

*Laboratory Medicine Practice Guidelines*

# Emerging Biomarkers for Primary Prevention of Cardiovascular Disease and Stroke

Edited by Gary L. Myers



NATIONAL ACADEMY  
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# **The National Academy of Clinical Biochemistry**

*Presents*

## LABORATORY MEDICINE PRACTICE GUIDELINES

# **EMERGING BIOMARKERS FOR PRIMARY PREVENTION OF CARDIOVASCULAR DISEASE AND STROKE**

**EDITED BY**  
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## Introduction

Gary L. Myers and Robert H. Christenson

The purpose of this document is to present the National Academy of Clinical Biochemistry (NACB) Laboratory Medicine and Practice Guidelines for utilization of emerging laboratory biomarkers of cardiovascular and stroke risk in a primary prevention setting. The NACB is the American Association for Clinical Chemistry's scientific academy. An important activity of the NACB is to develop laboratory medicine practice guidelines to assist clinical and laboratory practice decisions concerning patients at increased risk for specific diseases.

### BACKGROUND

For more than 20 years, heart disease and stroke have been the first and third leading causes of death and major causes of disability in the United States and other developed countries (1). Heart disease and stroke are estimated to be the first and second leading causes of death in the world today and are expected to remain so by the year 2020 (2). Despite significant reduction in all standardized mortality from cardiovascular disease (CVD) over the past 20 years, CVD remains the number one cause of death in the United States, out ranking all cancers by more than 60% (3). More than 70.1 million Americans have some form of CVD (3). Public health priorities for prevention of cardiovascular events and stroke as addressed in Healthy People 2010 are: prevention of risk, detection, and treatment of risk factors; early identification and treatment of heart attacks and stroke; and prevention of recurrent cardiovascular events (4,5). Thus the search for biomarkers that will better recognize patients with coronary disease who could potentially benefit from intensive primary prevention efforts is critically important.

The American Heart Association (6) and the National Cholesterol Education Program's (NCEP) Adult Treatment Panel III (ATP III) (7) have each issued recommendations designed to identify more people who are asymptomatic and clinically apparently free of coronary heart disease (CHD), but at sufficiently high risk for a future coronary event in order to justify more intensive risk reduction efforts. Within these recommendations are specific risk factors, including total cholesterol, low-density lipoprotein (LDL) cholesterol and high-density lipoprotein (HDL) cholesterol, that are typically used in risk prediction algorithms, such as the Framingham risk score (8), to estimate a global risk assessment for CVD. However, these predictive models based on conventional risk

factors are underutilized and have a lower than desired accuracy, thus providing a stimulus to search for new tools to refine CVD risk prediction (9). In recent years, the number of new candidate risk factors that have been proposed as significant predictors of CVD and its complications has grown considerably (Table 1). These biomarkers are termed emerging risk factors because they are associated with an increased risk for CVD, but their causative, independent, and quantitative contributions to CVD are not as well documented as dyslipdemia, high blood pressure, and smoking—major, longest established risk factors (10). An emerging marker may not be emerging in the sense that it is a newly discovered marker, but may be an existing marker for which evidence is only now available for establishing it as effective for independently identifying risk or for monitoring treatment.

While the guidelines issued by the NCEP's ATP III for global risk assessment using the traditional risk factors are based on strong evidence supporting their role in the pathogenesis of CVD, the role for the emerging risk factors in primary prevention is far less clear. Debate has taken place on whether a risk marker must be causally related to disease, or whether clinical utility can be advocated for a marker that might not be causal, but could indicate use of a different course of therapy or management strategy than would otherwise be considered. Additional guidance is needed to help clarify and define the

**Table 1. Emerging Risk Factors for Cardiovascular Disease**

C-Reactive Protein	Interleukins (eg, IL-6)
Serum amyloid A	Vascular and cellular adhesion molecules
Soluble CD-40 ligand	Leukocyte count
Fibrinogen	Plasminogen activator inhibitor 1
D-dimer	Tissue-plasminogen activator
Factors V, VII, VIII	Small dense LDL
Lipoprotein(a)	Apolipoproteins A1 and B
LDL and HDL subtypes	Oxidized LDL
Homocysteine	Lipoprotein-associated phospholipase A <sub>2</sub>
Microalbuminuria	creatinine (glomerular filtration rate)
Cystatin C	Infectious agents
Apo E genotype	Fibrinopeptide A
Remnant lipoproteins	von Willebrand factor antigen

**Table 2. American Heart Association/American College of Cardiology Classifications Summary of Indications**

I	Conditions for which there is evidence and/or general agreement that a given procedure or treatment is useful and effective
II	Conditions for which there is conflicting evidence and/or a divergence of opinion about the usefulness/efficacy of a procedure or treatment
IIa	Weight of evidence/opinion is in favor of usefulness/efficacy
IIb	Usefulness/efficacy is less well established by evidence/opinion
III	Conditions for which there is evidence and/or general agreement that the procedure/treatment is not useful/effective and in some cases may be harmful
<b>Weight of Evidence</b>	
A	Data derived from multiple randomized clinical trials that involved large numbers of patients
B	Data derived from a limited number of randomized trials that involved small numbers of patients or from careful analyses of nonrandomized studies or observational registries
C	Expert consensus was the primary basis for the recommendation

contribution that these emerging risk factors may have in identifying persons at risk for CVD. Benchmarks are needed against which new biomarkers can be evaluated. In evaluating the clinical potential of a new emerging biomarker we should ask; can the biomarker be measured, does the biomarker add information to or improve upon existing tests, and will the biomarker help in patient management (11)? The overall expectation is that a CVD biomarker will enhance the clinician's ability to appropriately manage the patient's disease status.

The NACB convened a multidisciplinary panel of experts to develop recommendations for the clinical utility and laboratory measurement of a selected number of these emerging risk factors for use in primary prevention of CVD and stroke. The selection of risk factors for evaluation and inclusion in this guideline was based on systematic expert consensus of the NACB guideline group after reviewing available evidence and evaluating criteria of clinical usefulness, consistency of epidemiologic data, improved predictive value, independence from other factors, and available analytical methods. The NACB expert panel defined the following risk factors as within the scope of this guideline: lipoprotein subclasses and particle concentration, lipoprotein (a), apolipoproteins A-I and B, C reactive protein, fibrinogen, white blood cell count, homocysteine, brain (B-type) natriuretic peptide (BNP) and N-terminal pro B-type natriuretic peptide (NT proBNP), and markers of renal function.

The current guidelines for these emerging risk factors have been developed based on the published evidence for their use in primary prevention to predict CVD and stroke risk in non-diseased populations compared to the ATP III recommendations based on the measurement of total cholesterol, low density lipoprotein cholesterol (LDL-C), and high density lipoprotein cholesterol (HDL-C). The scope for these guidelines is primary risk prediction. For this application, it is first necessary to calculate a 10-year predicted risk based on Framingham risk score (8) or other classification which incorporates a lipid profile (total cholesterol, HDL-C, triglycerides, calculated LDL-C, and non-HDL-C).

Specific recommendations in this NACB guideline are based, whenever possible, on relevant published information. For in-depth evaluation of each of the selected biomarkers,

we used all available literature from prospective observational studies of initially healthy populations published through February 2005. We did not consider retrospective studies or studies of populations with existing vascular diseases, except in the case of evaluating the use of biomarkers to direct secondary prevention after cardiovascular events (because less data are available in primary prevention settings). The strength of scientific data supporting each recommendation was characterized using the scoring criteria adopted from the American Heart Association/American College of Cardiology, as summarized in Table 2. For each recommendation, the designations I, IIa, IIb, and III describe the indications, and the upper case letters A through C describe the weight of evidence.

The draft guidelines were posted on the NACB website in September 2006 for public comment from individuals, organizations, and other interested parties. The guidelines were also presented at the 27<sup>th</sup> Arnold O. Beckman Conference in Baltimore, MD, October 2006. Public comments received through these channels were carefully reviewed by the committee and actions were taken to address them.

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## Chapter 2

# General Principles Concerning Laboratory Testing and Utility of Newer Biomarkers

*Peter W.F. Wilson*

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It is important to bear in mind several general principles when evaluating how biomarker measurements can help to advance science, guide risk screening strategies, and affect clinical care.

The first step in the evaluation of a biomarker is whether its concentration is different in persons affected by disease in comparison to those who are not affected. Initial studies have typically employed a case-control design and reporting of the results has focused on the risks related specifically to marker, with secondary consideration of statistical adjustments. Published reports at this phase may show relatively strong estimates of relative risk (RR) for persons with concentrations of the new biomarker that are significantly different in cases compared to controls.

A second step in the evaluation of a new biomarker and atherosclerosis is the development of a body of evidence from case-control and prospective studies that have evaluated the test. The prospective studies can include nested case-control studies and full cohort investigations. Evaluation is aided at this phase if the biomarker can be measured by thawing previous collected specimens that have been frozen, provided that measurements on aliquots yield accurate and precise data. The storage of samples at temperatures  $-70^{\circ}\text{C}$  or lower has greatly facilitated such investigations. A nested case-control study with adequate statistical power to address the question is an appropriate cost-effective strategy. An ancillary issue at this phase is to rigorously test whether the new biomarker effects are present in multivariate statistical analyses and appropriate corrections for variables including age, sex, ethnicity, underlying diseases, and the type and severity of CVD are performed.

A third step in the evaluation of a new biomarker for CVD is whether the measurement improves our ability to assess risk above and beyond the current approaches. In other words, can the new test improve the ability to discriminate between future cases and non-cases. Of course, accurate assessment of the new marker value can only be determined after correction for the appropriate confounders and the marker deemed useful if it offers additional value to existing risk algorithms such as the Framingham risk score (1). Unfortunately, many investigations lack such information and make it difficult to adequately evaluate the value of a new biomarker.

Finally, reliable analytical methods must be available for the measurement of the intended biomarker.

It is important to realize that insights into pathophysiology do not necessarily translate quickly into changes in screening procedures and clinical care in a field that already has developed strategies to accurately assess CVD risk. In the 1960s total cholesterol measurement was felt to be an adequate assessment of lipids and CVD risk; however, by the 1980s that assessment had moved to include LDL-C and HDL-C. Assessment of each of these markers has very consistently been shown to be important worldwide for assessment of CVD risk and for clinical care. Incorporating newer biomarkers promises to be more complex because of the experience developed to date.

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# Inflammation Biomarkers and Cardiovascular Disease Risk

Mary Cushman, Christie M. Ballantyne, Daniel Levy, Nader Rifai, Gerald R. Cooper, and Gary L. Myers

## RECOMMENDATIONS FOR INFLAMMATION MARKERS

Inflammation is involved in many disease processes and its contribution to the pathophysiology of CVD has become more widely appreciated in the last decade (1). Many markers of inflammation exist. Twenty-four analytes (Table 3) were discussed that have some supportive data from observational studies. These were rated by the Inflammation Working Group according to their practicality for clinical use, availability of a commercial assay that could be standardized, and whether the observational data were sufficient. From this rating process high sensitivity C-reactive protein (hsCRP), fibrinogen, and white blood cell count were selected for further evaluation.

Based on a thorough review of the published literature, the following are recommendations for the clinical use and measurement of hsCRP, fibrinogen, and white blood cell count in assessing risk for CHD and stroke.

## CLINICAL SCIENCE

### *Recommendation 1*

a. After standard global risk assessment, if the 10-year predicted risk is <5%, hsCRP should not be measured.

**Classification of recommendation: I**

**Level of evidence: A**

b. If the 10-year risk is 5% to <10%, it is expected that 10% might be reclassified to a higher risk group with the test. More information is needed on clinical application, particularly in relation to longer-term lifetime risk prediction and selection of an appropriate intervention (lifestyle/medical).

**Classification recommendation: II**

**Level of evidence: B**

c. If risk is intermediate (10% to 20%) and uncertainty remains as to the use of preventive therapies such as statins or aspirin, then hsCRP measurement might be useful for further stratification into a higher or lower risk category.

**Classification of recommendation: I**

**Level of evidence: A**

### *Recommendation 2*

Therapies prescribed based on hsCRP concentrations should be based on clinical judgment of the physician because benefits of such treatment are uncertain.

**Classification of recommendation: IIb**

**Level of evidence: B**

### *Recommendation 3*

There are insufficient data that therapeutic monitoring using hsCRP over time is useful to evaluate effects of treatments in primary prevention.

**Table 3. Markers of Inflammation**

Cytokines / Inflammation	Leukocytes / Platelets / Endothelium	Coagulation / Fibrinolysis
Serum amyloid A	Intercellular adhesion molecule-1 (ICAM-1)	Factor VIII
Sedimentation rate	Vascular cell adhesion molecule-1 (VCAM-1)	Von Willebrand factor
Interleukin-6	P-selectin	Plasminogen activator inhibitor-1
Interleukin-8	E-selectin	Tissue plasminogen activator
Interleukin-18	Myeloperoxidase (MPO)	D-dimer
Tumor necrosis alpha receptors 1 and 2	Lipoprotein associated phospholipase A2 (Lp-PLA <sub>2</sub> )	Fibrinogen
Tumor necrosis alpha	Monocyte chemoattractant protein-1	
Viscosity	CD40 ligand	
hsCRP	White blood cell count	

**Classification of recommendation:** III (against use)

**Level of evidence:** C

**Recommendation 4**

The utility of hsCRP concentrations to motivate patients to improve lifestyle behaviors has not been demonstrated.

**Classification of recommendation:** IIb

**Level of evidence:** C

**Recommendation 5**

Evidence is inadequate to support concurrent measurement of other inflammatory markers in addition to hsCRP for coronary risk assessment.

**Classification of recommendation:** IIb

**Level of evidence:** C

## POPULATION SCIENCE

The preponderance of evidence supports that higher hsCRP, fibrinogen, and white blood cell count are associated with increased risk of cardiovascular events after adjustment for other known risk factors.

