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PASSCLAIM[1] – Diet-related cardiovascular disease

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Summary Cardiovascular disease (CVD) has a multifactorial aetiology and many potential risk markers are known. As it was not feasible to discuss all markers and their possible interactions in relation to all aspects of CVD, selections had to be made in this paper. In the context of claims and functional foods, emphasis was placed on those aetiological processes and risk markers that have been shown previously to be modified by diet: lipid and lipoprotein metabolism, haemostatic function, oxidative damage, homocysteine metabolism, and blood pressure. Except for methodological and biological characteristics of these biomarkers, their relationships with the risk of CVD are discussed.

For LDL and HDL cholesterol, fasting triacylglycerol, homocysteine, and blood pressure well-validated, easy applicable, and generally accepted biomarkers exist. For haemostatic function and oxidative damage validation of markers with respect to CVD or intermediate clinical markers is recommended. For diet-related CVD, however, the ultimate question is whether changes in the biomarker are truly related to changes in risk. Only for LDL cholesterol and blood pressure does consensus exist among scientists for a possible application as enhanced function claims. For HDL, triacylglycerol, and homocysteine, and in particular for haemostatic function and oxidative damage, however, formal proof is lacking that diet-induced changes in these biomarkers alter the risk of CVD. At the same time, it should be emphasised that CVD is multifactorial. Therefore it does not seem justified that a change in one particular biomarker is enough evidence to substantiate a claim. There are examples of food components or drugs that one biomarker is changed in a favourable way, but at the same time another biomarker is changed in an unfavourable way. Therefore, studies to further validate generic predictors for the CVD risk should be initiated.

Key words diet – claims cardiovascular disease – cholesterol – triacylglycerol – HDL cholesterol – hemostasis – oxidation – homocysteine – blood pressure

1 Process for the Assessment of Scientific Support for Claims on Foods

Glossary

Aggregation	The formation of a loose thrombus
ATIII	Antithrombin III, a protein that may inhibit the coagulation cascade
CHD	Coronary heart disease
Coagulation	The process that ultimately leads to the formation of fibrin needed to stabilize a loose thrombus
CVD	Cardiovascular disease
Fibrinolysis	The process that ultimately leads to the dissolution of a thrombus
FVII	Factor VII, a protein involved in the coagulation cascade
HDL	High-density lipoproteins
LDL	Low-density lipoproteins
Lp(a)	Lipoprotein(a)
Lipoproteins	Particles that transport cholesterol and triacylglycerol through the blood
MDA	Malondialdehyde, a breakdown product of lipid oxidation
Meta-analysis	A statistical procedure that integrates the results of several independent studies which are combinable to arrive at a conclusion about a research question
MI	Myocardial infarction
PAI-1	Plasminogen activator inhibitor, a protein that may inhibit the fibrinolytic pathway
tPA	Tissue type plasminogen activator, a protein that may stimulate the fibrinolytic pathway
TBARS	Thiobarbituric acid reactive compounds, lipid oxidation products that react with thiobarbituric acid to produce a coloured pigment
VLDL	Very-low density lipoproteins

Introduction

Atherosclerosis, a main cause of cardiovascular disease (CVD), is a group of syndromes that can be divided into three main categories: coronary heart disease (CHD; angina pectoris, myocardial infarction (MI)), cerebrovascular diseases (stroke, transient ischaemic attacks), and peripheral arterial disease (gangrene, intermittent claudication). It has a multifactorial aetiology and many potential risk markers have been identified. Very likely, many of these risk markers were purely by chance associated with the disease and a causal relationship between these markers with CVD does not exist. In other words, modifying these markers will not affect the risk of atherosclerosis. Still, there are many potential risk markers that have been related in numerous studies to the risk of CVD. It is recognised that these risk markers may be interrelated and interact with each other. However, it is not feasible to discuss all markers and their interactions in relation to all aspects of CVD; thus selections had to be made in this paper. In the context of claims and functional foods, it is understandable that emphasis was placed on those aetiological processes and risk markers that can be modified by diet: lipid and lipoprotein metabolism, haemostatic function, oxidative damage, homocysteine metabolism and blood pressure.

The structure of each section is very similar. First some background information is given, followed by biomarkers and examples of potential claims. However, for this specific area, it is also relevant to discuss the strength of the relationship between biomarkers and CVD. A change in the concentration of high-density lipoproteins (HDL), for example, is by no means of interest if HDL cholesterol is not related to a reduced risk of disease. In this respect, special attention has been paid to human observational studies, marker-disease relationships including intervention studies, and mechanisms. Less attention has been paid to animal studies, as in the context of claims, such studies can be supportive, but not conclusive. In the final paragraph, the general conclusions are formulated.

The example of cholesterol

Background

The hallmark example of a biomarker for CVD is serum cholesterol [1]. It seems thus very appropriate to discuss briefly its scientific dossier as a benchmark for others. First and foremost, cholesterol is not a biomarker *per se*, in which its presence or absence reflects injury or damage. Rather, cholesterol is a natural and abundant metabolite present in blood, whose quantitative variation reflects various metabolic states that are in turn reflective of cardiovascular risk [2].

Biomarkers and claims

It is obvious that changes in serum cholesterol concentrations themselves can be used to validate a claim. The concentrations of cholesterol in serum do not vary dramatically over short periods, but do show significant responses to environment including diet, drugs, health status and exercise (Table 1). In no case has the serum concentration of cholesterol in a subset of the population been found to respond in a physiologically opposite way to the normal population. That is, in no population studied has an agent – drug or food substance – consistently lowered cholesterol concentrations in the majority of the population, but caused an elevation in choles-

European Journal of Nutrition, Vol. 42, Supplement 1 (2003)

Table 1 Methodological and biological characteristics of serum total, LDL and HDL cholesterol

Biomarker	Methodological characteristics	Biological characteristics
• Total, LDL and HDL cholesterol	• Excellent precision, validity and reproducibility • A variety of analytical methods exist • Chemically relatively stable and can therefore be analysed in properly stored, banked plasma samples	• Present in the blood of all humans at all times, in all physiological states, but its abundance varies • Levels vary as a function of many genetic, physiological and environmental variables • Relatively stable over time

terol in a subset of the population. Some methodological and biological characteristics of serum total cholesterol as biomarker are given in Table 1.

Existing claims include the following:

- Can help to lower cholesterol as part of a low-fat diet
- Can help to reduce cholesterol as part of a low-fat diet
- Can be beneficial in reducing cholesterol concentrations as part of a low-fat diet
- Lowers cholesterol
- Regulates cholesterol
- Controls cholesterol
- Reduces cholesterol concentrations
- Helps reduce cholesterol
- Helps maintain a healthy heart

The last claim suggests that diet-induced changes in serum cholesterol predict changes in cardiovascular risk and can therefore be considered as a type B claim.

Strength of relationship with cardiovascular disease

Human observational studies. The relationship between serum cholesterol and risk of CVD across populations, cultures and age groups has been evaluated extensively. These studies invariably show that populations with quantitatively high serum cholesterol are at greater risk of CVD than those with low serum cholesterol concentrations. In fact, the relationship is so widely studied and reported that the departure of populations from this relationship forms the basis for what has become known as the French paradox. Further, from normal through a high dynamic range of cholesterol concentrations, serum cholesterol concentrations accurately predict increased risk of CVD at all ages, across a wide range of populations and environmental backgrounds, including those with late stage CVD. This massive quantity of data has been examined in multiple meta-analyses and the striking relationship between serum concentrations of cholesterol even measured early in life, and the probability of morbidity and mortality from occlusive artery diseases has also been consistent across a wide range of populations and environmental backgrounds.

Marker-disease relationship. Very convincing for the strength of cholesterol as a biomarker for CVD is that – independent of the means by which cholesterol concentrations were altered – a rise in cholesterol increased CVD risk, and a decrease in cholesterol lowered CVD risk. Most of the data to support that intervention in lowering serum cholesterol translates into a proportionate reduction in morbidity and mortality has only emerged in the past few decades. Multiple studies using pharmacological [3], nutritional [4], and even organ transplant protocols [5] have now confirmed that when cholesterol in serum is targeted for therapeutic action, success in lowering the biomarker translates into predicted improvements in disease outcomes.

It is important to note that diet-induced decreases in cholesterol have not been shown to put even a subset of the population examined at increased risk of CVD. Recent studies, however, may actually put this statement into some question with the demonstration that very low-fat, high-carbohydrate diets may lower low-density lipoprotein (LDL) cholesterol, but reduce at the same time LDL particle size in a subset of the population [6]. There is evidence that not only the amount of cholesterol transported by LDL particles, but also its density affect CHD risk [7]. In fact, the few trials that studied replacement of saturated fatty acids by carbohydrates on cardiovascular risk were not conclusive [8].

