reasons: (a) to improve the discrimination of individuals who will or will not develop early CVD; (b) to identify patients with established subclinical disease; (c) to identify patients with established CVD who will develop recurrent events in the short or longer term; (d) to identify risk factors that may provide a target for pharmacological or lifestyle intervention.



**Fig. 1.** Receiver operator characteristics curves for men (a) and women (b) for the Framingham risk equation, blood pressure and cholesterol ratio (total cholesterol (TC)/HDL-cholesterol (HDL)). (■—■), Framingham 1991; (□—□), blood pressure; (●—●), age; (△—△), TC/HDL. The values shown are the risk cut-off points for each risk factor or the risk equation. (From Milne *et al.*<sup>(117)</sup>.)

#### Insights from the pathogenesis of atherosclerosis

According to the 'response to injury' hypothesis a subtle form of endothelial injury is the initiating event in atherogenesis, and a number of risk factors could be responsible including smoking, hypertension, hyperlipidaemia and infection<sup>(3)</sup>. Leucocyte recruitment to the injured endothelium is mediated by pairs of adhesion molecules that are up regulated early in atherogenesis<sup>(30)</sup>. Monocytes and

T lymphocytes subsequently accumulate within the sub-endothelial space<sup>(31)</sup>. The monocytes are converted to lipid-laden foam cells within the artery wall, giving rise to a lesion termed the fatty streak<sup>(32)</sup>, while T-cells may become activated and are initially involved in a T-helper 1-type response associated with the release of pro-inflammatory cytokines<sup>(33)</sup>. The existence of an alternative 'scavenger receptor' pathway that allows the unregulated uptake of cholesterol in the form of modified LDL has been proposed<sup>(34)</sup>. Cells of the arterial wall can cause LDL oxidation and the release of toxic LDL oxidation products<sup>(35)</sup>. These products may in turn react with apoB-100 within LDL particles causing cross-linking, altered antigen expression and subsequent recognition and uptake by scavenger receptors<sup>(36)</sup>. LDL modification also appears to lead to an autoimmune response, and auto-antibodies to oxidised LDL have been found in plasma of patients with atherosclerosis<sup>(37)</sup>. The formation of immune complexes of oxidatively-modified LDL and antibody may also allow the uptake of modified LDL by macrophages via their Fc receptors<sup>(38)</sup>. Oxidatively-modified LDL causes an induction of endothelial cell tissue factor and colony-stimulating factor gene expression and impairs the biological activity of NO<sup>(39–41)</sup>.

The conversion of the fatty streak into a fibrous plaque necessitates the recruitment and proliferation of vascular smooth-muscle cells<sup>(42)</sup>. This process is driven by the synergistic interplay of several growth factors<sup>(3)</sup>. The mature plaque is characterised by a fibrous cap composed of smooth-muscle cells and extracellular matrix that overlies a pool of lipid, cholesterol crystals and inflammatory cells<sup>(42)</sup>.

Unstable plaques, characterised by a large lipid pool, thin fibrous cap and large numbers of inflammatory cells located in the shoulder region of the plaque<sup>(43,44)</sup>, are thought to be particularly prone to fissuring and rupture. Activated macrophages within the plaque are a rich source of matrix metalloproteinases that have the ability to degrade extracellular matrix<sup>(45)</sup>, leading to localised regions of de-endothelialisation that may subsequently lead to focal thrombosis and plaque rupture. In most cases of fatal myocardial infarction at least one major coronary artery is narrowed by >70%, and this narrowing is usually associated with thrombi and plaque fissuring<sup>(46)</sup>. Some plaques may become stabilised through the development of a thick fibrous cap. Although such lesions may be sufficiently prominent to cause luminal narrowing, and hence angina or claudication, they are less likely to rupture and are therefore considered to be relatively safe<sup>(44)</sup>. Moreover, in many cases the formation of these lesions is associated with arterial remodelling<sup>(47)</sup> in which there is a compensatory increase in arterial diameter as the atherosclerotic lesion encroaches on the lumen, thereby maintaining luminal diameter.

#### *Inflammation and infection*

Atherosclerosis bears many hallmarks of a chronic inflammatory disease<sup>(3)</sup>. The possible stimuli to this inflammatory process include oxidised LDL, homocysteine, free radicals generated from cigarette smoking

and infectious micro-organisms. If the original insult is not adequately neutralised the inflammation may persist, causing the local and systemic release of growth factors and cytokines that can in turn lead to intimal thickening by stimulating smooth-muscle cell migration, proliferation and extracellular matrix elaboration<sup>(48)</sup>. The release of IL-1 $\beta$  and IL-6 from activated leucocytes may also lead to an induction of hepatic CRP synthesis<sup>(49)</sup>.