## Clinical Science/Laboratory Testing

**Recommendation 1**

Measurement of hsCRP should be done in the fasting or nonfasting state in metabolically stable patients free of infection or acute illness. If the hsCRP concentration is  $< 3$  mg/L, it does not need to be repeated. If the value is  $>3$  mg/dL, repeat the measurement at least 2 weeks later in metabolically stable state, free of infection or acute illness. The lower of the two results should be considered the patient's value. If hsCRP is  $\geq 10$  mg/L this might relate to CV risk. Other conditions such as acute infection or inflammation or inflammatory disorders might be responsible. Extensive evaluations with imaging tests or other testing for these patients is not recommended unless pertinent history and physical examination findings are present, or if pursuing normal practice for age-appropriate population screening.

**Classification of recommendation:** IIa

**Level of evidence:** A

### Laboratory Testing

**Recommendation 1**

Of the examined inflammatory markers for assessing CV risk, hsCRP has the analyte and assay characteristics most appropriate for use in clinical practice.

**Classification of recommendation:** I

**Level of evidence:** A

**Recommendation 2**

There are sufficient data that fibrinogen is an independent marker of CVD risk; however, because of analytical concerns, insufficient assay standardization, and uncertainty in identifying treatment strategies, measurement is not recommended for this application.

**Classification of recommendation:** III

**Level of evidence:** A

**Recommendation 3**

There are sufficient data that WBC is an independent marker of CVD risk; however, because utility in reclassifying risk level and identifying treatment strategies is not known, measurement is not recommended for this application.

**Classification of recommendation:** III

**Level of evidence:** C

**Recommendation 4**

hsCRP results regardless of the method used, should be expressed as mg/L.

**Classification of recommendation:** I

**Level of evidence:** C

**Recommendation 5**

hsCRP using standardized assays categorizes patients as follows:

- a. Low Risk  $< 1.0$  mg/L
- b. Average Risk 1.0 to 3.0 mg/L
- c. High Risk  $>3.0$  mg/L
- d. Very High Risk  $\geq 10.0$  mg/L

**Classification of recommendation:** IIa

**Level of evidence:** A

**Recommendation 6**

Manufacturers of diagnostic assays for hsCRP should follow approved value transfer protocols to assure that standardized assays are used for vascular risk assessment.

**Classification of recommendation:** I

**Level of evidence:** C

**Recommendation 7**

Caution is recommended in application of the hsCRP categorization in recommendation 5 for risk prediction in certain populations such as nonwhites and the elderly, as the clinical utility is less established.

**Classification of recommendation:** IIa

**Level of evidence:** C

## SUPPORTING EVIDENCE

### Introduction

The Inflammation Working Group first reviewed a list of possible inflammation-sensitive biomarkers that could be considered for more in-depth review for potential usefulness in clinical practice. The questions used included: were there results from prospective observational studies of initially healthy populations, demonstrating associations with coronary events and/or stroke; are there widely available commercial clinical methods for accurate and precise measurement; and was there high between-person, low within-person, and low analytical variability? Twenty-four analytes that had some supportive data from observational studies were considered. These were rated by the group according to their practicality for clinical use, availability of a commercial assay that could be standardized, and whether the observational data were sufficient.

Through this process we determined hsCRP, fibrinogen, and white blood cell count had adequate data to assess their clinical utility while the other analytes in Table 3 had insufficient data to assess their clinical utility, or they had analytical impracticalities.

Of the biomarkers not selected, intercellular adhesion molecule-1 (ICAM-1), D-dimer, lipoprotein-associated phospholipase A<sub>2</sub> (LpPLA<sub>2</sub>), myeloperoxidase (MPO), and CD40 ligand were thought to be promising in that there were epidemiologic data linking them to coronary or stroke outcomes in healthy populations, they had acceptable analytical properties, and because it may be possible to standardize the assays.

ICAM-1 is a member of the immunoglobulin gene superfamily that plays an important role in the development of inflammation. Levels of soluble ICAM-1 (sICAM-1) are increased with inflammation but the regulation of sICAM-1 is not well understood. Increased levels of sICAM-1 are associated with increased cardiovascular events (2,3). However, in a large meta-analysis, the RR was markedly diminished after adjustment for traditional risk factors and socioeconomic status (4). Assays for sICAM-1 are not approved for clinical use.

D-dimer measurement is used clinically for evaluation of disseminated intravascular coagulation and exclusion of venous thrombosis. Values at the higher end of the normal range have been associated with risk of future CVD in several studies, but associations were not always independent of other risk factors (5-9). Any role of interventions based on D-dimer to reduce CVD risk has not been studied.

Lp-PLA<sub>2</sub> is a serine-dependent lipase responsible for >95% of the phospholipase activity associated with LDL (10) and is associated predominantly with small, dense LDL (11) and electronegative LDL (12); 15% to 20% of total plasma Lp-PLA<sub>2</sub> activity is associated with HDL (10). Increased levels of Lp-PLA<sub>2</sub> mass or activity have been consistently associated with increased risk for cardiovascular events. Most studies have shown that either Lp-PLA<sub>2</sub> mass or Lp-PLA<sub>2</sub> activity remains associated with increased risk for cardiovas-

cular events(13-24), but this has not been seen in all studies (25). The mass assay is approved by the U.S. Food and Drug Administration (FDA) for clinical use for risk assessment for CVD and stroke, whereas the activity assay is for research only. There are a large number of ongoing studies that have been recently completed and are not yet published, and a collaborative meta-analysis of individual participant data was ongoing at the time of the literature review and preparation of these guidelines. Although this biomarker appears promising, complete evaluation is not possible at this time because of the large amount of pending data from large trials and the meta-analysis.

In summary, the analytes discussed above are in varied states of consideration for clinical use. They require further study for full assessment. Pursuit of analysis on automated platforms (D-dimer already available) might be appropriate. Collaborative analyses of individual-level data, as was done recently for fibrinogen (26) might be useful to build a rationale for their clinical use.

Fibrinogen, white blood cell count, and hsCRP (measured using high-sensitivity assays) are biomarkers that are widely available clinically and were chosen for consideration for further assessment for use in clinical practice. For in-depth evaluation of each of these three biomarkers we used all available literature from prospective observational studies of initially healthy populations published through February 2005. We did not consider retrospective studies or studies of populations with existing vascular diseases, except in the case of evaluating the use of biomarkers to direct secondary prevention after cardiovascular events (because less data are available in primary prevention settings). For fibrinogen, we based our analysis on the 2005 analysis of individual-level data by the Fibrinogen Studies Collaboration, which includes all the major observational studies on fibrinogen (26). For white blood count, we assessed the few additional prospective studies published since the most recent meta-analysis published in 1998 (27). For hsCRP, we assessed all available published studies through February 2005. In order to report the clinical utility, for each biomarker we specifically addressed:

1. The consistency of data among studies.
2. The status of analytical considerations.
3. The independence of risk prediction from other vascular disease risk factors.
4. The ability of the biomarker to add clinical information to global risk assessment with the Framingham risk score.
5. The ability of more than one inflammation biomarker to provide complementary information.
6. Whether there were predictable, safe, and effective interventions available to alter the risk factor to reduce the risk.

In the sections that follow, the epidemiologic and clinical data for each of these three biomarkers is reviewed, the role of using more than one biomarker is discussed, and analytical considerations are addressed.

## Fibrinogen

### *Rationale*

Fibrinogen, an abundant protein in the blood, is cleaved by thrombin to generate fibrin, which serves as the scaffolding in thrombus formation. Given its prominent role in coagulation and platelet aggregation there have been numerous studies of fibrinogen as a CVD risk factor and consideration of its modification as a means to prevent disease. In addition, like hsCRP, fibrinogen is an acute-phase reactant that increases in abundance in response to inflammation, which is a central element in atherosclerosis. In this way, fibrinogen may serve as a promoter of atherosclerosis through its effects on coagulation and as a marker of disease burden or activity because of its acute-phase response. Fibrinogen, however, is also closely correlated with other CVD risk factors including obesity, physical activity, lipid levels, and cigarette smoking. Studies of fibrinogen as a CVD risk factor must account for such confounding.

### *Interpretation of Epidemiologic Data*

For the past 20 years, a number of small and moderate-sized observational studies have examined the association of fibrinogen with CVD risk. Recently, the Prospective Studies Collaboration group conducted a meta-analysis of the relation of fibrinogen level to CVD risk based on individual data pooled from 31 prospective studies (26). With 6,944 coronary or stroke events during 1.38 million person-years of follow-up, a clear, continuous, and graded association of fibrinogen with CVD risk emerged. After adjustment for established risk factors the hazard ratios (per 1 g/L increment in fibrinogen) were 1.93 (95% CI, 1.79 to 2.08) for CHD and 1.75 (95% CI, 1.55 to 1.98) for stroke. A subset of studies in the pooled analysis had data on hsCRP, which was correlated with fibrinogen ( $r = 0.47$ ). The relation of fibrinogen to CVD risk was essentially unchanged after adjustment for hsCRP.

More recently, the Women's Health Study explored the joint contributions of fibrinogen and hsCRP on cardiovascular events in more than 27,000 women (28). Once again fibrinogen was correlated with hsCRP ( $r = 0.41$ ) and with multiple CVD risk factors. Hazards for CVD rose both with increasing levels of fibrinogen and hsCRP and each was predictive of risk. Compared with women in the bottom tertile for both fibrinogen and hsCRP, the age adjusted hazards ratios for those with top tertile values for both markers was 3.45 (95% CI, 2.60 to 4.57).

### *Intervention*

#### *A. Use of fibrinogen to identify which patients will respond to therapy*

Published studies have not evaluated the potential role of baseline fibrinogen as a predictor of response to preventive therapy with medications.

#### *B. Use of therapy to lower fibrinogen*

Bezafibrate reduced fibrinogen in the Bezafibrate Infarction Prevention (BIP) study (29) and the Bezafibrate Coronary Atherosclerosis Intervention Trial (BECAIT) (30) whereas gemfibrozil appeared to increase fibrinogen level (31). In most

studies, including the large West of Scotland Coronary Prevention Study (WOSCOPS) (32), statins have not been shown to reduce fibrinogen (33). Aspirin has no effect on fibrinogen levels (34). Rosiglitazone monotherapy has been reported to reduce fibrinogen in nondiabetic subjects with CAD (35), while results in diabetic subjects also receiving sulfonylureas have been inconsistent (36,37). Pioglitazone does not appear to affect fibrinogen levels (37,38)

#### *C. Relation between change in fibrinogen or fibrinogen level on therapy and CVD risk*

In the BIP study, change in fibrinogen was not associated with risk for cardiac events (39) or stroke (40). There were no other published studies identified relating drug-related changes in fibrinogen with vascular outcomes.

### *Emerging Considerations*

For fibrinogen, the emerging considerations primarily relate to assay standardization and whether this will allow feasibility for use of this biomarker in clinical practice. This consideration is discussed in Analytical Considerations. Ongoing and completed clinical trials of interventions for CVD risk reduction should strongly consider measurement of fibrinogen (with mass assays) to expand the research base.

## White Blood Cell Count

### *Rationale*

The white blood cell count has been used clinically for many decades and technological advances provide for automated, accurate and inexpensive measurement of total leukocyte count and its subcomponents. The white blood cell count is an inflammatory marker and it increases, often markedly, in response to localized or systemic infection. In addition to serving as a marker of inflammation, several additional potential mechanisms for the association of leukocyte count with coronary risk have been proposed (41). Leukocytes may promote the development or progression of atherosclerotic plaques and their rupture by virtue of their proteolytic capacity and oxidative properties. They release proteases and chemoattractants that activate adhesion molecules that promote platelet aggregation, monocyte recruitment, endothelial injury, and plaque rupture. Because leukocytes are larger and less malleable than red blood cells, they can promote hemostasis within the coronary circulation. White blood cell count is correlated with other coronary disease risk factors, most notable with cigarette smoking, but also with body mass index, cholesterol level, HDL-C (inversely), triglycerides, diabetes and blood glucose level, physical activity (inversely), and blood pressure. Consequently, it is essential that studies relating leukocyte count to CVD outcomes adjust for these risk factors.

### *Interpretation of the Epidemiologic Data*

A large body of data from prospective studies has established an association of leukocyte count with risk for CVD events. A meta-analysis of prospective studies published before 1998

(27) reported a consistent relation between leukocyte count and coronary disease risk. In 19 qualifying studies, which contributed 7,229 coronary events in aggregate, the risk ratio for individuals with white blood cell count in the top (mean  $8.4 \times 10^9/L$ ) vs. bottom (mean  $5.6 \times 10^9/L$ ) third of the distribution was 1.5 (95% CI, 1.4 to 1.6). Their results remained significant after adjustment for other CVD risk factors including cigarette smoking, which is associated both with elevated white blood cell count and coronary events.

Several reports published subsequent to this meta-analysis have come to similar conclusions about the association of white blood cell count with CVD risk. In the Atherosclerosis Risk in Communities Study, leukocyte count was associated with increased risk for coronary events, stroke, and cardiovascular mortality. After adjustment for multiple risk factors, individuals with top ( $\geq 7.0 \times 10^9/L$ ) versus bottom ( $< 4.8 \times 10^9/L$ ) quartile values had RR of 1.8 for coronary events, 2.0 for stroke, and 2.3 for cardiovascular mortality (all  $P < .001$ ) (42). Ostensibly similar results were reported in the National Health and Nutrition Examination Survey II mortality study (43). In the Women's Health Initiative, the relation of baseline leukocyte count to cardiovascular outcomes was investigated in a sample of more than 70,000 postmenopausal women (44). In multivariable adjusted models, women with top ( $\geq 6.7 \times 10^9/L$ ) versus bottom quartile ( $< 4.7 \times 10^9/L$ ) leukocyte counts had increased risks for coronary death (hazard ratio, 2.36), nonfatal myocardial infarction (hazard ratio, 1.41), and stroke (hazard ratio, 1.46). The coronary disease risk gradient across WBC count quartiles was similar to that observed for hsCRP and it remained significant after adjustment for hsCRP concentration in a subset of participants. In addition, women with highest quartile values for both leukocyte count and hsCRP had a coronary disease hazard of nearly seven-fold.

### *Intervention*

#### *A. Use of white blood cell count to identify which patients will respond to therapy*

In the secondary-prevention Long-Term Intervention with Pravastatin in Ischemic Disease (LIPID) study, patients with high white blood cell count at baseline had greater benefit with pravastatin therapy than patients with low white blood cell count (45). Similar results were reported in a retrospective case series (46)

#### *B. Use of therapy to lower white blood cell count*

There are no prospective trial data on the use of therapy to lower white blood cell count. Pravastatin did not lower white blood cell count in WOSCOPS, a primary-prevention trial in which baseline white blood cell count was a predictor of coronary events (32).