Mechanism. While scientific research continues unabated to resolve the many causes of heart disease and its severity, the basic mechanism that the absolute concentration of cholesterol in serum is mechanistically associated with its accumulation on artery walls has been supported. *In vitro* studies of the mechanisms of cholesterol absorption, biosynthesis and excretion have all been used to develop drugs and foods that have the potential to lower cholesterol *in vivo* according to the predicted mechanisms of action. For many cases, successful actions *in vitro* were demonstrated in order to achieve successful reductions in cholesterol *in vivo*, although the magnitudes of effect differed. However, in the context of clinical trials, relatively few have actually demonstrated a putative underlying mechanism of action for the alterations in cholesterol in humans. That is, only rarely was cholesterol absorption measured in trials using cholesterol absorption inhibitors, or cholesterol biosynthesis measured in trials using cholesterol synthesis inhibitors. Thus, while mechanistic studies have been

used, demonstration of mechanisms has not always been an essential component of the scientific dossier supporting cholesterol as a biomarker for functional ingredients.

Animal studies. Although cholesterol performs comparable functions in virtually all mammalians, there are no accepted animal models that accurately reflect the full development of CVD in humans. Furthermore, whereas in many animals serum cholesterol concentrations are sensitive to diet and other environmental variables, the response of humans and animals may differ significantly. As a result, the use of animals has led to both false positive and false negative conclusions as to the safety and efficacy of food ingredients for humans.

From the lack of coherence of animal models and animal trials to the body of knowledge of human arterial disease and the role of genetics and environment, it must be concluded that, in principle, animals studies and the availability of highly predictive animal models are not a necessary condition for the quality of a scientific dossier in support of claims for ingredients that decrease serum cholesterol. While supportive, the principle from cholesterol's example is that animals are not a necessity for the establishment of a scientific consensus to a functional food claim for lowering total cholesterol concentrations.

Conclusions and gap analysis

It must be concluded by the coherence of epidemiological studies and clinical intervention trials in humans that these two sources of information were key principles in the development of scientific consensus that the serum cholesterol concentration is a well-validated biomarker of CVD risk. It should be kept in mind, however, this may be only true, if cholesterol lowering is predominantly achieved by lowering LDL cholesterol. If lowering of HDL causes the cholesterol reduction, cardiovascular risk may actually increase. In this respect, it is also noteworthy that a "dose-response" mortality curve is evident for the total to HDL cholesterol ratio, rising exponentially over a value of six [9].

Lipid and lipoprotein metabolism

Background

Cholesterol and triacylglycerol play a major aetiological role in the formation of plaques in the arteries. Cholesterol represents a major component of the lipid core of the lipoproteins, and triacylglycerol a metabolic determinant of the structure and physical properties of the lipoproteins which transport cholesterol into and out of the plaque, namely LDL and HDL.

A link between LDL and CVD is well established and supported by several lines of evidence. Firstly, serum LDL cholesterol correlates with cross-cultural variance in CVD risk [10]. Secondly, oxidatively modified products of LDL (apoprotein B and cholesterol) have been isolated from plaques on post-mortem in amounts directly proportional to the concentration of serum LDL [11, 12]. Thirdly, prospective cohort studies show a positive, curvilinear relationship between serum LDL cholesterol and CHD mortality [13]. Most important, however, to establishing a causal relationship between LDL and CHD were the findings that decreasing serum LDL with pharmacological agents decreased CHD mortality [3, 14] and promoted plaque regression [15]. The evidence relating LDL cholesterol to CVD risk is generally accepted, has been addressed to some extent in the previous section, and will therefore not be addressed further.

Plaque regression involves the HDL-mediated transport of cholesterol away from the artery wall and back to the liver via reverse cholesterol transport, a process that underlies a powerful inverse association between the concentration of serum HDL cholesterol and CHD mortality [16]. On the other hand, the issue of raised serum triacylglycerol as a CHD risk factor has been more controversial, due mainly to the intimate metabolic relationships between triacylglycerol-rich lipoproteins, LDL and HDL [17]. The structure and composition of LDL and HDL have been shown to be an important determinant of their roles in atherosclerosis. Perturbation in the metabolic handling of triacylglycerol-rich lipoproteins, especially in the postprandial period [18], can promote pro-atherogenic changes in lipoprotein profile (predominance of small, dense LDL and low HDL) that may represent the most common source of increased, lipid-mediated CVD risk in free-living populations.

Biomarkers and claims

Changes in serum HDL cholesterol and triacylglycerol concentrations are of course the best markers to validate a claim. The concentrations of HDL cholesterol are fairly stable over time. Variation in fasting triacylglycerol concentrations is somewhat greater. Investigations of postprandial triacylglycerol concentrations are notoriously difficult to standardise, but the extent of postprandial lipaemia in response to a dietary fat load can be reliably predicted from the concentration of serum triacylglycerol in fasted individuals [19]. Some methodological and biological characteristics of these biomarkers are given in Tables 1 and 2.

Possible claims include the following:

- May increase HDL cholesterol concentrations

European Journal of Nutrition, Vol. 42, Supplement 1 (2003)

Table 2 Methodological and biological characteristics of fasting and postprandial triacylglycerol

Biomarker	Methodological characteristics	Biological characteristics
• Fasting and postprandial triacylglycerol	• Excellent precision, validity and reproducibility • A variety of analytical methods exist • Chemically relatively stable and can therefore be analysed in properly stored, banked plasma samples • Methodology and interpretation of fasting serum concentration may be confounded by the short-term intake of dietary fat which causes postprandial lipaemia • No generally accepted and validated procedure to measure postprandial triacylglycerol	• Relatively stable biologically, is present in blood at all times, in all people, and in all physiological states, but its abundance varies • Levels vary in blood as a function of many genetic, physiological and environmental variables

- May lower triacylglycerol concentrations (after a meal)
- May improve your lipoprotein profile
- May lower the risk of cardiovascular diseases/coronary heart disease

Strength of relationship with cardiovascular disease

HDL cholesterol

Human observational studies. A striking example of the potential cardioprotective role of HDL in humans is provided by gender and the fact that a higher concentration of HDL cholesterol confers decreased CHD risk in pre-menopausal women as compared to men of the same age [20]. In case-control studies, lower HDL cholesterol concentrations are a consistent finding in CHD patients versus CHD-free controls, while prospective cohort studies such as Framingham and the Caerphilly-Speedwell studies [16, 21] consistently showed serum HDL cholesterol to be a more discriminating measure of CHD risk than LDL cholesterol. These findings have since been confirmed in numerous prospective trials such as MRFIT and PROCAM [13, 22]. Low concentrations of HDL cholesterol are more predictive of vascular risk than LDL cholesterol in the elderly [23]. Young siblings of parents with a history of CHD express lower HDL cholesterol when compared to siblings with disease-free parents, suggesting that low HDL cholesterol may be an early marker for predicting the future onset of CHD.

Marker-disease relationship. No randomised clinical intervention trials have been specifically designed to examine the relationship between changes in CVD endpoints and HDL cholesterol. Trials that have resulted in significant increases in HDL cholesterol are interventions with fibric acid derivatives (fibrates), which also lower serum triacylglycerol. These studies include the Helsinki Heart Study [24] and the more recent Veterans Affairs-HDL Intervention Trial [25], both of which related significant drug-induced increases in HDL cholesterol with reduced CHD risk. Similar results were found with bezafibrate [26].

In primary and secondary intervention trials designed to lower serum cholesterol with statins [14], coronary events were decreased still further as the concentration of HDL cholesterol increased. It has also been observed that increasing HDL cholesterol has greater protective potency in patients with low LDL cholesterol (< 3.2 mmol/L). The majority of cholesterol-lowering trials which have measured plaque regression showed the regression of coronary lesions to be inversely related to HDL cholesterol concentrations [15, 27], with the concentration small dense HDL (non-cardioprotective HDL) emerging as a strong correlate of lesion progression.

Like LDL, HDL is highly responsive to environmental stimuli. In every case, the HDL response to these variables is consistent with an inverse association between HDL and CHD risk. Thus, weight loss and exercise increase HDL cholesterol, whereas weight gain, physical inactivity and smoking all exert powerful HDL cholesterol lowering effects.

CHD risk seems to decrease as HDL cholesterol increases across the entire HDL spectrum without a threshold, although a concentration < 1 mmol/L has been identified as being associated with a significantly increased risk. Serum HDL cholesterol also predicts CHD risk across LDL cholesterol and serum triacylglycerol spectra [16, 28]. However, since HDL cholesterol concentrations are intimately related (interdependent) on serum triacylglycerol, the strength of association has frequently been diminished by statistical manipulations to correct for these co-variates. The quality of circulating HDL cholesterol, i. e. the distribution of cholesterol between HDL particles of different sizes, density and composition, shows variable relationships with CHD risk. As HDL cholesterol concentrations are diminished, HDL particles become smaller and denser and more strongly associated with increased CHD risk [29].