Over the past few years there has been an increasing interest in the use of inflammatory markers to estimate the risks of acute events in patients with established coronary disease. In part, the predictive value of these markers may be related to their ability to identify patients with vulnerable plaques. The risk associated with a high serum CRP concentration is reported to be stronger than that associated with raised von Willebrand Factor or erythrocyte sedimentation rate, but weaker than increased cholesterol or a positive smoking habit<sup>(50)</sup>. It has also been found that serum levels of CRP are positively associated with sub-clinical carotid and femoral atherosclerosis and a number of established coronary risk factors in healthy middle-aged men, including smoking habit, indices of adiposity, blood pressure, TAG and HDL<sup>(51)</sup>. Of these factors, smoking habit shows the strongest association with serum CRP, and remains an independent determinant of femoral atherosclerosis on multiple regression analysis whereas CRP does not. Adipose tissue from obese individuals contains a substantial population of macrophages<sup>(52)</sup> that may be an additional source of inflammatory cytokines that could stimulate CRP production. Hence, smoking and indices of insulin resistance may confound an analysis of the relationship between serum CRP and atherosclerosis in healthy subjects. A positive relationship between CRP levels and antibody titres directed against modified LDL has also been reported<sup>(53)</sup>. It is possible that these two variables are linked by the presence of a heightened activation state of plaque macrophages.

Recent studies have shown that CRP is deposited in lipid-rich regions of human coronary arteries and that its accumulation may precede monocyte recruitment<sup>(54)</sup>. CRP is chemotactic for monocytes and may also be involved in the activation of complement<sup>(55)</sup>. A relationship between plasma concentrations of CRP and basal endothelial cell NO synthesis has also been reported<sup>(56)</sup>, suggesting that CRP may indeed be involved early in the pathogenesis of atherosclerosis.

Infective micro-organisms may stimulate a systemic inflammatory response and their role in the pathogenesis of atherosclerosis has been a matter of considerable debate over the last two decades. Several micro-organisms, e.g. *Chlamydia pneumoniae* and *Helicobacter pylori*, have been identified in human plaques and antibody titres to these organisms have been reported to be higher in patients with acute coronary syndrome<sup>(57)</sup>. It has been argued that eliminating these organisms by treating patients with antibiotics may therefore improve clinical outcomes<sup>(58)</sup>. However, antibiotic treatment has generally not proven to be beneficial in placebo-controlled trials. It has been hypothesised that regimens used to date may not eliminate the carriage of organisms by peripheral monocytes, which may explain the failure of these trials<sup>(59)</sup>. An autoimmune

mechanism has been proposed as one explanation for the link between infection, endothelial dysfunction and atherogenesis<sup>(60)</sup>. Some organisms elaborate large quantities of the chaperonin heat-shock protein<sup>(60)</sup>. A homologous protein is also produced by the endothelium. It is proposed that the immune response mounted against microbial heat-shock protein<sup>(60)</sup> also reacts with the endogenous heat-shock protein causing endothelial injury<sup>(61)</sup>. A recent prospective study has reported that anti-heat-shock protein antibody titres are predictive of cardiovascular events<sup>(62)</sup>.

#### *Endothelial function and its assessment*

Endothelial dysfunction is thought to precede atherosclerosis and is characterised by altered permeability barrier function, enhanced adhesion molecule expression, increased leucocyte adhesion and impaired endothelium-dependent vasodilator responses<sup>(63)</sup>. Endothelial dysfunction is also associated with enhanced thrombosis and impaired fibrinolysis. As there is no universally-accepted 'gold standard' measure of endothelial function, several aspects of endothelial function have been assessed, including the measurement of endothelium-dependent vasodilatation of the brachial or coronary arteries (flow-mediated dilatation) or indirect measures of function, e.g. plasma concentrations of endothelium-derived regulatory proteins (e.g. the soluble adhesion molecules) and possibly urinary factors such as microalbuminuria (for review, see Sattar & Ferns<sup>(63)</sup>). In atherogenesis the endothelium continues to elaborate NO, but its biological activity appears to be compromised<sup>(64)</sup>. The latter is likely in part to be a result of NO interaction with other molecular species such as the superoxide radical. These interactions not only neutralise the protective effects of NO, but also generate products, e.g. peroxynitrite, that may be cytotoxic. These changes in the properties of the endothelium have given rise to the concept of endothelial dysfunction.