#### *C. Relation between change in white blood cell count or white blood cell count on therapy and CVD risk*

There are no prospective trial data on the relation between change in white blood cell count or white blood cell count on therapy and CVD risk.

### *Emerging Considerations*

White blood cell count is a well-established, widely used, precise, and inexpensive laboratory test. Leukocyte count reflects systemic inflammation and may contribute to CVD risk through several potential mechanisms. Older and more contemporary reports from observational studies provide convincing evidence for a continuous and graded relation of leukocyte count to multiple morbid and fatal CVD outcomes in men and women. This relation has been observed in multiple subgroups and it is robust to adjustment for multiple established CVD risk factors. Clinical trials of interventions for CVD prevention with leukocyte count data available in their data sets should analyze this data to provide additional information as to the clinical utility.

### **hsCRP**

#### *Rationale*

hsCRP is the most widely studied biomarker of inflammation in CVD risk prediction. It is a nonspecific acute phase reactant that can rise several thousand-fold in response to infection or acute inflammation. It is produced by the liver and in endothelial cells and consists of a pentamer of 23-kDa identical subunits. Since the early 1990s with the development of highly sensitive assays for its measurement, detection of correlations of hsCRP with both CVD factors and future cardiovascular events has been possible. Higher hsCRP concentration is correlated with most major CVD factors, and is weakly or uncorrelated with the presence of measurable atherosclerosis in healthy subjects. Along with these epidemiologic studies, there is ongoing debate on the causal nature of the associations of hsCRP with vascular disease. Many possible biological activities for hsCRP itself have been suggested and debated based on *in vitro* and animal studies, as recently reviewed (47,48). Experiments by many investigators have supported roles for hsCRP in endothelial cell regulation, vascular smooth muscle, and monocyte / macrophage function, matrix biology, and coagulation. All of these effects could contribute to atherogenesis. It has been suggested that factors such as contamination of hsCRP preparations with azide and use of supraphysiological concentrations of hsCRP in experiments explain some of these associations. Regardless of whether there is a causal relationship of hsCRP with atherogenesis, akin to the association of cholesterol, it is possible that clinical utility could be derived from hsCRP measurement if higher values suggested a specific intervention that would not otherwise be considered.

A working group of the American Heart Association (AHA) and Centers for Disease Control and Prevention (CDC) issued a guideline statement concerning inflammation markers and vascular disease risk in 2003 which concluded that clinicians could consider hsCRP testing for men and women at intermediate risk of CHD (based on standard-risk prediction algorithms) where uncertainty remained as to the use of preventive treatments (49). It was suggested that values of  $< 1.0$  mg/L identify those at low

risk, values between 1.0 and 3.0 mg/L identify those at average risk, and values  $> 3.0$  mg/L identify high risk. These values represented approximate thirds of the healthy population distribution in primarily white populations available for examination at that time.

### *Interpretation of Epidemiologic Data*

We reviewed findings of 31 reports from prospective observational studies of baseline hsCRP and future first cardiovascular events including primarily CHD and stroke outcomes. Studies included a range of age groups and sufficient representation of both men and women, but relatively small numbers of nonwhite ethnic groups. Almost all the studies reported positive associations of higher hsCRP with future vascular events, with a range of adjusted RRs reported (1.45 to  $> 4.0$ ). There was no standardization in reporting in terms of the cutpoints considered for hsCRP or the covariates considered as potential confounders. Most studies since 2003 utilized the value of  $> 3.0$  mg/L to define elevated hsCRP based on the AHA/CDC guideline (49). Otherwise, there was a wide range of cutpoints used to define elevated hsCRP, corresponding to differing categorizations of the data (top tertile, quartile, or quintile for example, with some studies only reporting the RR associated with a standard deviation increment). In studies of women, the cutpoint defining elevated hsCRP was often higher (top quartile value 7.3 mg/L in the Framingham Study and the Women's Health Study, for example) (50,51). This may be accounted for by the fact that hsCRP levels are higher among women using hormone replacement therapy (52). Regardless, the large Women's Health Study reported that associations of hsCRP  $> 3.0$  mg/L with future vascular events were similar in women using or not using hormone replacement therapy at baseline (53). A few studies evaluated even higher hsCRP concentrations, demonstrating that elevations previously felt to represent acute or chronic illness at the time of the 2003 AHA/CDC guideline statement (eg, values  $>10$  mg/L or  $> 20$  mg/L) were also associated with future cardiovascular events, with RRs higher than those associated with hsCRP  $> 3.0$  mg/L (54,55).

In summary, the studies to date provide consistent evidence of an association of higher hsCRP with future vascular events (primarily CHD and stroke), independent of traditional CVD factors. Differences in study findings and lack of available data in some cases suggest future areas of study involving the proper cutpoint for clinical use in different groups (women) and the clinical applicability of hsCRP for nonwhite ethnic groups. We recommend standardization of reporting for future studies in terms of the cutpoints used to define elevated hsCRP (eg,  $> 3.0$  mg/L and  $>10$  mg/L) and the set of covariates used as potential confounders in multivariate analysis (eg, age, sex, race, blood pressure/hypertension status, total and HDL-C, diabetes, and smoking status) might help standardize results from study to study to allow more direct comparison.

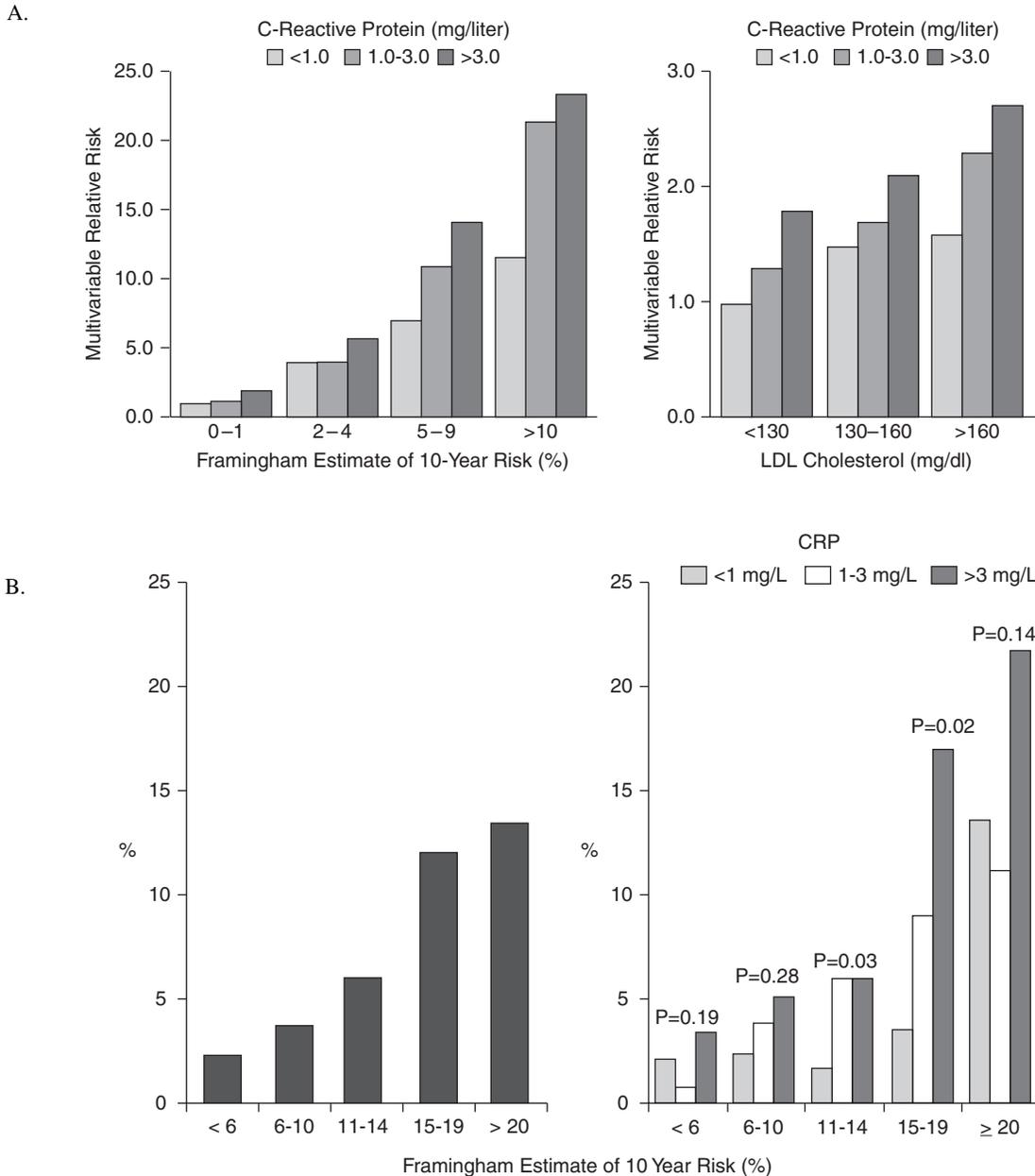
Independence of risk prediction in multivariable models is not the sole criterion for determining clinical usefulness of a new biomarker. We next evaluated the available data on the

clinical utility of hsCRP in studies that assessed the role of hsCRP in combination with 10-year predicted risk of CHD (based on Framingham risk score) or in low- and high-risk groups based on presence or absence of individual risk factors. For optimum clinical application, determination of hsCRP should be able to provide information over and above that provided by the standard risk assessment (such as with the Framingham risk score), specifically providing further stratification of risk in a clinically meaningful way for those with a predicted risk in the intermediate range (10% to 20% 10-year predicted risk, although some authors have suggested this range be 6% to 20%). Several studies have reported on this topic by use of receiver operator curves and C-statistics to assess whether there is improvement in risk prediction (56-67). Most studies have shown no improvement in the area under the curve or C-statistic, with at best modest improvement by the addition of hsCRP. It is apparent with this methodology that it is even difficult to show improved risk prediction by some standard risk factors, so other methods of assessing the role of novel risk markers have been considered. There were a few cohort studies reporting the combination of Framingham risk score and hsCRP in risk prediction using cross-classification of participants based on both factors (Figure 1). hsCRP determination added information over and above Framingham risk score in middle-age and older women and men (54-57). In middle-age US women, hsCRP provided improved risk prediction at all levels of Framingham risk (54,56). Among middle-age men in Germany, the clinical usefulness of hsCRP seemed greatest in men with a 10-year predicted risk  $>15\%$ , perhaps due to less discrimination of the Framingham risk score for future events in men at lower risk (57). Among elderly US men, hsCRP seemed to be most useful among those at intermediate (10% to 20%) or high ( $> 20\%$ ) predicted risk. Among elderly US women, it was most useful in those at high predicted risk, although very few women were classified as high risk, so overall utility was questionable.

A recent report from the Women's Health Study further addressed the clinical utility of hsCRP measurement by cross-classifying women at baseline by risk categories based on optimum risk prediction using Framingham variables in that cohort and hsCRP (68). They then compared the expected and observed incidences of cardiovascular events over 10 years without the information from hsCRP. The overall calibration of risk classification was statistically improved by adding hsCRP, however only 4% of women were reclassified after hsCRP assessment to a different risk category based on observed event rates (with 2.5% reclassified to a lower risk group and 1.3% to a higher risk group). In the intermediate risk group by traditional risk factors (10% to 20% 10-year predicted risk, comprising 3% of the cohort), 14% of the women were reclassified to a lower risk group and 5% to a higher risk group. Among women with a 10-year predicted risk of 5% to 10% (comprising 8% of the cohort), 12% were reclassified as lower risk and 10% as higher risk. Among women with a low predicted risk ( $< 6\%$ , comprising 88% of the cohort), hsCRP only resulted in reclassification as higher risk for 2% of

women. Thus, overall hsCRP assessment seemed more useful to reclassify middle-age women to a lower risk group; there is little role of hsCRP determination in low-risk women. Further, if measurement is limited to women at intermediate risk where therapeutic uncertainty exists, predicted risk will be upgraded

for some and downgraded for others. For these women who had low event rates over 10 years, the implications for lifetime risk prediction also warrant consideration. Similar analyses are needed in other cohorts for further evaluation of the clinical role of hsCRP testing.



**Figure 1 Combination of Framingham Risk Score and hsCRP in 10-Year Cardiovascular Risk Prediction in Three Studies.** Panel (A) Women's Health Study (56); © 2002 Massachusetts Medical Society. All rights reserved. Reprinted with permission. Panel (B) MONICA / KORA Augsburg cohort (57); © Lippincott, Williams and Wilkens. Koenig W, Löwel H, Baumert J, Meisinger C. C-reactive protein modulates risk prediction based on the Framingham score: implications for future risk assessment: results from a large cohort study in southern Germany. *Circulation* 2004;109:1349-1353; reprinted with permission). Panel (C) Cardiovascular Health Study (55); © Lippincott, Williams and Wilkens. Cushman M, Arnold AM, Psaty BM, Manolio TA, Kuller LH, BurkeGL, Polak JF, Tracy RP. C-reactive protein and the 10-year incidence of coronary heart disease in older men and women: the cardiovascular health study. *Circulation* 2005;112:25-31; reprinted with permission). Note: (A) reports the relative risk in groups, while (B) and (C) report observed incidence rates. Panel C. (A) Women; (B) men. No. of events/number at risk shown across the top of each panel.