There are no known adverse effects associated with raising HDL cholesterol.

Mechanisms. Landmark metabolic studies by Miller and Miller [30] showed that the concentration of HDL cholesterol decreased as the mass of total body cholesterol pool increased. This suggested a major role for HDL in the uptake of cholesterol from tissues and transport to the liver for excretion and thus a mechanism to explain the cardioprotective effects of HDL (reverse cholesterol transport). In fact, there is a wealth of evidence from *in vitro* and *in vivo* studies in animals and humans (including metabolic tracer studies and angiographic trials) to support a mechanism whereby small 'nascent' HDL particles (*pre*-β HDL) transport free cholesterol out of cells in peripheral tissues, including atherosclerotic lesions in the artery wall, down a cholesterol gradient maintained by the esterification of cholesterol by the enzyme LCAT (cholesterol efflux). More recent developments include the molecular characterisation of proteins involved in cholesterol efflux from cells and the selective uptake of HDL cholesterol in the liver [31]. It therefore becomes more and more accepted that the cardioprotective effects associated with a HDL cholesterol response may not be causally related to the circulating HDL cholesterol *per se*. HDL cholesterol seems to represent a cholesterol-enriched end product of reverse transport and other related metabolic processes and may therefore simply be a marker of the efficiency of this process. This may also explain the significantly increased CVD risk at a HDL cholesterol concentration below 1.0 mmol/L, as this may reflect a reduced capacity to perform reverse cholesterol transport.

Animal studies. Animals with a predominant proportion of their cholesterol in the form of HDL or HDL-like particles, such as horses and rats, express greater relative resistance to the development of coronary atherosclerosis as compared to animals whose principal lipoprotein is LDL, such as rabbits, pigs and primates [32]. Like for serum cholesterol, there are no animal models that reflect human HDL metabolism. Nevertheless, *in vivo* studies in the lymphatic system of the dog provided one of the first coherent models for the role of HDL in reverse cholesterol transport [33]. Also, certain transgenic animal strains are very useful to study specific aspects of HDL metabolism.

Triacylglycerol

As a marker for a health claim, serum triacylglycerol can be defined in either the post-absorptive (fasting) or postprandial states and with the aid of specialised biochemical techniques, be expressed in terms of its component triacylglycerol-rich lipoproteins of either intestinal (chylomicrons, chylomicron remnants) or hepatic (VLDL) origin.

Human observational studies. In cross-sectional studies, moderately raised serum triacylglycerol, as measured in the post-absorptive "fasting" state, was first implicated as a risk factor in MI survivors by Gofman [34] and in CHD cases by Albrink and Man [35]. Raised serum triacylglycerol has also been strongly associated with risk of MI more recently [36]. Prospective studies such as PROCAM [22] and the Physicians' Health Study [37] demonstrated a significant and independent association between serum triacylglycerol and the incidence of major coronary events. At about the same time, a landmark meta-analysis by Hokanson and Austin [38] showed that raised serum triacylglycerol was positively associated with the risk of CHD. Furthermore, in contrast to earlier studies which dismissed serum triacylglycerol as a risk factor because of its intimate association with HDL cholesterol, the significance of the relationship between serum triacylglycerol and CHD risk was shown to be maintained after correction for HDL cholesterol in both males and females.

Postprandial experiments in CHD cases and controls showed the diseased group to have an impaired capacity to remove triacylglycerol-rich lipoproteins from blood in the postprandial period with a resulting increase in the magnitude and duration of the postprandial triacylglycerol response. With increasing knowledge of the potential atherogenicity of the triacylglycerol-rich remnant lipoproteins, Zilversmit [39] proposed atherosclerosis to be a postprandial phenomenon. Subsequently, Patsch et al. [40] showed that measures of serum triacylglycerol during the postprandial period (6–7 hours post-meal) were more discriminating of CHD risk than concentrations of lipoproteins in the fasting state.

Marker-disease relationship. In common with HDL cholesterol, there have been no intervention trials specifically designed to examine the relationship between changes in serum triacylglycerol and CVD endpoints. Those that have shown benefit are again interventions with fibrates such as the Helsinki Heart Study which recorded a significant reduction in CHD events in response to a decrease in serum triacylglycerol of 35 % [24]. Although data on small, dense LDL were not available on this trial, 70 % of those who benefited had the 'lipid triad' of raised LDL/HDL ratio, low HDL and serum triacylglycerol > 2.3 mmol/L. In the Veterans' Affairs Study (VA-HIT), a significant reduction in non-fatal MI and CVD death was associated with a 31 % reduction in serum triacylglycerol.

A concentration of serum triacylglycerol of 1.5 mmol/L has been suggested as a critical threshold which, if exceeded, can lead to the formation of small, dense LDL and a low HDL cholesterol level [41]. The existence of this subclinical threshold was later confirmed as lying between 1.5 and 1.7 mmol/l in diabetic and non-diabetic populations [42]. Serum triacylglycerol con-

European Journal of Nutrition, Vol. 42, Supplement 1 (2003)

centrations are highly responsive to environmental stimuli, including drugs, diet, changes in weight and physical activity [43]. Serum triacylglycerol levels also increase with age and in postmenopausal women [44].

A concentration of serum triacylglycerol in excess of 10 mmol/L is recognised as a medical emergency due to the risk of acute pancreatitis. While a concentration of serum triacylglycerol in excess of 2.3 mmol/L is recognised as a clinical action limit for dietary and drug intervention, moderate increases in serum triacylglycerol levels below this limit can lead to the development of abnormalities in LDL and HDL which increase the atherogenic potential of these lipoproteins. The concentration of serum triacylglycerol at which triacylglycerol-rich lipoproteins may be directly damaging to the endothelium is unknown but will depend to a large extent on the duration and magnitude of postprandial lipaemia.

Mechanism. The potential atherogenicity associated with raised serum triacylglycerol levels has direct and indirect origins. There is evidence to suggest that remnants of triacylglycerol-rich lipoproteins (chylomicron remnants and VLDL) may be directly damaging and thus atherogenic to the vascular endothelium [45]. Other triacylglycerol-mediated risks are likely to be indirect and operate through pro-atherogenic changes in the structure and composition of LDL and HDL. The atherogenicity of small, dense LDL is explained by its unique physicochemical properties which, through a sequence of events, leads to the deposition of cholesterol [46]. The infiltration of the arterial intima by serum lipoproteins is a function of particle size, so that small, dense LDL can infiltrate the vessel wall to a greater extent than its larger counterparts. Arterial proteoglycans (extra-cellular tissue matrix of intima) show selective binding and sequestration of small dense LDL. Once bound, small, dense LDL shows relatively greater oxidative susceptibility than the larger and lighter LDL subclasses, increasing its uptake into scavenging macrophages. Low HDL is believed to reduce the capacity to perform the reverse transport of cholesterol out of arterial lesions. Evidence for the above has, in general, been derived from *in vitro* studies. However, there is evidence to show that LDL recovered from the artery wall has physiochemical properties that resemble those of small, dense LDL [46].

Raised serum triacylglycerol levels have also been associated with a pro-coagulable state due to an increased tendency of the blood to clot and by a decrease in fibrinolysis.

Animal studies. Evidence to support a role for remnant lipoproteins in atherosclerosis was obtained largely from animal data. However, as already discussed for serum LDL and HDL cholesterol, animal studies can only be supportive.

Conclusions and gap analysis

The evidence that HDL cholesterol is causally related to cardiovascular risk is strong and consistent. However, formal proof is lacking which may be obtained with placebo-controlled, clinical intervention trials designed specifically to examine the relationship between raising HDL cholesterol and CVD endpoints. More evidence is also needed to determine to what extent the anti-atherogenic effect of HDL is related to one of its subfractions or molecular components. It should also be examined whether diet-induced changes in the ratio of total serum cholesterol to HDL cholesterol, which strongly relates with cardiovascular risk, is a simple but valid predictor of CVD risk.

In the earlier studies, the positive relationship between serum fasting triacylglycerol and CHD risk was not independent of other variables, most notably HDL cholesterol. As a result, serum triacylglycerol has been down-graded as a risk factor because it failed to meet the criterion of independence that applied to other measures of risk such as LDL cholesterol [47]. Fortunately, this is no longer a consensus view because of improved knowledge and of appreciation of the metabolic interrelationships between the triacylglycerol-rich lipoproteins, LDL and HDL. The clustering of lipoprotein abnormalities associated with raised serum triacylglycerol levels, including a predominance of small, dense 'atherogenic' LDL and a low HDL has been associated with a 3-fold increase in risk in young MI survivors and is recognised as a common source of lipid-mediated risk in free-living populations [48]. As for HDL, definitive evidence is needed to link decreased serum triacylglycerol levels, changes in small, dense LDL and HDL with clinical endpoints of CHD.