#### *Coronary risk factors and endothelial dysfunction*

All the classical risk factors for coronary disease and several of those identified more recently appear to be associated with endothelial dysfunction<sup>(63)</sup>. The association between flow-mediated dilatation and cardiovascular risk is most clearly evident in subjects with low baseline risk in whom it has been estimated that there is a decrease of 1.42% flow-mediated dilatation for each 1% increase in risk<sup>(65)</sup>.

Hypertension is thought to cause direct mechanical damage, but is also associated with the release of free radicals from the endothelium and reduced NO bio-activity<sup>(66)</sup>. Hypertension also has pro-inflammatory effects on vascular smooth-muscle cells<sup>(66)</sup>.

Cigarette smoking causes morphological, biochemical and functional disturbances to the endothelium in experimental models<sup>(67)</sup>. Only a few components of cigarette smoke have been studied systematically. Nicotine is pro-inflammatory<sup>(68)</sup> but appears to be less damaging than whole smoke<sup>(69)</sup>. Clinical and experimental-animal studies suggest that arterial endothelium-dependent acetylcholine relaxation is impaired by smoking and this effect may be

attenuated by treatment with the antioxidant vitamin C or smoking cessation<sup>(70,71)</sup>. Excessive endothelial apoptosis may contribute to cigarette smoke-induced endothelial injury<sup>(72)</sup>.

Endothelial dysfunction is seen early in subjects with severe hypercholesterolaemia, and has been described in young children with familial hypercholesterolaemia<sup>(24)</sup>. These effects may be mediated in part by the oxidation products of LDL. There is evidence that plasma HDL-cholesterol concentrations are positively related to endothelium-dependent relaxation<sup>(73)</sup>. In patients with type 2 diabetes mellitus decreased HDL-cholesterol has been shown to be the best predictor of impaired vasodilatation to acetylcholine, even after adjustment for all other lipid and lipoprotein concentrations and LDL size<sup>(74)</sup>. The protective effects of HDL may relate to its antioxidant properties, as it protects LDL against oxidative modification<sup>(75)</sup>. The antioxidant properties of HDL may be associated with its protein constituents, which include intrinsic antioxidative enzymes such as paraoxonase<sup>(76)</sup>.

Raised serum CRP concentrations are associated with impaired endothelial NO production in peripheral arteries, impaired coronary endothelial function and elevated pulse pressure<sup>(77)</sup>. CRP, either directly or via inflammatory cytokines such as IL-6 or TNF $\alpha$ , may mediate endothelial dysfunction<sup>(78)</sup>. Inflammatory cytokines cause a selective impairment of endothelium-dependent relaxation that may be abolished by previous treatment with hydrocortisone or high-dose aspirin<sup>(79)</sup>.

The association between very high concentrations of plasma homocysteine and atherosclerosis<sup>(80,81)</sup> may be related to a free-radical mechanism affecting the endothelium<sup>(82)</sup>. However, although folate and B-vitamin supplements have been found to lower plasma homocysteine concentrations, they have no significant effect on flow-mediated dilatation and blood pressure<sup>(83,84)</sup> or cardiovascular outcomes<sup>(85)</sup>.

#### **Reversal of endothelial dysfunction by risk factor intervention**

In patients with familial hypercholesterolaemia apheresis (extracorporeal removal of LDL) is associated with a rapid improvement in endothelial responsiveness in the brachial artery<sup>(86)</sup>. Treatment with hydroxymethylglutaryl-CoA reductase inhibitors such as lovastatin and pravastatin, which lower LDL, has also been shown to have a favourable effect on vasomotor tone<sup>(87,88)</sup>. Recent studies have demonstrated beneficial effects of the antidiabetic glitazone drugs on endothelial function in patients with diabetes<sup>(89)</sup>, and oestrogen-replacement therapy can improve endothelial function in healthy post-menopausal women, although not if they already have CVD<sup>(90,91)</sup>. Antioxidants such as vitamin E, C and probucol appear to improve endothelial dysfunction<sup>(92,93)</sup>. Epidemiological studies support the benefits of dietary antioxidants, although the results of clinical intervention studies are inconsistent. It appears that some of the discrepancies in these studies may be associated with the complex mechanisms of action of the antioxidants<sup>(94)</sup>.