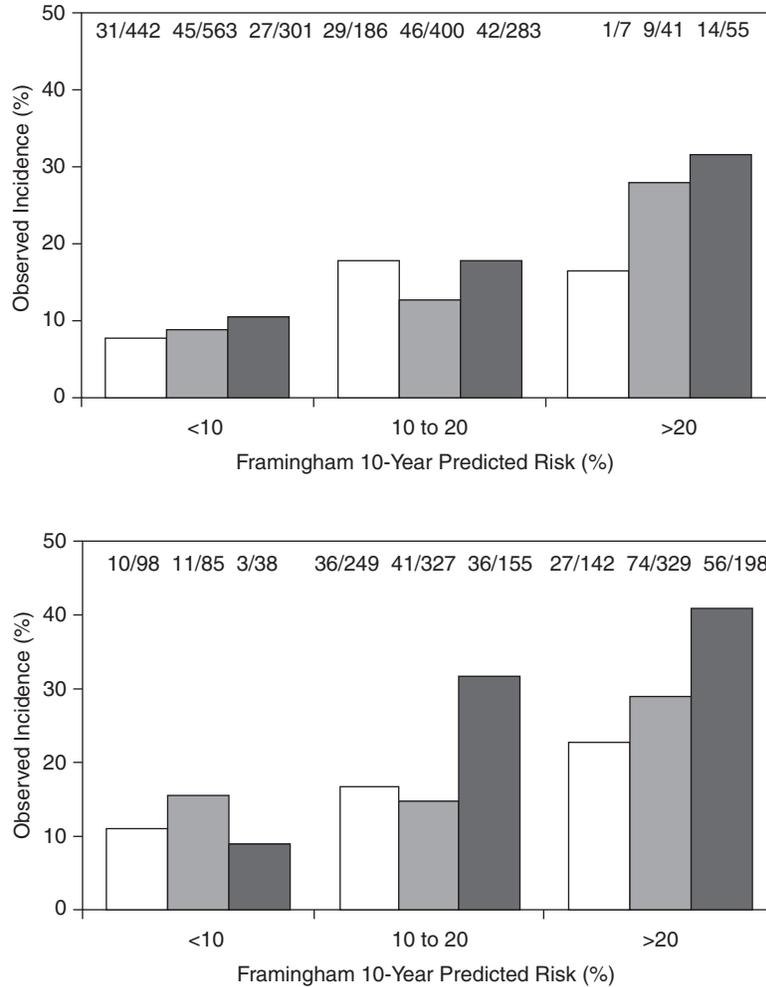


Figure 1 Continued

**Use of Combinations of Inflammation Biomarkers for Risk Prediction**

*Intervention*

*A. Use of hsCRP to identify which patients will respond to therapy*

hsCRP levels at baseline may help identify patients who are likely to respond to therapy. In the Physician’s Health Study, men with higher hsCRP had the greatest risk reduction from aspirin therapy (56% RR reduction in the top quartile of hsCRP compared to 14% in the bottom quartile) (64). In a post hoc analysis from the Air Force/Texas Coronary Atherosclerosis Prevention Study (AFCAPS/TexCAPS), in patients without known CHD, those with LDL-C below the median and hsCRP above the median and those with LDL-C above the median and hsCRP below the median had significant reductions in acute coronary events with lovastatin therapy (42% and 62%, respectively), whereas those with both LDL-C and hsCRP values below the median or both above the median did not have significant benefit (65). In a post hoc analysis from the secondary-prevention Cholesterol and Recurrent Events (CARE) study, patients with inflammation (hsCRP and serum amyloid A  $\geq$

95th percentile) had the highest risk for coronary events on placebo, but pravastatin treatment reduced risk to that of placebo patients without inflammation (hsCRP and serum amyloid A < 90th percentile) (66). However, in results reported from the Prospective Study of Pravastatin in the Elderly at Risk (PROSPER), baseline hsCRP was not significantly associated with response to statin therapy for total CVD or CHD events (69). There are no prospective trials designed specifically to determine whether higher versus lower levels of hsCRP can be used to identify individuals who should be treated differently in primary prevention.

*B. Use of therapy to lower hsCRP*

Multiple prospective trials have shown the hsCRP-lowering effects of therapies that are commonly used in the treatment of patients who are at increased risk for CVD. These include diet and exercise (67,70), weight loss (71), and lipid-lowering therapies such as statins (72) and fibrates (73,74). Higher doses of highly effective statins are associated with greater reductions of hsCRP, and the combination of ezetimibe added to a statin leads to further hsCRP reduction, whereas ezetimibe alone does not

lower hsCRP (75). The angiotensin II receptor blocker valsartan was also shown to reduce hsCRP level in the Valsartan-Managing Blood Pressure Aggressively and Evaluating Reductions in hsCRP (Val-MARC) trial, and the reduction was independent of blood pressure reduction (76). The peroxisome proliferator-activated receptor- $\gamma$  (PPAR- $\gamma$ ) agonists pioglitazone (77) and rosiglitazone (78) have been shown to reduce hsCRP; the reduction in hsCRP with pioglitazone was independent of the effects of pioglitazone on glucose metabolism (77). In the Rimonabant in Obesity-Lipids (RIO-Lipids) study, rimonabant 20 mg significantly reduced hsCRP in conjunction with weight loss (79). Removal of subcutaneous fat by liposuction did not lower hsCRP in one study (80), but did in another (81).

### *C. Association of change in hsCRP or level of hsCRP on therapy with CVD risk*

In one recent clinical trial in patients with acute coronary syndrome, the Pravastatin or Atorvastatin Evaluation and Infection Therapy-Thrombolysis in Myocardial Infarction 22 (PROVE IT-TIMI 22) study, patients whose hsCRP was  $< 2$  mg/L after 30 days on statin therapy had significantly reduced coronary event rates compared with patients with hsCRP  $\geq 2$  mg/L on therapy; patients with LDL-C  $\geq 70$  mg/dL and hsCRP  $\geq 2$  mg/L had the highest event rate, whereas patients with LDL-C  $< 70$  mg/dL and hsCRP  $< 2$  mg/L had the lowest event rate (82). Similar findings were reported from the Aggrastat-to-Zocor trial (83). Baseline hsCRP levels were not accounted for as possible confounders in these studies to confirm that the drug effect and not the baseline level was the relevant factor in risk reduction. There are no similar published data from primary-prevention trials.

### *Emerging Considerations*

To address the question of clinical utility of hsCRP measurement in primary prevention the Justification for the Use of statins in Primary prevention: an Intervention Trial Evaluating Rosuvastatin (JUPITER) is evaluating the role of a statin versus placebo in primary prevention in men and women with normal lipid values and hsCRP  $> 2$  mg/L (84). Work is also ongoing in basic research settings to identify possible causal roles for hsCRP in atherogenesis. Consideration of inflammation as a target for novel strategies in primary and secondary prevention settings is also ongoing. There are remaining analytical issues for hsCRP, discussed below (in Sources of Variability in hsCRP Testing section). The 2003 AHA/CDC guideline statement suggested that studies be undertaken to evaluate whether a patient's knowledge of an elevated hsCRP would affect behavior modification among patients with lifestyle risk factors such as obesity or physical inactivity (49). This research has not yet been published and we continue to recommend it.

### **Use of More Than One Inflammation Biomarker in Risk Assessment**

Studies were reviewed in which inflammation markers were studied simultaneously in relation to CVD outcomes. These included studies that addressed confounding of one inflammation marker

by another (or others considered together) and where the combined influence of elevations of more than one marker was evaluated. In the ARIC study (85), simultaneous addition of fibrinogen, intercellular adhesion molecule-1, and leukocyte count to multivariable models predicting future CHD resulted in some attenuation of the association of hsCRP with events. In the WOSCOPS trial (86), there was also modest attenuation of the association of hsCRP with incident CHD after individual adjustment for interleukin-6 or D-dimer. In the PRIME study with inclusion of hsCRP, interleukin-6 and fibrinogen in a multivariable model only interleukin-6 remained statistically associated with CHD risk over 5 years (87). Similar results were seen in the Quebec Cardiovascular Study (61) where the association of hsCRP with future CHD was not independent of traditional cardiovascular risk factors but interleukin-6 was. In the Fibrinogen Studies Collaboration meta-analysis, adjustment for established risk factors and for hsCRP measurements in a selected subgroup with both measurements did not weaken the association of fibrinogen with incident coronary events; the hazard ratio associated with hsCRP was not reported (27).

A few studies have evaluated the joint influence of elevations in biomarkers with subsequent risk (eg, complementary). In the Women's Health Study (28), higher fibrinogen (measured by a mass assay) and hsCRP were associated with future CVD events independent of each other and traditional risk factors. Further, the risk was highest when both biomarkers were elevated, suggesting possible complementary use of these markers. Assessment of the joint influence of biomarker reflecting different biology may prove useful. In the Caerphilly and Speedwell studies, hsCRP and D-dimer provided additive information in CHD risk prediction, suggesting the possibility that risk markers from different biological domains might be combined (88). In the ARIC study among subjects with LDL-C  $< 130$  mg/dl (but not among those with higher values) there was additive risk prediction of future CHD by hsCRP and Lp-PLA<sub>2</sub> in the top tertile (89). Similar modest additive associations of hsCRP and LpPLA<sub>2</sub> were observed in the MONICA Augsburg cohort study (90). For stroke as the outcome in ARIC, regardless of LDL-C, the complementary risk prediction for stroke using hsCRP and Lp-PLA<sub>2</sub> was striking, with an 11-fold increased risk if both factors were elevated, compared to if both low (91). Based on these early findings, more work is needed on the utility of combinations of biomarkers in risk prediction, especially when these biomarkers reflect different biology.

### **Analytical Consideration for Inflammation Biomarkers**

As indicated earlier, prospective studies have shown an association between primary inflammatory mediators, such as cytokines and adhesion molecules and CVD (92-95). The laboratory measurement of these markers can be problematic since they are present in circulation at very low concentrations and are typically measured by enzyme-linked immunosorbent assay (ELISA) techniques that are labor intensive, currently not standardized, often lack the desired level of sensitivity, not approved by the FDA for clinical use, and not available in routine clinical laboratories. In addition, most of these analytes are very unstable after collection

thus requiring immediate testing after blood collection or freezing the sample at -70 °C until analysis. For these reasons, the measurement of cytokines and adhesion molecules has remained in the research setting. In contrast, the measurement of WBC count is accurate, precise, and readily available in all clinical laboratories. However, for other than analytical reasons described earlier, this marker is not recommended for CVD risk prediction.

The association of down stream inflammatory markers such as fibrinogen, serum amyloid A and hsCRP with CVD has been established (95-97). Clinical assays are currently available for these three proteins. However, fibrinogen is measured in clinical laboratories using approximately 40 different functional assays. Although these methods are relatively precise (approximately 10% reproducibility), they are not standardized and great variability in measured fibrinogen in a particular sample is seen among various laboratories. For example, according to a College of American Pathologists Survey, fibrinogen values reported from different laboratories in a single sample varied from 121 to 437 mg/dL. Since patients' results will be interpreted in the context of nationally established cutpoints, the use of standardized assays for risk assessment of heart disease is mandatory. An alternative approach is to use a mass immunoassay, which is standardized and commercially available, for risk assessment of CVD. A single serum amyloid A assay is currently available but is not approved by FDA or accessible to most clinical laboratories in the US (98). In contrast, more than 30 high sensitivity assays that can reliably measure hsCRP in apparently healthy subjects to determine their future CVD risk are available (99). The amplification of the light-scattering properties of the antigen-antibody complex by covalently coupling latex particles to specific antibody has been the most successful in measuring low hsCRP concentration ( $\leq 0.3$  mg/L) since it enables the adaptation of the assay to automated chemistry analyzers (98).

For the reasons presented in this document, it was concluded that of all the examined inflammatory biomarkers only hsCRP has a clinical utility in CHD risk prediction and therefore, specific issues related to its measurement and interpretation are presented below.

## SOURCES OF VARIABILITY IN hsCRP TESTING

In order to determine hsCRP reliably for CVD risk prediction, the various sources of pre-analytical and analytical variability should be appreciated and controlled.

## Preanalytical Variability

### Physiologic and Clinical

#### A. Age

Although most studies showed no relationship between age and hsCRP concentration (98, 100), some have reported a slight increase of hsCRP concentrations with age (101,102). This increase might be due to the higher incidence of obesity that is associated with aging (101). In a subset of the Women's Health Study (15,770 participants), only a slight association of hsCRP with age was seen: median hsCRP concentrations for individuals 45 to 54, 55 to 64, 65 to 74, and  $\geq 75$  years of age were 1.31, 1.89, 1.99, and 1.52 mg/L, respectively (103).

#### B. Population distribution

Several studies have shown that the distribution of hsCRP concentrations in both sexes was non-Gaussian when evaluated for skewness and kurtosis (98,100). Findings from American and European studies have shown comparable distribution of hsCRP concentrations among men and women, not receiving hormone replacement therapy (35,930 subjects) (103,104) (Table 4). Other data from the Multi-Ethnic Study of Atherosclerosis reported higher levels in women than men after accounting for hormone use and risk factor level differences (105). The National Health and Nutrition Examination Survey III (NHANES III) also showed slightly higher hsCRP concentrations in women compared to men, however, this difference did not affect the subjects' classification into the CDC/AHA risk categories (106). Ethnic group differences in hsCRP distribution have also been reported. In the NHANES III, there was no significant difference in the distribution of hsCRP concentration among white, African American, and Mexican American men (107). A comparable hsCRP distribution was seen in Japan among men but not women, who had slightly lower values (108). This was not the case in Japanese-American men in Hawaii, whose 75th percentile hsCRP value was 1.00 mg/L (109). Values above this low threshold were predictive of future vascular events over many years of follow-up. Other recent data were suggestive of lower hsCRP among Chinese subjects (105). The available data indicate that sex- or ethnic-specific cutpoints for hsCRP are not needed for CHD risk prediction, although, as stated in the CDC/AHA guideline (49), this question requires further study using data from cohorts with men and women of various ethnic groups and clinical outcomes.

	Percentile						
	5th	10th	25th	50th	75th	90th	95th
American women†	0.2	0.3	0.6	1.5	3.5	6.6	9.1
American men	0.3	0.4	0.8	1.5	3.2	6.1	8.6
European women†	0.3	0.4	0.9	1.7	3.4	6.2	8.8
European men	0.3	0.6	0.8	1.6	3.3	6.5	8.6

\*Data from Rifai (103) and Imhof (104).  
 †Only women not taking hormone replacement therapy.