Hemostatic function

Background

Haemostasis can be represented as a process in which endothelium, platelets, and coagulation and fibrinolytic factors are in constant interaction with each other. The endothelium maintains a non-thrombogenic surface for blood flow, prevents platelet and leukocyte adhesion to the vessel surface, modulates cellular composition of the arterial wall, and promotes dilator tone of arteries and veins, homeostatic properties regulated in part by the local synthesis of nitric oxide. Platelets take part in haemostatic and thrombotic events by adhering to and aggregating on the site of injury, releasing several regulatory compounds and providing activated membrane phospholipid surfaces for coagulation complexes. Under normal physiological conditions coagulation and fibrinolytic factors balance each other. Many of these factors

are present in the plasma in an inactive form and become activated as a result of vascular injury. A prothrombotic condition may arise in situations in which the balance between the different parts of haemostasis becomes disrupted [49]. Hemostatic function may be affected to some extent by dietary factors such as total dietary fat, n-3 and *trans* fatty acids, alcohol, vitamins E and C, and folic acid, as well as L-arginine [50].

Biomarkers and claims

The endothelium is a highly specialised, metabolically active interface between blood and underlying tissues. It maintains vascular tone, thromboresistance, and a selective permeability to cells and proteins. Moreover, under the stimulation of agents such as interleukin 1, the endothelium undergoes changes, which allows it to participate in the inflammatory responses. This process is known as endothelial cell activation and is characterised by a loss of vascular integrity, expression of leukocyte adhesion molecules, change in phenotype from antithrombotic to prothrombotic, cytokine production, and up-regulation of HLA molecules. Two stages of endothelial cell activation exist. Activation type I does not require *de novo* protein synthesis or gene regulation and it occurs rapidly (P-selectin and von Willebrand factor), and activation type II, which proceeds through gene transcription and protein synthesis (adhesion molecules, cytokines, and tissue factor). Diverse pathways of endothelial cell activation, however, activate a cytoplasmic nuclear factor κB, which is currently regarded an interesting target for pharmacological and dietary manipulation.

Endothelial dysfunction may arise either from enhanced and maintained endothelial activation or impaired endothelial-dependent vasodilatation. Endothelial activation is detected by increased plasma concentrations of soluble adhesion molecules, which are shed and released into plasma from activated endothelium and macrophages. Defects in endothelium-dependent vasodilatation can be detected by a noninvasive ultrasound technique, which measures the vasodilator responses of conduct vessels after infusion of an agonist such as acetylcholine or serotonin, or, more commonly, in response to increased flow induced by reactive hyperaemia [50, 51].

During arterial thrombogenesis *platelets* will be activated with subsequent biochemical and morphological changes, which lead to expression of cell surface receptors, adhesion and to the platelet release reaction, and finally to platelet plug formation. The principle method in platelet studies has been the *in vitro* aggregation test because of the lack of proper ways to measure platelet activation *in vivo*. The rationale behind platelet aggregation studies *in vitro* is to mimic the situation during the thrombotic process. The shortcome of this method is that the aggregation test only measures the ability of platelets to react to a single external stimulus, a situation clearly different from the conditions in the human body. The method is not easy to standardise and difficult to introduce into epidemiological studies; thus there are not much data on the role of platelet aggregation pattern as the risk marker for CVD [49]. Nowadays, however, other methods to measure platelet function are also used, e. g. measuring the activation status of fibrinogen receptor, P-selectin expression and shedding of microparticles. *In vivo* bleeding time is considered to represent a combined action of both endothelial function and platelet reactivity.

The activation of the *coagulation system* has often been determined using the concentrations of fibrinogen and prothrombin fragments F_{1+2} as well as concentrations of the total antigen or activated factor and/or *in vitro* activity of FVII and other coagulation factors or antithrombin III (ATIII). Tissue type plasminogen activator (tPA), its inhibitor PAI-1, and D-dimers have been measured as markers of fibrinolysis. Lipoprotein Lp(a), an apolipoprotein homologous with plasminogen, impairs fibrinolysis and thus can be considered as a marker for blood coagulation. New promising risk marker in this field may be the tPA/PAI-1 complex [52]. Some methodological and biological characteristics of these biomarkers are given in Table 3.

Table 3 Methodological and biological characteristics of hemostatic function

Biomarker	Methodological characteristics	Biological characteristics
• Endothelial function	• Not yet enough data to evaluate precision, validity and reproducibility	• A physiological variable which is present in human body at all times, in all people, and in all physiological states, but its intensity varies
• Platelet function	• Low reproducibility, reliability questionable since not enough data to understand how in vitro/ex vivo results are related to in vivo situation	• Present in human body at all times, in all people, and in all physiological states, but its intensity varies as a function of many genetic, physiological and environmental variables
• Coagulation and fibrinolysis	• Good precision, validity and reproducibility for several antigen measurements • Precision, validity and reproducibility for activity measurements questionable	• Present in human body at all times, in all people, and in all physiological states, but concentrations/activities vary as a function of many genetic, physiological and environmental variables

European Journal of Nutrition, Vol. 42, Supplement 1 (2003)

Potential claims include the following:

- Can have a beneficial effect on bleeding time
- Can have a beneficial effect on blood clotting (or on specific coagulation factors)
- Can have a beneficial effect on the fibrinolytic system (or on specific fibrinolytic factors)
- May help to keep the blood thin
- May improve endothelial function
- May lower the risk of cardiovascular diseases/coronary heart disease

Strength of relationship with cardiovascular disease

Human observational studies. There are several observational and some case-control studies showing that patients with CVD or diabetes have impaired endothelium-dependent vasodilatation or enhanced adhesion molecule expression [53–55]. Furthermore, elevated concentrations of adhesion molecules may predict further risk of MI [56]. Nearly all known risk factors for CVD (smoking, hypercholesterolaemia, hypertension, insulin resistance) may cause endothelial dysfunction [50]. Thus strategies such as cholesterol lowering agents and increased physical activity all improve endothelial function. Even though endothelial activation and/or dysfunction may arise as a consequence of other metabolic disorders it may be a promising target for dietary strategies due to its early role as a marker for CVD.

Markers of platelet activation like platelet volume, platelet specific proteins, and spontaneous platelet aggregation have in certain situations shown a predictive value for the risk of CDH or MI, but these markers have not been tested in large scale prospective studies [57].

Fibrinogen has been identified as a major independent risk marker for CVD [58, 59], while the plasma concentration of tPA/PAI-1 has recently been shown to be a novel risk marker for recurrent MI [52]. Furthermore, lipoprotein Lp(a), an apolipoprotein homologous with plasminogen, impairs fibrinolysis and thus enhances blood coagulation. Its clear association with CHD was recently confirmed by a meta-analysis of the 27 prospective studies [60].

Marker-disease relationship. There have been no intervention trials specifically designed to examine the relationship between changes in haemostatic function and risk for CVD. There is however evidence that suppression of platelet activation by drugs is associated with decreased CHD events [61].

Mechanism. Because angiographic trials with lipid lowering therapy have shown a reduction in CVD risk but only little reduction in atherosclerotic plaque size, an improvement in endothelial vasodilator function was suggested as an alternate mechanism. Unknown from all reported studies on the effects of statin therapy on endothelial function, however, is whether improved dilator responsiveness to any dilator agonist or flow-mediated dilation predicts a reduction in the risk of CVD. Several studies actually suggest that therapeutic improvement in endothelial vasodilator function of patients with CHD does not necessarily translate into reduced ischaemia or lower risk of CVD events, thus questioning the relevance of improved endothelial vasodilator function as a therapeutic benefit at least for statin therapy [62]. An impaired haemostatic function may further lead to thrombus formation. This may ultimately occlude a coronary vessel, which may lead to ischaemia and a heart attack. Fibrinogen itself may increase cardiovascular risk in several ways. It takes part in platelet aggregation and fibrin formation, and changes plasma viscosity. Furthermore, it is an acute-phase protein that is increased in inflammatory states [63].

Animal studies. Many studies with transgenic mice have emphasised the importance of proper haemostatic function in the aetiology of CVD. However, less is known as to whether these factors are responsive to dietary changes and whether such changes translate into a reduced risk of CVD.

Conclusions and gap analysis

Since experiments with several dietary components have revealed that *in vitro* effects on the expression of adhesion molecules are not always confirmed in *in vivo* situations, more mechanistic studies in *in vivo* models are needed. Furthermore, positive effects on endothelial vasodilatation are so far restricted to high-risk patients [50] and thus the relevance of endothelial function as a risk marker for CVD in healthy subjects should be studied in more detail. More information is also needed on the relationships between postprandial vs. long-term effects of diet (such as the effects of a high fat diet and of vitamins) on endothelial function. Furthermore it is hardly known how quickly adaptation to a new situation happens, which could be answered by trials with much longer duration. Thus, studies should be designed to validate markers for endothelial function related to cardiovascular outcome. These studies could also address the question of whether nuclear factor kB could become a common novel marker for endothelial function.