### Markers of instability of coronary artery lesions

Careful morphological and immunocytochemical studies have revealed the complexity of the atherogenic process and the sequence of cellular events that generally take place over decades. However, the clinical end points, marked by myocardial infarct or stroke, happen abruptly and are the result of an acute occlusive event<sup>(25,26)</sup>. This factor has led to the concept of the unstable plaque, a lesion characterised by a large lipid pool and thin fibrous cap<sup>(95,96)</sup> and having a high inflammatory cell and metalloproteinase content<sup>(2)</sup>. It has been proposed that systemic markers of inflammation such as CRP, IL-6 and lipoprotein-associated phospholipase A<sub>2</sub> may reflect or contribute to the local pro-inflammatory milieu<sup>(97)</sup>. The nature of the plaque may also contribute to the outcome following percutaneous coronary interventions such as angioplasty<sup>(98)</sup>.

Plaque inflammation and local thrombus formation are major determinants of coronary artery plaque instability<sup>(25)</sup>. It has been proposed that plaque disruption causes a release of cytokines from activated monocytes and macrophages at the disrupted site, and that these cytokines promote hepatic synthesis of the acute-phase proteins, CRP and serum amyloid A<sup>(49)</sup>. Increased serum concentrations of these markers may provide a means of identifying patients with unstable coronary plaques, and they have been reported to be higher in patients with unstable angina and to carry prognostic importance in these subjects<sup>(99)</sup>. CRP levels have also been investigated for their ability to predict unfavourable outcomes and impairment of left ventricular function in patients with acute coronary necrosis or previous myocardial infarction<sup>(100)</sup>.

Platelet activation is an important feature of thrombus formation, and hence indicators of platelet activation such as P-selectin may help to assess a patient's risk of intracoronary thrombosis. P-selectin is an adhesion molecule expressed on the surface of platelets when activated by exposure to collagen and other agonists released following plaque disruption<sup>(101)</sup>. Soluble fibrin and fibrin degradation products may also indicate a recent thrombotic process or risk of an impending event<sup>(102)</sup>. Elevated levels of these markers are pathognomonic of procoagulant fibrinolytic activity and may help to identify patients at an increased risk of myocardial infarction-related complications<sup>(103)</sup>. Other indicators of activation of the coagulation system or inhibition of fibrinolytic activity are also elevated in patients with unstable angina<sup>(104)</sup>. However, the use of most of the biochemical markers of inflammation and activation of the coagulation cascade for the detection of unstable coronary artery disease is limited by their low specificity and lack of standardisation of the analytical procedures<sup>(105)</sup>.

### The rationale for autoimmune biomarkers

Monocytes and lymphocytes have both been detected within atherosclerotic lesions<sup>(106)</sup> and auto-antibodies directed against modified LDL and heat-shock proteins 60 and 27 have been identified in the blood of individuals with atherosclerosis<sup>(60,107,108)</sup>. It is proposed that LDL

oxidation that occurs in the absence of adequate anti-oxidant protection promotes an autoimmune response that may itself lead to progressive disease. These data have contributed to the formulation of the 'lipid-oxidation hypothesis'<sup>(36)</sup> and provide the basis for the measurement of markers of oxidant stress in assessing coronary risk. Furthermore, autoimmune conditions such as rheumatoid arthritis<sup>(109)</sup> and systemic lupus erythematosus<sup>(110)</sup> are associated with a substantially increased coronary risk, suggesting that a heightened state of immune activation may be pro-atherogenic.

### Direct evaluation of the vasculature

Plasma biomarkers only provide an indirect assessment of vascular health and may be confounded by other comorbidities. More direct methods of evaluating vascular dysfunction have been advocated, such as imaging and functional vascular assessment. In addition to flow-mediated dilatation (a measure of endothelial function), these methods include carotid artery intima media thickness, multislice computed tomography (for quantifying coronary artery Ca accumulation) and coronary thermography (measuring temperature differentials as an indicator of localised inflammation), and they appear to predict clinical events and may improve the discrimination of conventional risk scores<sup>(111–114)</sup>. These methods now require further evaluation for clinical utility. The coronary artery Ca score is an independent predictor of CHD that may be of particular value in patients at intermediate cardiovascular risk. Invasive assessments of coronary disease, such as angiography and intravascular ultrasound, are not indicated in the absence of symptomatic disease; however, ultrafast MRI with intravenous contrast now makes 'virtual angiography' a possibility<sup>(115)</sup>.

### Conclusion

The use of risk 'engines', e.g. the Framingham coronary risk score, has facilitated the identification of individuals at high risk, but these algorithms do not adequately differentiate individuals at moderate risk. Biomarkers of early disease and plaque instability have therefore both been sought. Although some of these markers have been shown individually to be associated with a significant hazard ratio, no substantial improvement in discrimination has been demonstrated when they are incorporated into a risk 'engine'<sup>(116)</sup>. Direct measures of disease burden using other modalities such as imaging may prove to be more useful.

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