### C. Biological variability

It is well-established that hsCRP has a relatively large biological variability. Therefore, the impact of this variability on its utility in CVD risk assessment has been the subject of great interest. In two studies, the within subject coefficient of variation (CV) ranged from 42% to 63% and the between-subject CV ranged from 76% to 92% (110,111), leading investigators to question the reliability of this marker in CVD risk assessment. However, another study showed that the rather large intraindividual CV (averaging 30%) was acceptable when the estimated composite CV for the group of individuals was 12% (112). However, multiple blood sampling to establish an individual's baseline hsCRP is needed; three independent determinations were recommended (111). However, findings from the SEASON trial showed that two independent measurements of hsCRP or total cholesterol, 3 months apart, enabled classification in up to 90% of subjects into the exact or immediately adjacent quartile (113). Additional analyses of these data have shown that more than 95% of subjects would be classified in the exact tertile of risk or vary by one tertile using the newly recommended cutpoints (114) (Figure 2). Furthermore, in the Cholesterol and Recurrent Events trial, the age-adjusted correlation between two hsCRP measurements from blood samples drawn 5 years apart was 0.6—again, a value comparable to that of cholesterol and other lipid parameters (66). Recently, data from the Reykjavik Heart Study further confirmed this finding and demonstrated that the decade-to-decade variability for hsCRP ( $r = 0.59$ ; 95% CI, 0.52 to 0.66) is identical to that of total cholesterol ( $r = 0.60$ ; 95% CI, 0.54 to 0.66)(60). Variability may be higher among patients with established coronary disease (115). Although continued skepticism surrounding the issue of intraindividual variation remains (116), somewhat similar recommendations to those made by the

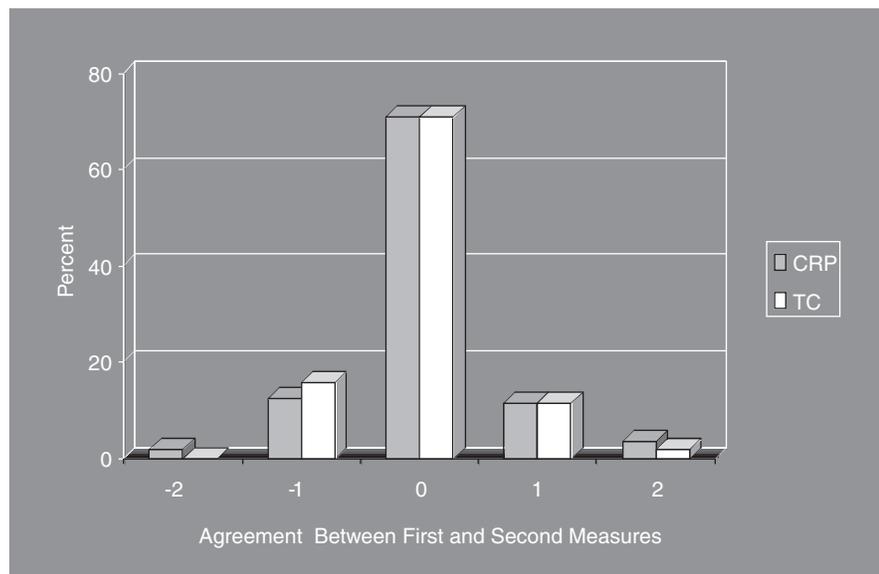
CDC/AHA expert panel (49) are proposed here; two independent measurements (fasting or nonfasting) of hsCRP, taken at least 2 weeks apart, with the lowest used to establish someone's CVD risk. Although it was recommended by the CDC/AHA panel to repeat the measurement when hsCRP concentration exceeds 10 mg/L (49), recent evidence suggests that the association of hsCRP with risk extends well beyond that range of concentration (54,55).

### D. Lifestyle behavior

Exercise, obesity, cigarette smoking, and alcohol consumption are known to influence hsCRP concentration. Strenuous exercise was shown to decrease hsCRP concentrations and an inverse association between hsCRP concentration and levels of cardiorespiratory fitness was also reported (117). In addition, a higher frequency of physical activity was associated with significantly lower odds of having increased hsCRP (118).

A positive association between hsCRP concentrations and body mass index has been clearly demonstrated (119, 120). The relationships between hsCRP concentrations and measures of obesity were reported to be consistent with *in vivo* release of interleukin (IL)-6 from adipose tissue. Significant weight reduction was associated with decreased concentrations of hsCRP and several cytokines and adhesion molecules indicating a reduction in the entire inflammatory state of an individual (121, 122).

Numerous studies have documented an increased hsCRP concentration with cigarette smoking (123, 124). This association was independent of cessation, suggesting that some of the smoking-related damage may be irreversible. In the Physicians' Health Study (64), the Women's Health Study (124), and the Cardiovascular Health Study (55), hsCRP was a good predictor of future myocardial infarction in both smokers and nonsmokers.



**Figure 2** Within-Person Variability: hsCRP Versus Total Cholesterol From Ockene et al (113)

Abbreviations: CRP, C-reactive protein; TC, total cholesterol. © *Clinical Chemistry*. Used with permission.

Moderate alcohol consumption is associated with lower hsCRP concentration compared to no or occasional alcohol intake suggesting that alcohol may attenuate CVD risk in part through anti-inflammatory mechanisms. Furthermore, data from prospective studies have shown that IL-6 and tumor necrosis factor (TNF)- $\alpha$  receptors 1 and 2 are lower in moderate drinkers than non-drinkers, further suggesting the anti-inflammatory effects of alcohol (125).

#### *E. Drugs*

Several pharmacologic agents and treatment modalities influence hsCRP concentrations. Randomized clinical trials and cross sectional studies have shown that hormone replacement therapy increases serum hsCRP concentrations by 2- to 3-fold (52, 126). Selective estrogen receptor modulating drugs such as raloxifene and tamoxifen do not have this effect (127-129). In addition, no effect of exogenous androgen therapy in men was observed on serum inflammatory markers including hsCRP (130). Clinical consequences of the above-mentioned hormone drug effects on hsCRP are unknown. Although, the effect of aspirin on reducing the incidence of MI in men with increased hsCRP (approximately 60%) is clear (64), its effect on hsCRP concentration remains uncertain (131-134). As described earlier, both lovastatin and pravastatin reduced coronary events in subjects with increased hsCRP concentrations (65,66), suggesting a possible anti-inflammatory effect of statins. In addition, all other statin drugs lower hsCRP by about 15% to 20% independently of the reduction seen in LDL-C (65,133,135).

#### *F. Specimen collection and handling*

Although samples collected in the fasting state are not required for the measurement of hsCRP (136), certain assays are affected by optical clarity and fasting before sampling may be needed in the presence of severe hypertriglyceridemia. Furthermore, hsCRP does not exhibit a circadian rhythm and, therefore, there is no need to standardize the time for sample collection to assess CHD risk (137).

Either serum or plasma is suitable for the measurement of hsCRP. As expected, due to the osmotic shifting effect of the anticoagulant on erythrocytes, the use of EDTA or citrated plasma specimens resulted in significant biases (>10%) in hsCRP concentration when compared to serum (138). In contrast, no difference in hsCRP values were seen using heparinized and serum samples (139). Another report, however, showed no significant differences when serum, heparin, and EDTA-plasma samples were simultaneously collected from a single venipuncture in 25 patients (140). Additional studies are needed to clarify this important issue.

hsCRP has been shown to be stable at 4°C for 60 days (141) and no significant changes in its concentration were seen in samples stored at -70°C for more than 20 years (136) or in liquid nitrogen for up to 6 months (103).

#### *G. Analytical variability*

Since subjects would be classified into categories of risk using specific cutpoints, assays must be able to reliably measure hsCRP at least at the lowest cutpoint (1 mg/L).

Assays used for population-based studies and clinical research, however, should be able to measure hsCRP concentrations at much lower concentrations such as 0.15 mg/L (2.5th percentile of the reference population). An evaluation of nine second generation hsCRP assays showed that all had a sensitivity of  $\leq 0.3$  mg/L (143). The reliability of a particular assay is in part a function of its reproducibility. It was recently suggested that for hsCRP, the within-laboratory total imprecision should be lower than 10% across the linear range of the assay (144). However, in the above-mentioned evaluation, only five of nine hsCRP methods examined met this criterion indicating the need for more precise assays (143).

As indicated earlier, more than 30 hsCRP methods are currently available with differing performance (99). Several studies have shown significant discrepancy in reported results among methods and emphasized the need for additional standardization (143, 144). In the above-mentioned study, four of the hsCRP methods were in excellent agreement with the comparison method in classifying subjects into quartiles of risk (143). In fact, 92% to 95% of subjects were classified by these methods into the exact quartile and the remaining 5% to 8% fell almost equally in the adjacent two quartiles. Using the other four methods, however, only 65% to 75% of subjects were classified into the exact quartile and the remaining 25% to 35% all fell in the adjacent upper quartile indicating a problem with standardization. Agreement among the various hsCRP methodologies is essential considering that the individual patient result is interpreted within the context of nationally established cutpoints. To address this issue, the CDC initiated a standardization program to which manufacturers of all hsCRP reagents worldwide have been invited to participate (99). The goal of this project is to identify a suitable reference material and then use this common calibrator among the various assays to harmonize patients' results. Currently there is no reference method for hsCRP.

In this document, we further support the strong recommendation made in the CDC/AHA guideline (49) and the FDA (145) for using mg/L as the unit of choice for result reporting for hsCRP when used in CVD risk prediction. It is imperative that hsCRP values be reported in a single unit. If some laboratories report hsCRP concentration in mg/L and others in mg/dL, confusion will result in the medical community in terms of using this marker and patients will be misclassified and mismanaged.

#### *Interpretation of hsCRP Results*

In this document, we concur with the CDC/AHA guideline recommendation of using specific cutpoints for clinical interpretation that correspond with the approximate tertiles of the population distribution of hsCRP (49); hsCRP concentrations < 1.0 mg/L are considered low risk, 1.0 to 3.0 mg/L are considered average risk, and > 3.0 mg/L are considered high risk with the addition of  $\geq 10$  mg/L considered as very high risk.

## Conclusions

In future studies of biomarkers, investigators should consider standard reporting that utilizes:

1. the CDC/AHA cutpoints for hsCRP (with values of both > 3.0 mg/L and >10.0 mg/L assessed),
2. the same set of covariates (traditional cardiovascular risk factors; age, sex, race, blood pressure/hypertension status, total cholesterol and HDL-C, diabetes, and smoking status),
3. reporting of results in sex- and ethnic-specific subgroups, and
4. evaluating the number of participants that would be reclassified into a different risk group based on combining results from hsCRP testing with risk prediction algorithms developed within the test population.

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# Lipoprotein Subclasses and Particle Concentration and Cardiovascular Disease Risk

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## RECOMMENDATIONS FOR LDL SUBCLASSES AND PARTICLE SIZE

Based on a thorough review of the published literature, the following recommendations for the clinical use and measurement of LDL subclasses and particle concentration in assessing risk for CHD and stroke in primary prevention.

### **Recommendation 1**

Lipoprotein subclasses, especially the number or concentration of small, dense LDL particles, have been shown to be related to the development of initial CHD events, but the data analyses of existing studies are generally not adequate to show added benefit over standard risk assessment for primary prevention.

**Classification of recommendation:** III (lipoprotein subclass determination is not recommended)

**Level of evidence:** A

### **Recommendation 2**

There are insufficient data that measurement of lipoprotein subclasses over time is useful to evaluate the effects of treatments.

**Classification of recommendation:** III

**Level of evidence:** C

### **Recommendation 3**

Several methods are available to assess lipoprotein subclasses. Standardization is needed for this technology.

**Classification of recommendation:** IIa

**Level of evidence:** C

was able to separate very low density lipoproteins (VLDL), LDL, intermediate density lipoproteins (IDL), and HDL(1). The separation into only these major subclasses was relatively arbitrary. It was recognized that many more subclasses could be identified and that they could be characterized according to their density, electric charge, and the concentration of phospholipid, cholesterol, and triglyceride, and specific proteins. This section on lipoprotein subclasses places a focus on the utility of such information in the prediction of CHD when the subclasses are identified according to density, electric charge, and other physical chemistry aspects of the particles such as nuclear magnetic resonance.

## Methodology

Initial methodology for determination of lipoprotein subclasses was based on LDL alone and the assignment of pattern A or pattern B (2). This technique was followed by the development of methods that provided for assessment of particles in the VLDL, IDL, LDL, and HDL ranges. There are now a number of commercial methods available to measure LDL subfractions and particle concentration. One of the first methods developed used gradient gel electrophoresis (3). This technique serves as the basis for the method used by Berkley HeartLab (Alameda, CA) for LDL fractionation (4,5). A vertical automated profile (VAP) technique derived from density gradient ultracentrifugation was developed by Atherotech (Birmingham, AL) (6,7) and a tube gel electrophoresis method is used in the Quantimetrix Lipoprint LDL System (Quantimetrix, Redondo Beach, CA) (8). Liposcience (Raleigh, NC) employs nuclear magnetic resonance to estimate lipoprotein particle concentration (9). The methodologic standard or benchmark for determining lipoprotein subfractions is analytical ultracentrifugation (10).

## CLINICAL RATIONALE/EVIDENCE

### Cross-Sectional and Case-Control Studies

Early clinical reports using techniques to identify lipoprotein subclasses showed that small, dense LDL particles were highly associated with the occurrence of CHD (2,11). This report from the Boston Area Health Study included 109 patients with CHD and 121 controls from metropolitan Boston and showed that

## SUPPORTING EVIDENCE

### Introduction

Enthusiasm to identify the role of lipoprotein subclasses dates to the mid 1960s when scientists described how ultracentrifugation

small, dense LDL particles were more commonly found in the case group. The laboratory technique used by the scientists generally identified persons as having either pattern A (large, buoyant LDL particles) or pattern B (small, dense LDL particles). The authors concluded that “the metabolic trait responsible for this LDL subclass pattern results in a set of interrelated lipoprotein changes that lead to increased risk of CHD.”(2) This conclusion was prescient, as reports since that time have tended to show similar results—atherosclerotic vascular disease is more commonly associated with small, dense LDL particles, but such particles are more commonly seen when triglycerides are elevated and HDL-C is low.

Similar results in cross-sectional and case-control studies that investigated the association of small LDL particles with clinical CHD were reported by Campos and Sherrard (11,12). It was noted that high triglycerides typically accompanied the small, dense particles, diet may affect particle composition, and persons with familial hypercholesterolemia did not necessarily make small, dense LDL particles (12).