Platelet function responds to dietary changes, especially changes in dietary fat quality [64]. For example there is a considerable amount of evidence that platelet activation can be suppressed with long-chain n-3 fatty acids, but the interpretation of the changes with regard to prevention of CVD is still lacking. Even less well understood is the role of other dietary compounds, e. g. flavonoids and other polyphenols. In some epidemio-

logical studies the intake of flavonoids was inversely associated with mortality from CHD and flavonoids also reduce platelet function *in vitro*. *In vivo* controlled interventions have, however, failed to show any effect on platelet function [65]. Early studies with long-chain n-3 fatty acids as well as results from the supplementation studies with alpha-linolenic acid suggest a possible use of increased bleeding time as a claim [66, 67]. However, similar to the platelet aggregation data, there are no data on the relationship of this parameter and the risk of CVD. Since no prospective studies are available to show increased platelet activation as measured with conventional *in vitro* assays or decreased bleeding time to be causally related to the risk of CVD, it can therefore be concluded that available measures of platelet function are not reliable indicators of the prothrombotic state in humans. New markers of the prothrombotic state are needed and their relevance should be tested in well-controlled clinical trials and large-scale prospective studies before they could be used as a basis for claims.

Even though fibrinogen and Lp(a) are associated with the risk of CVD, their relative unresponsivenesses to dietary changes weaken their use for claims. Furthermore, plasma concentrations of a single coagulation and/or the fibrinolysis proteins/activities may not be sensitive enough to indicate the prothrombotic state. This again underlines the need of new sensitive markers to measure changed coagulation and fibrinolytic patterns in humans.

Oxidative damage

Background

Oxygen-dependent metabolism is closely related to the generation of reactive oxygen species, which may oxidise lipids, DNA, proteins, or carbohydrates, processes linked to the pathobiochemistry of several degenerative diseases [68, 69]. There is evidence, for example, that oxidative modification of lipids and proteins from LDL plays a crucial role in the pathogenetic pathway of fatty streak formation [70]. Different defence systems are operative against lipid oxidation, which include dietary antioxidants such as tocopherols, carotenoids, polyphenols and vitamin C. Most of these dietary antioxidants exhibit biological activities beyond their antioxidative properties, which could also be important in context with the prevention of cardiovascular events. In fact, tocopherols, flavonoids and carotenoids have an impact on regulatory pathways, which might play a role in atherogenesis [71–73]. Evidence from *in vitro* and *in vivo* experiments indeed suggests that dietary antioxidants are relevant in the prevention of CVD [74–76]. Epidemiological studies also provide strong evidence that the consumption of a diet rich in fruit and vegetables – major sources of dietary antioxidants – is associated with a lower risk of CVD [77]. Together with the concept of LDL oxidation, the idea of antioxidants as protectants against CVD has developed [78, 79].

Biomarkers and claims

Markers of lipid peroxidation. The extent of lipid peroxidation *in vitro* and *in vivo* can be followed by the analysis of biomarkers [80–83]. A detailed review of the available markers and an evaluation of the validity of the methods is given in a consensus paper from the EU concerted action EUROFEDA [84, 109]. Most frequently, oxidative damage to lipids is determined by measuring breakdown products as thiobarbituric acid reactive substances (TBARS) or malondialdehyde (MDA). Both assays, however, lack specificity. Endogenous compounds may interfere and oxidative damage can be overestimated [85]. The assays are also sensitive to oxidative conditions *in vivo* [86]. In some intervention studies with antioxidants a response of these biomarkers was observed [87, 88], while others found no significant effects [89]. Another relatively unspecific method is based on the measurement of conjugated dienes, which increase as a result of lipid peroxidation [90].

Lipid hydroperoxides or oxidised cholesterol derivatives are generated during lipid peroxidation. Separation of these oxidation products by HPLC combined with selective detection methods increases the specificity of the assay [90]. In the process of lipid peroxidation volatile products like ethane and pentane are formed. They are exhaled and their concentrations are elevated under conditions of oxidative stress [91]. Interference of pentane with endogenous compounds such as isoprene has been reported [92].

The analysis of isoprostanes has been introduced as a measure of the extent of lipid peroxidation [93, 80]. It has been shown that urinary excretion of F_2-isoprostanes is increased under conditions of oxidative stress. At present the concentration of isoprostanes is thought to be the most reliable biomarker indicating lipid peroxidation. Intervention with antioxidant supplementation decreased the concentration of F_2-isoprostanes in plasma from smokers [94]. The consumption of green tea, rich in polyphenols, did not affect isoprostane excretion [95, 96] although plasma MDA concentrations were lowered [95].

Markers of LDL modification. Depending on the nature of the prooxidant, the protein part or lipid core of an oxidised LDL particle may be modified in various ways. Changes in the composition, function or susceptibility towards prooxidants are used to characterise LDL particles [97]. Markers of lipid peroxidation (see above) or immunological methods are applied to measure the

level of modified LDL. Resistance of LDL towards an oxidative challenge is determined by measuring the lag-phase [98, 99]. It has been shown that the intervention with dietary antioxidants or dietary sources of antioxidants may increase the resistance of LDL towards oxidation and influences the lag-phase [100–102]. It should be noted, however, that these experiments are *in vivo*/*ex vivo* studies and associated with several technical problems [97].

Markers of antioxidant status. The analysis of circulating antioxidants can be used to evaluate the antioxidant network, but also conditions of oxidative stress. It is known that serum concentrations of antioxidants can be influenced by a variety of parameters related to disease, nutrition and lifestyle. Since the antioxidant defence system represents a complex network with interactions, synergisms but also specific tasks for a given antioxidant, the measurement of the total antioxidant potential of plasma may not be considered as an alternative to the assay of individual plasma antioxidants [103]. Some methodological and biological characteristics of these biomarkers are given in Table 4.

Possible claims for antioxidants include the following:

- May inhibit lipid peroxidation
- May lower oxidative damage
- May decrease LDL oxidation/modification
- May lower the risk of cardiovascular diseases/coronary heart disease

Strength of relationship with cardiovascular disease

Human observational studies. Epidemiological studies have shown an inverse association between a high intake of dietary antioxidants and the CVD risk in humans [104–108]. These studies provide evidence that people with a high intake of antioxidant vitamins have a lower risk of MI and stroke than people with a lower consumption of antioxidant vitamins. Those correlations have been found for carotenoids, ascorbic acid as well as for tocopherol. The levels of single antioxidants or differences in the antioxidant pattern have also been associated with differences in the risk of CVD. With a few exceptions, there is a substantial lack of studies investigating whether plasma antioxidants or antioxidant patterns may be used to assess oxidative stress in atherosclerosis or its risk.

Marker-disease relationship. There is evidence that malondialdehyde-modified LDL and oxLDL are present in atherosclerotic lesions. Increased levels of MDA-modified LDL have been found in the plasma of patients with ischaemic heart disease. Case control studies have further provided evidence for a relationship between oxidation resistance of LDL and both severity of MI and carotid atherosclerosis. However, there is also a number of human studies which showed no correlation between markers of lipid peroxidation and/or LDL modification and the risk of atherosclerotic diseases. The inconsistency in the present data can as yet not be explained and these markers can therefore not be considered as fully validated with respect to the marker-disease relationship [109].

Table 4 Methodological and biological characteristics of oxidative damage

Biomarker	Methodological characteristics	Biological characteristics
• Lipid peroxidation		
– TBARS and MDA	• Not very specific and possible formation artefacts • TBARS: not stable	• TBARS: highly variable and interferences with other biological compounds
– Conjugated dienes	• Not specific • Interferences with other components	• Not suitable for the determination of lipid peroxidation *in vivo*
– Lipid peroxides	• Very specific, but lack of stability	• Specific indicator for lipid peroxidation *in vivo*
– Pentane/ethane exhalation	• Stable, but sampling is difficult • Specificity is questionable • Interferences with biological compounds (isoprene)	• Indicator for lipid peroxidation *in vivo*. Only limited data for comparison are available.
– Isoprostanes in urine	• Specific, stable, reproducible and few interferences with other biological compounds when HPLC or GC-MS is applied	• Specific indicator for lipid peroxidation *in vivo*
• LDL modification	• Artifacts during isolation • Lack of specificity • Problems associated with measurements of lipid peroxidation in LDL (see above)	• Mainly used for *ex vivo* experiments
• Antioxidant status		
– Total antioxidant capacity as measured with LDL lag phase	• Not specific and variable with respect to the test system • Interferences with other biological compounds	• Ex vivo method and not specific for lipid peroxidation
– Analyses of the antioxidant pattern	• Specific and reproducible	• Broad interindividual variation

Despite the amount of evidence from observational studies, antioxidant vitamins as food supplements have shown no beneficial effects in the primary prevention of MI and stroke in randomised controlled trials such as the HOPE, the CHAOS, the ATBC, and the GISSI studies [106] as well as the MRC/BHF Heart Protection Study [110]. It should be noted, however, that vitamin E supplementation resulted in a RR of 0.53 for cardiovascular death and nonfatal MI in the CHAOS trial, while in the ATBC trial vitamin E and β-carotene supplementation led to a small reduction of fatal CHD incidence. In the GISSI-Prevenzione Trial vitamin E supplementation was associated with a significantly decreased risk of CVD death including cardiac, coronary and sudden death.