### Subclinical Disease

Assessment of subclinical atherosclerotic disease has been available for more than a decade and a variety of techniques have been used, such as carotid intima medial thickness, coronary angiography, and vascular calcification of various arterial beds. Several case-control studies compared LDL particle sizes in persons with and without angiographic coronary artery disease (CAD). Campos reported a predominance of large LDL particles in normolipidemic men with angiographic CAD (14), Rajman described smaller LDL particles in men with angiographic CAD in Britain (15), and Freedman showed that smaller LDL particles were more common in persons with angiographic CAD but the effect was not statistically significant in multivariate analyses that included traditional CHD risk factors. More recent studies have that increased carotid intima medial thickness is associated with small LDL particle concentration in the Bogalusa Study (16) and decreased LDL size is related to greater prevalence of coronary calcification in the Women’s Health Initiative (17).

### Longitudinal Studies

Longitudinal studies have generally shown that greater concentrations of small LDL particles are associated with increased risk of CAD events as reported from the Quebec Heart Study (18), Veterans Administration HDL Intervention Trial (VAHIT) (19), Cardiovascular Health Study (CHS) (20), Women’s Health Study (21), Atherosclerosis Risk in Communities (ARIC) (22), and Framingham (23). Some longitudinal studies have not shown that greater concentrations of small LDL are related to greater risk for CHD, as in the Strong Heart Study (24) and Cholesterol and Recurrent Events (CARE) (25). Data tends to support stronger relations between LDL particle number and events than small LDL particle size.

The multivariable adjusted effects for LDL measures in models that include traditional CHD risk assessment are generally not statistically significant.

Information on the relation between concentration of IDL and HDL particles and the occurrence of CHD has generally been reported less frequently, but smaller sizes of these moieties are generally associated with greater risk of CHD events (19,22,25,26).

## DISCUSSION

Great progress has been made in the development of refined lipoprotein assessment and such measurements have helped in our understanding of the atherosclerotic process. The general utility of these measurements as adjuncts for screening persons at risk for CVD or monitoring responses to lipid lowering regimens is not well understood. In particular, we do not know if this additional information helps the health care provider to identify with greater precision and accuracy the person who will develop clinical or subclinical CVD. Longitudinal studies with serial determinations of lipid subclasses would definitely improve our understanding. Unfortunately, we lack reference standards for the measurement of lipid subclasses and there is little information on head-to-head comparisons of the various lipid measurement methods across electrophoretic and nuclear magnetic resonance platforms (27,28).

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# Lipoprotein (a) and Cardiovascular Disease Risk

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## RECOMMENDATIONS FOR LIPOPROTEIN (a)

Based on a thorough review of the published literature, the following are recommendations for the clinical use and measurement of lipoprotein (a) in assessing risk for CHD and stroke in primary prevention.

### *Recommendation 1*

Lipoprotein (a) screening is not warranted for primary prevention and assessment of cardiovascular risk.

**Classification of recommendation:** III (against measurement)

**Level of evidence:** A

### *Recommendation 2*

If risk is intermediate (10% to 20%) and uncertainty remains as to the use of preventive therapies such as statins or aspirin, then lipoprotein (a) measurement may be done at the physician's discretion.

**Classification of recommendations:** IIb

**Level of evidence:** C

### *Recommendation 3*

After global risk assessment, lipoprotein (a) measurements in patients with a strong family history of premature CVD may be useful for identifying individuals having a genetic predisposition of CVD.

**Classification of recommendation:** IIb

**Level of evidence:** C

### *Recommendation 4*

The benefits of therapies based on lipoprotein (a) concentrations are uncertain. If both lipoprotein (a) and LDL-C are highly increased, an attempt can be made at the physician's discretion to lower lipoprotein (a) level by lowering the elevated LDL-C.

**Classification of recommendation:** IIb

**Level of evidence:** C

### *Recommendation 5*

There is insufficient evidence to support therapeutic monitoring of lipoprotein (a) levels for evaluating the effects of treatment.

**Classification of recommendation:** III (against measurement)

**Level of evidence:** C

### *Recommendation 6*

Population routine testing for small size apolipoprotein (a) is not warranted.

**Classification of recommendation:** IIb

**Level of evidence:** C

## INTRODUCTION

Lipoprotein (a) is composed of the unique glycoprotein (a) which is attached to a disulfide-bound apo B-100. It has a higher density and higher sialic acid content than LDL. Lipoprotein (a) is naturally in human plasma in very low concentrations between < 0.1 to > 180 mmol/L. Studies predominantly have found that lipoprotein (a) is a risk factor for CHD. The lipoprotein (a) striking structural homology with human plasminogen suggests a function for lipoprotein (a) in thrombogenesis. Lipoprotein (a) strongly contributes to CHD risk when LDL-C and lipoprotein (a) are concomitantly high in concentration. Small apo size is associated with greater strength prediction of CHD and independence than lipoprotein (a) concentration. Inaccuracy arises in laboratory measurements because antibodies that recognize the kringle 4 type 2 repeated epitopes will have variable immunoreactivity with the size of apo (a). Even though lipoprotein (a) appears to be high pathogenic for CHD, homocysteine (Hcy) screening for primary prevention, and assessment of CHD risk is not warranted.

## CLINICAL RATIONALE/EVIDENCE

Seven hundred fifty-nine lipoprotein (a) candidate publications from 1996 to 2005 on primary intervention were obtained from Ovid Embase. After subsequent review by the

Lipoprotein (a) Working Group, the number of appropriate and eligible citations decreased to 32 representative publications which were used in preparation of a spreadsheet summary. The summary tabulated the following information: author, year, name of study, title, citation, study type, follow-up, age range, end point, number of cases, number of controls, hazard or odds ratio, multivariate adjustment, adjustment factors, assay used, cutpoints, mean in noncases, ethnic makeup, sex, comparison to lipids in cohort, and comments.

### Lipoprotein (a) and CHD Risk

Evidence from reviewed publications indicate lipoprotein (a) is independently associated with CHD, is a risk factor for premature CHD in persons < 50 years of age and in the elderly (older than 70 years), and elevated lipoprotein (a) increases risk for CHD in combination with other CHD risk factors. A meta-analysis of prospective studies conducted before 2000 concluded that evidence has clearly established a moderately strong association of lipoprotein (a) with CHD and that the effect was independent of the standard vascular risk factors (1). High lipoprotein (a) concentration has been shown to predict risk of angina and the risk is substantially increased with concomitant high LDL-C concentration (2). Small apo(a) size predicted angina with greater strength, independently more than lipoprotein (a) concentration (2,3).

A prospective case-control study nested within the Northern Sweden Health and Disease Cohort found high plasma levels of lipoprotein (a) were independently associated with subsequent development of a first myocardial infarction in men (4). A prospective study in Framingham of men up to age 55 years found elevated plasma lipoprotein (a) to be an independent risk factor for the development of premature CHD in men (RR = 1.9; 95% CI, 1.2 to 2.9) comparable in magnitude and prevalence to a total cholesterol level of 6.2 mmol/L (240 mg/dL) or an HDL-C level lower than 0.9 mmol/L (35 mg/dL) (5).

In the PROSPER study of elderly subjects between 70 and 82 years of age, after adjustment for baseline characteristics, a statistically significant association was found between baseline lipoprotein (a) and risk of both CHD death and vascular disease but not between lipoprotein (a) and cognitive function (6). In the 2002 Austrian Study of 100 patients referred for possible CHD, a lack of association was observed between plasma lipoprotein(a) concentration and the presence or absence of CHD (7). RR for the fifth quintile of plasma lipoprotein (a) as compared with the first quintile was 0.87 (95% CI, 0.66 to 1.34), and after adjustments, RR became 1.06 (95% CI, 0.81 to 1.39). In the 5-year 1991 Helsinki Heart Study of men age 40 to 55 years who were devoid of CHD at the beginning of the trial, the odds ratio for the subjects in the higher lipoprotein (a) category compared with those in the lower was 1.06 (95% CI, 0.64 to 1.76), but when using the lower limit of the highest lipoprotein (a) tertile as the cutpoint, became 1.32 (95% CI, 0.77 to 2.00) (8). They concluded that in the Helsinki Heart Study cohort the serum lipoprotein (a) level was not a predictor of future coronary events (8).

### Lipoprotein (a) and CHD Risk in Women

The prospective study in Framingham of women by multivariable-adjusted RR estimates found for outcomes in the Hcy band present versus band absent groups: total CVD, RR = 1.44(95% CI, 1.09 to 1.91), thus giving evidence that lipoprotein (a) is a strong predictor of CVD in women (9). In a study of a general population in 5 California cities, strong evidence linked lipoprotein (a) level as a prospective independent risk factor for developing CHD in men, but this relationship was not statistically significant in women (10). Among middle-age women in the Nurses Health Study, lipoprotein (a) levels > 30 mg/dL were associated with twice the risk of CHD events, independent from lipid and non-lipid risk factors (11). In the 12-year follow-up of subjects in western Sweden in 2002, sex-specific analysis revealed that in women, but not in men, the risk for total mortality was significantly increased among subjects in the high lipoprotein (a) quartiles (RR = 1.21(95% CI, 1.0 to 1.5; *P* = 0.05) (12).

### Lipoprotein (a) Interaction With Other CHD Risk Factors

Lipoprotein (a) becomes associated often with more than an expected increase in risk for CVD when an elevated lipoprotein (a) accompanies another significant CVD risk factor or metabolic condition. CVD risk is substantially increased when a high lipoprotein (a) concentration is present with a high LDL-C concentration (2). Findings of the Physicians' Health Study showed that lipoprotein (a) concentration strongly contributed to CHD risk when LDL-C was concomitantly increased, consistent with the PROCAM, Prime and Bruneck studies (2). Lipoprotein (a) concentration can be used to identify a small group of people with high LDL-C possessing especially high CHD risk and who might benefit from more intensive risk management.

In the 1997 Scandinavian Simvastatin Survival Study (4S) 48-month follow-up of subjects with elevated total cholesterol levels between 5.5 and 8.0 mmol/L, it was observed that lipoprotein (a) levels were strikingly higher in Scandinavian CHD subjects than in healthy controls and numbers of deaths differed significantly between quartiles of lipoprotein (a) levels (13). Coexistent elevated lipoprotein (a) and smoking show significantly increased risk for CVD (5). A positive association was observed between small size lipoprotein (a) isoforms and CHD deaths only among smokers (14). In Quebec City, Canada, high lipoprotein (a) levels together with high fibrinogen levels significantly increased the risk of CVD (15). In the ARIC study, population-based samples of residents 45 to 64 years old from four different state communities with no evidence of CHD at baseline, a very small gain in overall prediction of CHD was observed from adding lipoprotein (a) to other lipids even though lipoprotein (a) was independently significant with an RR of 1.17 (16). In the 2004 reported Indian Moradabad Lifestyle and Heart Study of apparently healthy males age 45 to 60 years, the incidence of lipoprotein (a) excess (> 30 mg/dL, 42.6 vs 24.7%; *P* = .05) was significantly greater

in the CHD group than in the controls (17). Lipoprotein (a) was significantly higher during the acute cardiac event and showed a significant decline at 4 weeks when the acute cardiac incident had been controlled. A large breakfast and a large dinner, significantly associated with metabolic reactions, such as nitrite deficiency, or greater levels of proinflammatory cytokines in conjunction with obesity, hyperglycemia, and hyperinsulinemia, were observed to be related to a significantly higher incidence of cardiac events in the second and fourth quarters of the day, respectively (17). It is possible that subjects consuming large fatty breakfasts and dinners enhance the release of catecholamines, glucose, insulin, and triglycerides, which may have an adverse effect on cardiovascular function (17). In the University of Florence study of vascular aortic aneurysm, plasma lipoprotein (a) concentration levels were significantly elevated and significantly contributed in the evaluation of the need for an elective surgical intervention (18). A logistic regression analysis with multivariate analysis found a significant association of high levels of lipoprotein (a) with peripheral vascular abdominal aortic aneurysm disease (OR = 2.6; 95% CI, 1.7 to 3.9;  $P < .0001$ ). In addition, the contemporary presence of high levels of lipoprotein (a) and elevated Hcy led to an increased risk of having peripheral vascular abdominal aneurysm (OR = 22.7; 95% CI, 5.0 to 102.5) (18).

OR or RR for CHD risk in relation to lipoprotein (a) levels have been reported in 14 representative studies using multivariate analysis since the year 2000 (Table 5).

Although elevated lipoprotein (a) concentrations have shown positive associations with CHD risk, an elevated lipoprotein (a) concentration is relatively uncommon in the population (14) and there is general uncertainty concerning the laboratory measurement, which has led to the recommendation that lipoprotein (a) determinations should not be performed when routine testing the population to assess risk for initial CHD events (25, 26).

## Analytical Considerations

### Lipoprotein (a) Reference System

Primary reference method: No IDMS method is available.

Secondary reference method: A designated comparison method measures the lipoprotein (a) protein concentration with a double monoclonal antibody (MAB)-based ELISA with the capture MAB directed to epitope K4 type 2 in lipoprotein (a) and with the detecting MAB directed toward a unique epitope kringle 4 type 9 in lipoprotein (a) (26).

Primary reference material: A freshly purified lipoprotein (a) with protein mass concentrations determined by amino acid analysis.

Secondary reference material: IFCC proposed lipoprotein (a) serum reference material (IFCC-primary reference material- lipoprotein (a)) (25).

Stability: Studies on the effect of long-term storage found no significant changes in lipoprotein (a) concentration in sera stored at  $-20^{\circ}\text{C}$  for up to 8.5 years (8).

### Clinical Laboratory Assays

In the CAP Chemistry Surveys 2005 C-C (2006 C-C), all immunoturbidimetric methods showed a mean CV of 20.0% (19.24%) and all immunonephelometric assays gave a mean CV of 25.3% (25.1%) on proficiency testing samples (27). Laboratories using reagents from three major distributors in 2005 (and from two major distributors in 2006) showed a range of CV from 5.9 to 13.5% (4.7% to 11.2%) for the immunoturbidimetric method and in 2006, an average CV of 9.1% for two laboratories using two different distributor reagents and for two nephelometric methods, with a range of CV 5.8% to 13.3%. At this time, a practical within laboratory CV and bias goal for clinical laboratories appears to be  $< 10\%$ . A desirable goal and reasonable analytical performance for research and dedicated laboratories for CVD studies seems to be a bias and CV of 5%.

### Lipoprotein (a) Measurement Issues

The major problems faced in Hcy analytical performance in the clinical laboratory arise from existence of multiple isoforms, attached carbohydrate complexes, and formation of lipoprotein (a) with an apo B molecule (26). Antibodies that recognize the kringle 4 type 2 repeated epitopes will have variable immunoreactivity depending on the size of apo(a) (26). The size heterogeneity of apo(a) among individuals presents a unique challenge to the determination of accurate lipoprotein (a) concentrations (2). Monoclonal antibodies reagents should be used that are directed to apo(a) antigenic sites other than kringle 4 type 2 that are sensitive to the size of apo(a). The epitope present in apo(a) kringle 4 type 9 was found to be insensitive to apo(a) size (26). At the present time, the commercial latex methods use polyclonal antibodies that recognize kringle 4 type 2 and tend to underestimate lipoprotein (a) in individuals with apo (a) of a smaller size and overestimates lipoprotein (a) in individuals with larger apo(a) particles. The fact that almost all commercially available methods for lipoprotein (a) are affected by the kringle 4 type

**Table 5. Relative Risk for Coronary Heart Disease in Relation to Lipoprotein (a)**

Author (Reference No.)	Relative Risk (95% CI)
Danesh (1)	1.7 (CI 1.4-1.9)
Auer (7)	1.06 (0.87-1.39)
Cantin (15)	1.09 (0.69-1.73)
Lundstam (12)	1.21 (0-1.5)
Rifai (2)	2.57 (1.09-6.07)
Jansen (19)	1.50 (1.20-1.79) female and 2.82 (2.37-3.36) male
Rajasekhar (20)	1.98 (0.007-1.18)
Frolich (22)	1.002 (1.001-1.003) for women
Hong (21)	4.36 (1.76-10.85)
Gaw (6)	1.06 (1.005-1.12)
Thogersen (4)	7.21 (1.31-39.8)
Longenecker (23)	1.38 (1.06-1.79)
Holmes (24)	3.19 (1.79-5.69)
Shai (11)	2.09 (1.16-3.77)

2 variably repeated epitopes, and thus underestimates the predicting power risk power of lipoprotein (a), reaffirms the need for developing accurate lipoprotein (a) methods for the clinical laboratory (2). In the Physicians Health Study, the lipoprotein (a) ELISA method currently uses a monoclonal antibody specifically directed to an epitope present in apo(a) kringle 4 type 9 (2). It seems likely that lipoprotein (a) methods previously used by the Physicians' Health Study before using kringle 4 type 9 antibody reagents, may have underestimated or even obscured the true relationship between lipoprotein (a) concentration and CHD (2).

## DISCUSSION

Uncertainty over reliability of the lipoprotein (a) measurement is one major factor against routine testing in the population (25, 26). In a nested case-control study of predominantly middle-age white male participants in the Physicians Health Study, no evidence was found of association between lipoprotein (a) and risk of future myocardial infarction (27). They concluded that these data did not support the use of lipoprotein (a) level as a routine testing tool to define CVD risk among the population (27). Routine testing for stroke in the population also is not recommended in primary intervention in the population since no association was observed between baseline plasma concentration of lipoprotein (a) and future risk of total or thromboembolic stroke (28). No recommendation for routine testing was made by the meta-analysis of the results of 18 population-based cohorts before 2000 with a combined RR of 1.7 (95% CI, 1.4 to 1.9) (1).

Routine testing for lipoprotein (a) can be considered under the following circumstances: patient or family history of premature atherosclerotic heart disease, familial history of hyperlipidemia, (c) established atherosclerotic heart disease with a normal routine lipid profile, (d) hyperlipidemia refractory to therapy, and (e) history of recurrent arterial stenosis (29). High levels of lipoprotein (a) in 6-year-old children show an association with a grandparent history of CVD and stroke, and with high levels of LDL-C and apolipoprotein B (30). In India, evidence was found that lipoprotein (a) was associated with CHD (OR 1.98; 95% CI, 0.007 to 1.18), suggesting a genetic predisposition (20). In the Honolulu Heart Study, the mean lipoprotein (a) value of 403 controls was 13.7 mg/dL, the OR in men with a prior MI was 2.5 times that for men in the lower three quartiles for a recurrent MI, and the population-attributable risk was 25% in men younger than 60 years of age (31). Conclusions of the Scandinavian Simvastatin Survival Study (4S) were that it is desirable to measure lipoprotein (a) concentration in epidemiologic studies as well as in families with aggregation of cases of CHD, in individuals with premature CHD in the absence of traditional risk factors, and in the relatives of such patients (13).

A 6-month follow-up interval is suggested for evaluation of serum lipoprotein (a) as a predictor for acute vascular events, particularly in association with inflammatory conditions since lipoprotein (a) showed a delayed response to various conditions eliciting inflammation unlike markers such as hsCRP

(21). Lipoprotein (a) can be serially followed at the physician's discretion, especially in subjects with the synergistic reactions of high hsCRP concentrations and high plasma cholesterol (21) or with high lipoprotein (a) and high fibrinogen (15).

To determine intermediate risk by Framingham risk score, a form such as one published on page 361 and sample on page 362 of Anderson et al can be used (32).

The risk for CHD associated with lipoprotein (a) levels is uncertain since subjects vary with different lipoprotein (a) kringles and types (2,3). Cutpoints reported for multiple different reviewed publications varied from 13 to 56.8 mg/dL, indicating that uncertainty exists at the present time about reliability of selecting therapies based on lipoprotein (a) levels.

The individual's lipoprotein (a) serum level varies with the number of kringles and types among subjects (26). Accurately labeled calibrators and reagents needed for accurate analysis are not readily available for lipoprotein (a) analysis at the present time.

Although small apo(a) size predicts angina with greater strength and independence than lipoprotein (a) concentration (2,3), routine testing is not recommended since neither small size apolipoprotein measurement or lipoprotein(a) concentration assays have readily available standardized commercial assays validated to accurately measure lipoprotein (a) size or lipoprotein (a) concentration independent of apo(a) size (2, 26). Routine testing of the population is not recommended generally because of infrequency of elevated small size lipoprotein (a) concentrations even though low molecular weight lipoproteins (a) are significantly associated with development of CHD as demonstrated by the following publications. In the prospective nested case-control four cohorts of German MONICA studies of white men and one cohort of the German Glostrup population study of white men younger than 60 years, low molecular weight apo (a) isoforms are significantly associated with the development of CHD with an OR of 3.83 (95% CI, 1.18 to 12.4) (3). High plasma levels of low molecular lipoprotein (a) isoforms are associated with increased risk of death in patients with CHD with an RR of 2.2 (95% CI, 1.3 to 3.7) (12). In a Gulf Arab Kuwait population study, unlike in certain other populations, no simple relationship or consistent trend was observed between the lipoprotein (a) isoform pattern and serum lipoprotein (a) concentration levels (30). Similar lipoprotein (a) isoform patterns were found in the CHD patients and the healthy population controls (33). Measurements of lipoprotein (a) size was not recommended by the Stanford Five-City Project since they found that apo(a) size did not add to the predictive power of lipoprotein (a) concentration (10). The results of the PROSPER study casts doubt on the clinical utility of plasma lipoprotein (a) measurements in assessing vascular risk in elderly subjects (6).

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# Apolipoproteins A-I and B and Cardiovascular Disease Risk

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## RECOMMENDATIONS FOR APOLIPOPROTEINS A-I AND B

Based on a thorough review of the published literature, the following are recommendations for the clinical use and measurement of apolipoproteins B and A-I in assessing risk for CHD and stroke in primary prevention.

### *Recommendation 1*

The first step to monitor efficacy of lipid lowering therapies is to measure LDL-C (and non-HDL-C in patients with elevated triglycerides).

**Classification of recommendation:** I

**Level of evidence:** A

### *Recommendation 2*

Although apoB measures atherogenic lipoproteins and is a good predictor of CVD risk (equal at least to LDL-C and non-HDL-C), it is only a marginally better predictor than the current lipid profile and should not be routinely measured at this time for use in global risk assessment.

**Classification of recommendation:** IIa (against measurement);

**Level of evidence:** B.

### *Recommendation 3*

Measurement of apo-B can be used to monitor efficacy of lipid-lowering therapies as an alternative to non-HDL-C.

**Classification of recommendation:** IIb

**Level of evidence:** B

### *Recommendation 4*

The apo B/apo A-I ratio can be used as an alternative to the usual total cholesterol/HDL-C ratio to determine lipoprotein-related risk for CVD.

**Classification of recommendation:** IIa

**Level of evidence:** A

### *Recommendation 5*

Manufacturers of apo-B and apo-AI assays should establish traceability to accepted standards to assure reliable and comparable results.

**Classification of recommendation:** IIa

**Level of evidence:** C

## SUPPORTING EVIDENCE

### Introduction

apo A-I and B are structural and functional components of lipoprotein particles that serve as transporters of cholesterol. apo B transfers cholesterol and triglyceride from sites of production to tissues where they are utilized for energy production, storage, membrane assembly, or hormone synthesis. On the other hand, Apo A-I plays an important role in the reverse cholesterol transport by transferring cholesterol from tissues back to the liver. Epidemiologic research and clinical event trials have provided evidence that both apo A-I and B could play an important role in the initial assessment and ongoing monitoring of CVD for coronary events and stroke.

## CLINICAL RATIONALE/EVIDENCE

### apo A-I

The A lipoproteins form the major proteins found in HDL-C. The name A lipoproteins is a result of HDL formerly being referred to as alpha migrating lipoprotein on electrophoresis. Of the two major apo As in human plasma (apo A-I and apo A-II), the concentration of apo A-I concentration is roughly three times greater than for apo A-II. The ability of HDL to predict coronary risk has been confirmed in a number of studies linking low levels of serum HDL-C to increased CHD morbidity and mortality (1-7). Conversely high HDL-C levels provide a protective effect and convey reduced risk. Since apo A-I is the predominant apo associated with HDL-C, it seems reasonable to assume that apo A-I levels would behave

similarly to HDL-C with anti-atherogenic properties. However, with a few exceptions such as the very large AMORIS Study, apo A-I was found to have no statistically significant association with CHD risk when evaluated in multivariate analysis that included HDL (8-11). These studies generally showed little advantage over HDL-C levels and do not support the premise that apo A-I measurements may provide more information than HDL-C levels in the assessment of CHD risk.

### apo B

apo B is essential for the transport of all cholesterol carrying lipoproteins emanating in the liver and gut, including secretion of triglyceride-rich lipoproteins, VLDL and IDL. It plays a central role in the lipoprotein transport system carrying cholesterol and triglyceride from the liver and gut to the sites of utilization or storage and while the lipid content of these lipoproteins is continually changing the apo B content remains stable. It drew its name from its former identification as beta migrating lipoprotein on electrophoresis. apo B is present as a single molecule in LDLs, IDL, and VLDL, respectively, and many experts feel that it is potentially the most biologically and analytically superior marker for all atherogenic particles (11-16). However some controversy remains as to whether apo B provides any additional information to improve the prediction of risk over and above LDL-C and non-HDL-C—the usual lipid parameters.

Some early case-control and cohort studies reported better predictive properties for apo B than for total and LDL-C (10,17-19). However, other studies did not confirm these findings (20). For example, in the Physician's Health Study apo B levels were not found to add significantly to risk after multivariate analysis for conventional risk factors (9). In the Reykjavik study apo B was a highly significant risk factor in a univariate analysis, but not in a multivariate analysis when serum cholesterol was included (21). Thus apo B did not contribute further to CHD risk prediction over measurement of traditional factors and serum total cholesterol. Likewise, in the ARIC study, apo B was strongly predictive for CHD in univariate but not in multivariate analysis (8). The 10-year follow-up results from the Goettigen Risk, Incidence and Prevalence Study (GRIPS) established LDL-C as the strongest predictor of MI (22). apo B was less effective in predicting risk than LDL-C and did not contribute independently to the estimation of MI risk (22). In a prospective nested case-control study among 32,826 middle-age women in the Nurses Health Study, the usefulness of multiple lipid parameters in predicting future CHD was evaluated (23). Here, apo B was more strongly associated with CHD than LDL-C, but when evaluated in a multivariable-adjusted model the association of apo B with CHD was more attenuated by lipid and nonlipid risk factors than was LDL-C and did not add significant information beyond LDL-C (23).

Numerous studies have supported the importance of apo B as a predictor of risk. Results from the Quebec study indicated that elevated apo B levels were strongly associated with

an increased RR of ischemic heart disease, even after adjustment for triglyceride and HDL-C (10). In another study Talmud et al compared the pairwise combinations of total cholesterol, triglycerides, apo B, HDL-C, LDL-C, and apo A-I on CHD risk prediction in middle-age men in the United Kingdom. They found that apo B was a better predictor of risk than total or LDL-C (11). In a prospective cohort study of 15,632 initially healthy women enrolled in the Women's Health Study, apos A-I and B, non-HDL-C, standard lipid measures, lipid ratios, and hsCRP were directly compared to evaluate their clinical utility as predictors of future cardiovascular events (24). The observed magnitude of the association with cardiovascular risk was greater for apo B (HR, 2.50; 95% CI, 1.68 to 3.72) than for either total cholesterol (HR, 2.32; 95% CI, 1.64 to 3.33) or LDL-C (HR, 1.62; 95% CI, 1.17 to 2.25) (24). Study results also indicated that apo B and non-HDL-C were highly correlated ( $r = 0.87$ ) and that the strength of association for non-HDL-C (HR, 2.51; 95% CI, 1.69 to 3.72) was clinically equivalent to that of apo B (24). In the Apolipoprotein-Related Mortality Risk (AMORIS) study, a large prospective study in Swedish men and women, increasing concentrations of both apo B and LDL-C were related to a greater risk of fatal MI (25). After multivariate analysis apo B proved to be a stronger predictor of MI than LDL-C. In a later study of stroke mortality from the AMORIS study, apo B was also shown to be significantly related to the risk of stroke (26). More recently the population-based MONICA/KORA Augsburg cohort study showed apo B to have a strong association with incident coronary events in both sexes, even after adjustment for conventional risk factors (27).