In patients with CHD and low plasma HDL cholesterol levels, antioxidant supplementation resulted in a 1.8% average stenosis progression as compared to the 3.9% stenosis progression of the placebo group, although the difference did not reach statistical significance [111]. Furthermore, vitamin E plus vitamin C supplementation slowed down the early progression of transplant-associated coronary arteriosclerosis [112], and probucol – a potent antioxidant – stabilised the plaque and lowered the incidence of cardiac events in hypercholesterolaemic patients [113].

Animal studies. Studies with animal models sensitive to diet-induced atherosclerosis have shown that modulation of the antioxidant defence system via administration of dietary antioxidants is correlated with delayed progression or regression of this process [114–116]. In addition experiments with transgenic mice models in combination with reliable markers of lipid peroxidation provided evidence for a functional role of oxidative stress in atherogenesis [117].

Mechanism. The general basis of the theory that dietary antioxidants may contribute to the prevention of CVD is related to formation of atherogenic oxLDL *in vivo* under conditions of oxidative stress [70]. Our understanding of the impact of oxLDL on the mechanism of atherogenesis and the underlying biochemical pathways is however limited. Further research is therefore needed to identify the specific kind of oxidative damage that affects the LDL and to relate the extent of damage to pathogenicity. The signalling properties of dietary antioxidants, which are not or only partly related to antioxidant effects, may also provide alternative mechanisms of action and deserve further research activities [71–73].

Conclusions and gap analysis

There is a great body of evidence that antioxidants inhibit lipid peroxidation and prevent the formation of oxLDL. In addition, numerous studies support the idea that oxLDL play a role in atherogenesis as an early event in the development of CVD. Furthermore, epidemiological studies support the idea that an increased intake of antioxidants helps prevent CVD. Unfortunately, contradictory results from intervention trials do not allow for a final conclusion. However, the beneficial effect of a diet rich in antioxidant-containing fruit and vegetables in disease protection is still obvious [118–120], and – regardless of current level of intake – almost all individuals are expected to benefit from increasing their intake of fruits and vegetables [121, 122]. Thus, the oxidation hypothesis is still viable, but has been challenged [123–126]. For this, validation of the markers with respect to the endpoint or intermediate clinical markers must be performed.

The discrepancies between observational studies and intervention trials outcomes may have several explanations, although one recurrent feature of the intervention studies is the absence of a biochemical or nutritional basis for patient inclusion [127]. It has been suggested that the biological activity was assigned to the wrong dietary constituents and that high concentrations of plasma antioxidants are just a surrogate marker. It has been speculated that the dose concentrations of the supplements were too low to provide effects in a selected population, which is at a high risk of CVD. On the other hand, adverse effects have been attributed to nonphysiologically high doses. A biochemical oxidative stress-, nutrition- or clinical marker outcome-related baseline evaluation should constitute critical issues for intervention trial designs. Finally none of the intervention studies available to date was designed to reveal clear relations between dose and efficacy or toxicity.

Pro-oxidant effects of antioxidants, which can be observed under specific *in vitro* conditions, have also been addressed as a possible reason for the failure of intervention. The relevance of pro-oxidative activities of antioxidants *in vivo*, however, is yet unclear. There is increasing evidence that the antioxidant defence system is a complex network of many partners, endogenous antioxidants, dietary components, and antioxidant enzymes as well as repair systems. Co-operative interaction provides synergistic effects of protection. A nonphysiological disturbance of the delicate balance between the components may not only result in a loss of synergy but might also weaken the impact of other components to total antioxidant defence. The aspect of synergistic effects in a balanced antioxidant network must be investigated in detail, especially keeping in mind the positive epidemiological data related to a balanced diet rich in dietary antioxidants and the negative outcome of intervention studies with high doses of a single antioxidant. Several interactions between antioxidant systems have already been demonstrated. Epidemiological as well as experimental studies suggest that such interactions result in synergistic effects.

European Journal of Nutrition, Vol. 42, Supplement 1 (2003)

Studies on the bioavailability of antioxidants are needed. The availability of antioxidants from the diet and from supplements is influenced by numerous parameters, which have an impact on the availability of the active compound in the systemic circulation, and at the target site. Most of the studies on antioxidants have been performed with isolated compounds. More information is needed about the availability from dietary sources.

Animal studies should be applied to confirm concepts derived from *in vitro* and cell culture studies. Suitable animal models are needed to investigate dose-relationships and the quality of markers. This refers to markers of exposure as well as to markers of response. Animal models are essential for dose finding.

In vitro and cell culture experiments are warranted to identify further antioxidants present in the diet. Protective effects of diets rich in antioxidants are apparently more pronounced than supplementation with a single component. This suggests that either yet unknown factors are of importance or that synergistic activities of combinations of antioxidants are relevant.

The ultimate goal of course must be to collect all the relevant data for a risk-benefit analysis to examine the effects of dietary antioxidants on CVD. For this, human intervention studies with various population groups are needed measuring validated markers of exposure, function and intermediate endpoints. The design of such intervention studies with respect to doses, selected antioxidants or mixtures, duration and characteristics of the participants should be based on previous animal studies or small-sized intervention studies with humans.

Homocysteine metabolism

Background

Homocysteine, an intermediary metabolite of intracellular methionine metabolism, has no known physiological function. It is produced by demethylation of methionine and metabolised through two alternative pathways: transsulphuration by the vitamin B_6-dependent cystathionine-beta-synthase producing cystathionine or remethylation to methionine by methionine synthase in a reaction with methyltetrahydrofolate as methyl donor and vitamin B_{12} as a cofactor, or with betaine as a methyl donor.

Plasma homocysteine concentrations are influenced by dietary intakes of folates, vitamins B_6 and B_{12}, and betaine. Of these, folate (folic acid) intake seems to be the most important. At the population level, folate intake is inversely associated with plasma homocysteine concentrations. TT homozygosity for the methylenetetrahydrofolate reductase (MTHFR) gene is related to hyperhomocysteinaemia – commonly defined as plasma homocysteine concentrations above 14 µmol/L – particularly when folate intake is low. Folic acid supplements reduce plasma homocysteine concentrations on average by 25 %. The effect is largest in persons with high plasma homocysteine concentrations and the lowest folate intakes. The maximal effect is achieved by doses of 0.4–0.8 mg or more folic acid [128, 129]. The bioavailability of dietary folates is 35–50 % lower than that of folic acid [130]. Increasing the intake of vitamin B_{12} has a smaller reducing effect (about 7 %) on plasma homocysteine, whereas the effect on plasma homocysteine of increasing vitamin B_6 intake is questionable [128]. High doses of betaine (6 g/d) have been used in combination with folic acid in the treatment of homocystinuria. Plasma homocysteine concentrations of healthy individuals can also be reduced by betaine supplementation [131,132].

Biomarkers and claims

The most obvious marker to evaluate homocysteine status is the plasma total homocysteine concentration. However, other markers are used to evaluate dietary intakes of specific determinants of homocysteine metabolism.

Very low concentrations of plasma folate indicate folate deficiency, but are less accurate in distinguishing differences in intakes that are within the normal range. Low plasma folate combined with high homocysteine concentrations is a better indicator of mild folate deficiency than folate alone. Red blood cell (RBC) folate is a better indicator of long-term exposure. Low plasma vitamin B_{12} concentrations often indicate deficiency, but false positive results are common. Low plasma vitamin B_{12} combined with high plasma homocysteine or methylmalonic acid concentrations is a more reliable indicator of B_{12} deficiency. Plasma pyridoxal phosphate (vitamin B_6) concentrations reflect tissue stores and change rather slowly in response to changes in dietary intakes. RBC aminotransferase activity has been used to examine vitamin B_6 status. Methionine loading tests the activity of the transsulphuration pathway. In epidemiological studies fasting plasma homocysteine concentrations have generally produced the same evidence as values recorded after a methionine load. Betaine intakes from food are probably too low to affect homocysteine metabolism, but more information is needed. Some methodological and biological characteristics of homocysteine as biomarker are given in Table 5.

Possible claims include the following:

- May reduce blood homocysteine concentrations
- May improve endothelial function
- May lower the risk of cardiovascular diseases/coronary heart disease

Table 5 Methodological and biological characteristics of homocysteine

Biomarker	Methodological characteristics	Biological characteristics
• Homocysteine	• Excellent precision, validity and reproducibility • A variety of analytical methods exist • Chemically relatively stable and can therefore be analysed in properly stored, banked plasma samples	• Present in the blood of all humans at all times, in all physiological states, but its abundance varies • Levels vary as a function of many genetic, physiological and environmental variables • Relatively stable over time

Strength of relationship with cardiovascular disease

Human observational studies. Cross-sectional case-control studies have found consistent associations between high plasma homocysteine concentrations and risk of vascular diseases. Subsequent studies also showed that subjects with CHD, stroke, and venous thrombosis have higher mean plasma homocysteine concentrations than healthy subjects [128, 133]. Prospective cohort studies, however, have found less consistent results. In a meta-analysis of studies published from 1966 to January 1999, the risk of CHD in prospective studies was half as strong as in retrospective studies and was attenuated with increasing time to event [134]. In subjects with no CVD at baseline, plasma homocysteine did not predict the risk of CVD, but in patients who had CHD already at baseline, hyperhomocysteinemia was associated with increased risk of CHD [135]. In a population-based cohort study from Norway, plasma homocysteine was a strong predictor of CVD only in elderly individuals and among those with pre-existing CVD [136]. In patients with CHD, high plasma homocysteine concentrations are associated with high risk of recurrent MI and predict poor prognosis [137]. Thus hyperhomocysteinaemia seems to be at least an indicator of the severity of atherosclerosis.

Low plasma folate concentrations or a low folate intake have been inversely associated with increased risk of CVD. The association may reflect the combined effects of nutrients provided by a diet high in fruit and vegetables, but does not rule out the possibility of a direct effect of folates. A relationship has been observed between plasma folates and risk of CVD, irrespective of homocysteine concentrations [138, 139].

Marker-disease relationship. In homocystinuria, plasma homocysteine concentrations are grossly increased and premature thrombotic events are common [140]. Treatment with vitamin B_6, folic acid and betaine reduces both plasma homocysteine concentrations and risk of vascular disease. Based on this experience it was postulated that hyperhomocysteinaemia might be causally related to CVD [141]. Supplementation with folic acid and vitamins B_6 and B_{12} failed to prevent intimal thickening of arteries and development of vascular dysfunction in monkeys [142], but supplementation with folic acid plus vitamins B_6 and B_{12} reduced the rate of restenosis after coronary angioplasty in humans [143, 144].

Another approach to examine the possible causal role for homocysteine in the risk of CVD is to use polymorphisms. If a certain polymorphism is from birth onwards related to higher levels of a possible risk factor and these subjects are at a later stage of life indeed at increased risk for a certain disease, then it is obvious that the cause preceded the disease. Hyperhomocysteinaemia associated with the TT genotype of the C677T variant of the MTHFR polymorphism was not associated with increased risk of CVD in one meta-analysis [145], whereas in another analysis a significant association was found among Japanese people but not in other populations [146].

Mechanism. There is no commonly accepted concept of the mechanism of action of homocysteine. Impaired renal function reduces homocysteine clearance and increases plasma homocysteine concentrations. The association between plasma homocysteine and risk of CVD has therefore been explained by impaired renal function due to hypertension and atherosclerosis [147]. As plasma homocysteine concentrations are increased by acute MI, it may also be an acute phase reactant reflecting severity of inflammatory processes associated with atherosclerosis [148]. Further, homocysteine is associated with a variety of thrombotic factors, but it may also cause direct toxic endothelial damage, reduce the production of endothelium-derived nitric oxide resulting in endothelial dysfunction, stimulate smooth muscle proliferation, and increase susceptibility to oxidation of low-density lipoprotein. It has been suggested that in healthy endothelium the effects of homocysteine are neutralised by nitric oxide whereas failure of diseased endothelium to neutralise homocysteine leads to production of hydrogen peroxide by auto-oxidation of homocysteine and to oxidative damage [133]. Therefore, data are needed of effects on both intact and affected endothelium. An antioxidative mechanism is supported by two studies showing that endothelial dysfunction associated with high plasma homocysteine concentrations can be reversed by the administration of vitamin C [147].

European Journal of Nutrition, Vol. 42, Supplement 1 (2003)

Conclusions and gap analysis

At present homocysteine is just a marker associated with CVD, and it is not known whether reduction of plasma homocysteine concentrations affects risk of CVD or has other positive health effects. Claims related to plasma homocysteine concentrations may become relevant if evidence for beneficial effects is obtained from ongoing clinical trials. There are at least nine ongoing clinical intervention trials in patients with CVD on effects of lowering plasma homocysteine concentrations by dietary supplements, started between 1997 and 1999 [128]. The supplements include folic acid in doses of 0.2 to 5 mg/d, either alone or in combination with vitamins B_6 and B_{12}. These trials, however, are secondary prevention trials and include only patients with existing vascular disease, but do not answer the question if the findings can be extrapolated to the general population. Also, more information is needed on the effects of homocysteine-lowering after coronary angioplasty.

It is also important to examine if folates have direct effects on CVD risk or if these effects are mediated by homocysteine. Folic acid may have a rapid action on endothelial function apparently independent of homocysteine lowering [149]. In this respect, studies with betaine can be useful. If lowering of plasma homocysteine by betaine produces similar effects on CVD risk as folic acid, this would suggest that homocysteine is the mediator of cardiovascular endpoints. Finally, more information is needed on the mode of action of homocysteine.

Blood pressure

Background

Blood pressure is one of the most important modifiable risk factors of CVD and dietary factors that may influence blood pressure are excessive energy intake, alcohol, sodium, potassium, calcium, dietary fats, protein, fibre, and coffee. For adults, hypertension is defined as a systolic blood pressure of 140 mmHg or greater, a diastolic blood pressure of 90 mmHg or greater, or taking antihypertensive medication [150]. The prevalence of hypertension is about 25 % in the general population and amounts to 60–70 % in the age group above 60 years [151, 152]. Aggregation of high blood pressure with other risk factors of CVD such as abdominal obesity, glucose intolerance, hyperinsulinaemia, dyslipidaemia, and hyperuricaemia has been a consistent finding in Westernised populations [153], probably because of a common aetiology. Therefore, risk factor clustering should be kept in mind if a blood pressure reducing agent or therapy is evaluated in relation to CVD.

Increased blood pressure may result in cardiac, cerebral, retinal or renal complications. The heart responds to hypertension with compensatory growth of the myocardium, thus resulting in left ventricular hypertrophy and, if left untreated, followed by dilatation and heart failure. Besides affecting the myocardium directly, hypertension is also one of the risk factors of CVD, including stroke. Hypertension also increases the risk of all types of dementia, including Alzheimer's disease, in the elderly [154, 155]. Microangiopathic changes due to hypertension may further result in retinopathy. The kidney may be primarily engaged in the development of essential hypertension by an increased sodium reabsorption or a change in sodium homeostasis. On the other hand, the kidney is also a major target for organ damage due to high blood pressure. Hypertension may lead to a fall in glomerular filtration rate, a reduction in renal blood flow and eventually renal failure. In this summary document, however, only complications related to CVD will be discussed.

Biomarkers and claims

Guidelines on blood pressure measurements, target blood pressure, risk stratification, parameters for target organ damage and treatment strategies, which could be helpful in the design of clinical trials, are summarised in the 1999 WHO/ISH document [156] and in the Sixth Report of the Joint National Committee on Prevention, Detection, Evaluation and Treatment of High Blood Pressure [150].

Blood pressure is usually measured by conventional sphygmomanometry. Although apparently simple, this procedure is fraught with many potential sources of error. Detailed guidelines for the measurement of office blood pressure are available from the British Hypertension Society [157] or the Joint National Committee on Prevention, Detection, Evaluation and Treatment of High Blood Pressure [150]. Standardisation of the method (choice of the arm, position of the arm, position of the subject, rate of inflation, etc.) and the equipment (cuff size, calibration of the sphygmomanometer, etc.) are important criteria. Moreover, blood pressure should be an average value of readings obtained on at least 2 office visits with preferably 3 readings per visit separated by 2 minutes. Digit preference and inter-observer bias should be estimated at regular intervals, especially in multi-centre trials with a large number of observers. In addition to the problems of digit preference and observer bias, conventional blood pressure has raised environmental concerns (the call for a 'ban of mercury') and is subject to the so-called 'white-coat effect', i. e. the transient rise of the blood pressure in response to the surroundings of the clinic or the presence of an observer.

Recently, automated techniques to measure blood pressure have gained increasing interest [158], since

they might overcome these problems. The automated technique of ambulatory blood pressure monitoring (24-hour blood pressure monitoring) has been validated over the last decade. Ambulatory daytime systolic blood pressure is a significant predictor for the risk of CVD over and beyond conventional blood pressure [159]. The main disadvantages, however, are the high costs and the relative inconvenience to the patient. A cheaper and more comfortable automated technique is the self-measurement of the blood pressure by the patient at home for which validated devices have become available. Clinical validation of home blood pressure appears promising, but is still under investigation. In conclusion, conventional blood pressure remains the golden standard in epidemiological research and in clinical trials.

Systolic and diastolic blood pressure show a high degree of colinearity: the correlation between systolic and diastolic blood pressure is 0.7 to 0.8, which is about the same as the correlation between repeated measures of either systolic or diastolic pressure [160]. However, systolic blood pressure can be measured more accurately and increases much more with age than diastolic pressure. The latter even tends to fall above 50 years of age [151]. Consequently, systolic blood pressure is the measurement of choice in the elderly in whom the predominant type of hypertension is isolated systolic hypertension [161]. Data from the Framingham Heart Study show that from the age of 60 years on, systolic blood pressure and pulse pressure (i. e. the difference between systolic and diastolic blood pressure) become superior as predictor of CHD [162]. Pulse pressure is a pulsatile component of the blood pressure wave and is determined by left ventricular ejection, arterial stiffness and wave reflections. Some methodological and biological characteristics of blood pressure as biomarkers are given in Table 6.

Possible claims include the following:

- May lower systolic blood pressure
- May lower diastolic blood pressure
- May reduce left ventricular hypertrophy
- May lower the risk of stroke
- May lower the risk of heart failure
- May lower the risk of cardiovascular diseases/coronary heart disease

Strength of relationship with cardiovascular disease

Marker-disease relationship. As substantial data are available from randomised clinical trials, results from observational studies will not be summarised in a separate section. These randomised clinical trials provide consistent data on the effect of blood pressure reduction on CVD, but follow-up on randomised treatment is usually not longer than 3 to 5 years. In 1990, Collins et al. [163] published an overview of 17 randomised trials of antihypertensive treatment including 47,653 patients (56 % men) with a mean age of 56 years and a median follow-up of 4.9 years. The average blood pressure reduction was 5–6 mmHg diastolic and 10–14 mmHg systolic. This decrease in blood pressure was associated with 38 % (95 % CI: 31–45 %) reduction in stroke, 16 % (8–23 %) reduction in CHD and 21 % (13–28 %) reduction in total mortality. In a recent meta-analysis of eight outcome trials in the elderly including 15,693 patients of 60 years or older with isolated systolic hypertension and a median follow-up of 3.8 years, active treatment in comparison with placebo resulted in a blood pressure reduction of 4.9 mmHg diastolic and 10.4 mmHg systolic. Active treatment was associated with a 30 % (95 % CI: 18–41 %) reduction in stroke, 23 % (10–34 %) reduction in coronary events and 13 % (2–22 %) reduction in total mortality [161].

In a collaborative project [164] using data from nine prospective studies including 418,343 individuals between 25 and 84 years, mainly (96 %) men, baseline blood pressure was associated with disease outcome after a follow-up period of on average 10 years. A 5 mmHg lower diastolic pressure, accompanied by a 9 mmHg lower systolic pressure conferred a 35–40 % lower risk of stroke and 20–25 % lower risk of CHD. The Framingham Heart Study showed that, after a 30-year follow-up, hypertensives compared to normotensives had a sixfold higher risk for heart failure and a twofold higher risk for intermittent claudication [165].

In epidemiological studies, the effect sizes of blood pressure differences on the risks of stroke or CHD were similar in men and women, for fatal and non-fatal disease or in normotensives and hypertensives [164]. In clinical trials, the relative benefit of treatment did not differ according to sex, age, initial blood pressure, previ-

Table 6 Methodological and biological characteristics of blood pressure

Biomarker	Methodological characteristics	Biological characteristics
• Blood pressure	• Precision and reproducibility depend on the standardisation of the equipment and the method • Assessment of digit preferences and inter-observer bias is essential in multi-centre trials • Can be falsely high due to the 'white-coat effect'	• Levels vary as a function of many genetic, physiological and environmental variables • In older people (60 +), systolic blood pressure or pulse pressure (and not diastolic blood pressure) is the measurement of choice • Blood pressure increases with age

ous cardiovascular complications or smoking habits [161]. Nevertheless, in absolute terms, i. e. in terms of the number of patients to treat to prevent one event, antihypertensive treatment was more effective in men, in patients aged 70 years or older, and in patients with previous cardiovascular complications [161].

Diabetic patients are at greater risk of developing hypertension. Moreover, coexistence of diabetes and hypertension is a synergistic risk factor for cardiovascular morbidity and mortality and for developing end-organ damage, especially of the kidney and the retina [166].

A log-linear relationship was found between blood pressure and cardiovascular events, in epidemiological studies in the blood pressure range 70–110 mmHg diastolic and in the clinical trials in hypertensive patients [163]. Yet, in 1987, Cruickshank et al. [167] were the first to lance the idea of a J-shaped curve: lowering blood pressure below 80–90 mmHg diastolic in patients with pre-existing coronary sclerosis was associated with an increased risks of cardiac morbidity and mortality. In the randomised double-blind placebo controlled EWPHE trial [168] a J-curve was also found, both in the placebo and in the active treatment group, but patients in the lower third of blood pressures were characterised by decreases in body weight and haemoglobin suggesting some deterioration of general health. The discussion on whether or not there is a threshold in the risk between blood pressure and risk of CVD is still inconclusive. Recently, Port et al. [169] proposed a new model with an age- and sex-dependent systolic blood pressure threshold and a steadily rising risk of deaths due to CVD when the threshold is exceeded. Data from the Framingham Heart Study have shown that people with borderline hypertension have an increased risk of developing hypertension in the following years and thus intensive monitoring and primary prevention of hypertension are recommended [170].

Mechanism. Hypertension is associated with hypertrophy of the resistance vessels. Chronically raised intraluminal pressure results in synthesis of matrix proteins, mediated by growth factors and cytokinines secreted by the endothelium and the smooth muscle cells themselves. Moreover, hypertension exacerbates atherosclerosis, possibly through shear or stress on the artery wall resulting in a proliferative response of vascular smooth muscle cells. The narrowing of the lumen and other risk factors such as increased transport of lipoproteins, oxyradical formation, intravascular coagulation and immunological mechanisms contribute to an enhanced risk for MI, stroke and peripheral vascular disease. At the cerebral circulation, hypertension also resets the autoregulation of cerebral blood flow, thus impairing the tolerance to pressure changes and increasing the risk for transient ischaemic attacks and stroke. At the heart, arterial blood pressure is the most common cause of left ventricular pressure overload. It results in left ventricular hypertrophy and later in dilatation and heart failure [171].

Conclusions and gap analysis

Epidemiology, clinical research, and physiology have provided substantial information about the factors contributing to hypertension. From these studies it is clear that hypertension is a multifactorial disease resulting from many genetically and environmentally controlled factors interacting with each other. Furthermore, there is consistent evidence that decreasing blood pressure in hypertensives reduces cardiovascular morbidity and mortality. However, there is still incomplete knowledge on the exact pathophysiological mechanisms. The evidence for a J-shaped curve between blood pressure and adverse effects is still inconclusive and raises questions on whether or not it is necessary to treat borderline hypertension.

A challenge for the future will be to identify candidate genes which predispose to hypertension and its cardiovascular complications. A variety of candidate genes have been tested for linkage and associations, including loci involving the renin-angiotensin-aldosterone system, sodium epithelial channel, catecholaminergic/adrenergic function, renal kallikrein system, beta3-unit of the G-protein, alpha- and beta-adducin, and others involving lipoprotein metabolism, hormone receptors, and growth factors [172].

Final conclusions

For LDL and HDL cholesterol, fasting triacylglycerol, homocysteine, and blood pressure well-validated, easy applicable, and generally accepted biomarkers exist. For diet-related CVD, however, the ultimate question for both the consumer and the industry is whether changes in the biomarker are truly related to changes in risk. As discussed in the previous sections, only for LDL cholesterol and blood pressure does consensus exist among scientists for a possible application as enhanced function claims. Important issues, however, not addressed in this manuscript, are if claims needs to be re-evaluated and if it is necessary to define when an effect is relevant from a health point of view. This issue is further complicated by the fact that some people are more responsive than others to dietary changes. It should further be realised that CVD is multifactorial. It seems therefore not justified if – for example – a reduction in LDL cholesterol alone is enough evidence to substantiate a claim. There are examples of food components or drugs that lower LDL cholesterol, but at the same time decrease

HDL cholesterol or promote thrombosis. Such examples can also be given for the other biomarkers. This problem can be overcome to some extent by animal studies, but such studies can never substitute human studies with hard end points, such as morbidity and mortality. At the same time, however, it should be acknowledged that studies with clinical endpoints are difficult to perform. Therefore, studies to validate generic predictors for the CVD risk like C-reactive protein, carotid artery wall intima, coronary calcification, and endothelial function, should be initiated.

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European Journal of Nutrition, Vol. 42, Supplement 1 (2003)

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