In a recent literature-based meta-analysis of prospective studies to assess associations of apo A-I, apo B, and apo B/A-I ratio with risk of incident CHD, Thompson and Danesh reported for apo B a RR of 1.99 (95% CI, 1.65 to 2.39) for a comparison of apo B concentrations of individuals in the top tertile versus those in the lowest tertile (28). This was based on a combined analysis of 6,320 CHD cases from 19 studies reviewed. Overall, prospective studies published to date do indicate a strong independent association of apo B and the risk for CHD and stroke.

### apoB/apoA-I Ratio

Risk for CHD increases in proportion to total cholesterol and LDL-C levels and inversely according to HDL-C concentration. Based on these relations, cholesterol ratios such as total cholesterol/HDL-C and LDL-C/HDL-C are considered by some investigators as a simple approach for lipid risk assessment (29-32). The ratio reflects two powerful components of risk and provides a tool to express the balance between the proatherogenic and the antiatherogenic lipoproteins. More recent work however provides growing evidence that apo B and apo A-I are more effective indicators of CHD risk. A number of reports have been published that support apo B/apo A-I as a better overall indicator of risk for vascular disease (11, 33-35). More recently two studies, the AMORIS study

and the INTERHEART study, have reported findings that the apo B/apo A-I ratio is a significantly better predictor of CHD and stroke risk than any of the conventional cholesterol indices (15, 36). As a result many investigators are recommending that the apo B/apo A-I ratio be accepted as an alternative to the total cholesterol/HDL-C ratio for risk assessment (37).

## ANALYTICAL CONSIDERATIONS FOR apo B AND apo A-I MEASUREMENT

In most routine clinical laboratories in 2006, the measurement of apos were made using immunonephelometry or immunoturbidimetry assays. Prior use of radialimmunodiffusion (RID) and electroimmunoassay (EID), two techniques subject to inherent analytical problems, have largely disappeared from laboratories. Acceptance of apo measurements has suffered from a lack of standardization across laboratories (38). Efforts to standardize and improve apo B and apo A-I measurement were initiated in 1981 through a collaborative effort between the CDC and the International Union of Immunological Societies. A survey of international laboratories found that among-laboratory CVs were about 15% for apo A-I and 24% for apo B. An analysis of the results from this survey suggested the major sources of variation resulted from the use of different calibrator materials, lack of assay linearity and parallelism, matrix effects, and inaccuracy of dilutions (39). Further work to standardize apo B and apo A-I measurements were conducted by the Northwest Lipid Research Laboratories (NWLRL) at the University of Washington, Seattle, WA, under the auspices of the IFCC. The main objective of this collaborative effort to standardize apo B and apo A-I measurement was to identify suitable reference materials to be used by assay manufacturers to assign accuracy-based values to their calibrators. As a result of this work common reference mate-

rials for apo B (SP3-07) and apo A-I (SP1-01) were prepared, evaluated, and subsequently accepted by the World Health Organization (WHO) as the WHO/IFCC International Reference Materials for apo B and apo A-I measurement (40,41). SP1-01 had an assigned value of 1.50 g/L using the European Community Bureau of Reference (BCR)-Certified Reference Material (CRM) 393 as the primary reference material and a CDC RIA consensus method (42). An apo B value of 1.22 g/L was assigned to SP3-07 using ultracentrifuged purified LDL as the primary reference material and an immunonephelometric assay at NWLRL (42).

A recent CAP survey for apos reported average CVs of 8.0% for apo B and 9.4% for apoA-I, respectively (43). In a study within the framework of the Dutch project "Calibration 2000", apo measurement was assessed as part of an effort to prepare and characterize commutable frozen human serum pools as secondary reference materials (44). For apo B and apo A-I target value assignment of the reference materials was established using an immunoturbidimetric method on a Hitachi 911 (Roche Diagnostics) with calibration traceable to the WHO/IFCC International Reference Materials for apo B and apo A-I. Fifty-three percent of the laboratories participating in the study used an immunonephelometric method, while 47% used an immunoturbidimetric method. The overall interlaboratory variation found was approximately 7% for apo B and approximately 9% for apo A-I, respectively. The imprecision observed in this study was very similar to the interlaboratory variation reported in the CAP survey.

As with other lipoprotein determinations, apo measurements are influenced by numerous preanalytical factors that must be taken into account when interpreting results (45-47). apo A-I biological variation averages 7% to 8%, while apo B has an average within-day biological variation of 6.5% and a day-to-day variation of 8% to 10% (48). Factors having a potential influence on apo measurement are summarized in Table 6. The NCEP

**Table 6. Preanalytical Factors Affecting Apolipoprotein Measurement (45)**

	Effect on apo B	Effect on apo A-I
Diurnal variation	About 5%. Peaks at mid-day and midnight	About 4%. Peaks in late evening
Seasonal variation (47)	Increase in fall/winter	Increase in spring/summer
Sex	Males > females	Females > males
Age	Increases with age	Some reports indicate fall with age
Menstrual cycle	Lower values or no change	Lower values or no change
Pregnancy	Increase by 60%	Increase by 30%, sustained until delivery
Diet	Acute change: no effect Chronic change: fall	Acute change: no effect Chronic change: increase
Exercise	Not known	Not known
Alcohol	Not known	Higher values
Smoking	Not known	Lower values
Coffee	Probable increase by 15%	No change
Posture	5% fall	9% fall
Venous occlusion	5% increase	5% increase
Storage at -70°C	7% decrease*	No effect*
Storage at 4°C	5% increase*	No effect or increase*

\*Method dependent.

**Table 7. Decision Cutpoints for LDL Cholesterol, Non-HDL Cholesterol, and Total apo B (55)**

Risk Status	Decision Cutpoints, g/L		
	Primary Target: LDL Cholesterol	Secondary Target: Non-HDL Cholesterol	Secondary Target: Total apo B
High risk: CHD or CHD risk equivalents	< 1.00	< 1.30	< 0.90
Moderate risk: $\geq 2$ risk factors	< 1.30	< 1.60	< 1.10
Low risk: 0-1 risk factors	< 1.60	< 1.90	< 1.30

Abbreviations: LDL, low density lipoprotein, HDL, high density lipoprotein; apo, apolipoprotein; CHD, coronary heart disease.

Laboratory Standardization Panel provided recommendations for minimizing the effect of preanalytical factors on lipid and lipoprotein testing but made no specific recommendations concerning apo testing. However, steps similar to those described for lipids and lipoproteins should be taken to help minimize preanalytical sources of variation in apomeasurement.

### Reference Intervals and Cutpoints

Reference intervals for apo B and apo A-I have been published for a variety of regions, including a Finnish population (48), a Swedish population (50), a French Canadian population (51), and for the US population in the Framingham Offspring Study (52, 53) and the National Health and Nutrition Examination Survey (NHANES) (54). Measurements for the Framingham Offspring Study reference interval data and the NHANES national probability estimates were both made with commercial assays that were standardized and traceable to the WHO-IFCC International Reference Materials for apo B and apo A-I. In the Framingham study, Contois et al chose cutpoints for apo B and apo A-I that correlated to the accepted cutpoints for LDL-C and HDL-C, respectively (52, 53). For apo B, they proposed cutpoints of 1.00 g/L and 1.20 g/L, the approximate 50th and 75th percentiles, which correspond to the accepted LDL-C cutpoints 1.30 g/L and 1.60 g/L (53). For apo A-I, a concentration of 1.20 g/L falls at roughly the 25th percentile of the distribution in men and the 5th percentile in women, similar to the percentile distribution for HDL-C at the cutpoint of 0.35 g/L (52). Similarly an HDL-C value of 0.60 g/L, which is considered protective for CHD, corresponds to an apo A-I cutpoint of 1.60 g/L (52). Table 7 summarizes ATP III's cutpoints for LDL-C and non-HDL-C (55) and suggested cutpoints for apo B as proposed by Grundy in 2001 (55). Proposed goals for apo B are also shown, which are derived from the known relationship between non-HDL-C and apo B (55).

## DISCUSSION

Longstanding debate concerns the role apolipoproteins A-I and B (particularly apo B) should play in risk assessment for CHD and stroke in primary prevention (55-59). There has been reluctance to endorse the use of the apolipoproteins because almost all

of the evidence used to support apo use was derived from cross-sectional studies. For apo A-I, conventional opinion is that alone it does not add any additional predictive power above that of HDL-C and thus its inclusion in global risk management and independent measurement is not considered beneficial. However, for apo B support has steadily grown as evidence from prospective studies has accumulated indicating that apo B is a strong predictor of CHD risk (28). The position taken in this document, consistent with the recommendations of the ATP III (60), is that apo B is a good predictor of CHD risk, but only marginally better than the standard lipid profile and thus should not, at this point in time, be routinely measured for use in global risk assessment of CHD and stroke in primary prevention. The incremental benefit of additionally knowing apo B concentrations for risk prediction in primary prevention does not justify the additional cost for apo B measurement. ATP III also felt that measurement, standardization, and availability of apo B was not sufficiently robust. The ATP III considered whether apo B should be measured and included in global risk assessment strategy, but decided in 2001 it was not preferred as a target for therapy and thus did not include its use as part of the ATP III recommendations (60). However as an attempt to capture risk associated with other apo B-containing lipoproteins in addition to LDL-C they introduced the concept of nonHDL-C.

However, while the measurement of apo B for primary risk prediction is not recommended, the results from a growing number of studies do indicate a potential role for apo B as a clinical target for monitoring patient therapy (33-35, 61-63). Cholesterol lowering based on LDL-C continues to be the focus of the Adult Treatment Panel's treatment therapy (60). There is also considerable evidence on the predictive power of non-HDL-C (LDL + IDL + VLDL cholesterol) to predict major coronary events (64-66). As a result, ATP III designated non-HDL-C as a secondary target of therapy for persons who have elevated serum triglyceride levels  $\geq 200$  mg/dL, while for those who have lower triglyceride levels, LDL-C is still considered a sufficient target alone (60). As outlined earlier in ATP III, non-HDL-C was selected as a surrogate measurement for all apo B lipoproteins since in routine clinical practice it is more easily available (subtracting HDL from total cholesterol) since total and HDL-C are already measured, less expensive, and also can be measured in non-fasting patients. Studies indicate that non-HDL-C levels and apo B levels are strongly correlated—apo B being the major apolipoprotein of all atherogenic lipoproteins (24,54). Therefore, in this guideline apo B

is recommended as a possible alternative to non-HDL-C for use to monitor efficacy of lipid-lowering therapies in patients with elevated triglycerides.

Whether LDL-C is someday replaced by other lipoproteins as the primary target for risk assessment and therapy will depend on a clearly demonstrated need and benefit for a major paradigm change in lipid management.

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# Markers of Renal Function and Cardiovascular Disease Risk

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## RECOMMENDATIONS FOR RENAL MARKERS

Based on a thorough review of the published literature, the following are recommendations for the clinical use and measurement of markers of renal function in assessing risk for CHD and stroke in primary prevention.

### *Recommendation 1*

Chronic kidney disease (CKD) testing is not routinely recommended if the 10-year predicted risk is < 5% without specific CKD or CVD risk factors, either for CKD detection or CVD risk assessment.

**Classification of recommendation:** III (against routine measurement)

**Level of evidence:** C

### *Recommendation 2*

CKD testing, including serum creatinine for glomerular filtration rate (GFR) estimation and microalbuminuria, for primary prevention should be performed for all individuals with hypertension, diabetes mellitus, family history of CKD, and those at intermediate risk (10% to 20%) for CVD. In addition, measurement of serum creatinine for GFR estimation should be performed in all individuals > 65 years old. Individual decisions are recommended in those with other CKD risk factors.

**Classification of recommendation:** IIa

**Level of evidence:** B

### *Recommendation 3*

Manufacturers of creatinine assays should comply with the most recent recommendations for standardization and other performance characteristics recommended by the National Kidney Disease Education Program (NKDEP). Calculation of estimated GFR from creatinine values should be consistent with the most recent NKDEP recommendations.

**Classification of recommendation:** I

**Level of evidence:** C

### *Recommendation 4*

Cystatin C may be a more powerful predictor of cardiovascular events than estimated GFR calculation based on creatinine. Research should be conducted to examine if interventions based on cystatin C measurements for risk stratification in individuals with diminished estimated GFR will provide added clinical benefit.

**Classification of recommendation:** IIa

**Level of evidence:** C

### *Recommendation 5*

Properly designed studies focusing on the role of kidney disease markers (microalbumin, creatinine, estimated GFR, and cystatin C) should be conducted to characterize the utility of these markers in the global assessment of CVD risk in the primary prevention setting.

**Classification of recommendation:** I

**Level of evidence:** C

## SUPPORTING EVIDENCE

### Introduction

The results from recent studies reported in the literature have demonstrated that renal function predicts independently cardiovascular mortality and morbidity in high-risk populations such as individuals with CKD or CVD, persons with cardiovascular risk factors, diabetes, and hypertension (1-5). However, the risk for CVD complications associated with less severe degrees of renal impairment within asymptomatic ranges for the general population is unclear. The question of whether a similar association exists for patients with less severely impaired renal function is important because the incidence of renal insufficiency is rapidly increasing (6).

This guideline will address the potential role of renal impairment as a risk factor for CVD and stroke in the general population.

## Clinical Rationale/Evidence

### *Renal Insufficiency As Reduced GFR*

Relatively few studies have reported on the relationship between renal function and the risk of incident cardiovascular events and stroke in the general population. In some studies, renal insufficiency has been shown to be associated with CVD and stroke while in other studies no independent risk prediction was found after adjustment for cardiovascular risk factors.

### *Serum Creatinine*

In clinical practice, the serum creatinine concentration is often used as an index of renal function and is widely interpreted as a measure of GFR (7). In 1926, Rehberg studied the renal clearance of exogenously administered creatinine and thus originated the use of serum creatinine as a marker of GFR (8). Rehberg found that with increasing serum creatinine concentration between 1.2 to 2.5 mg/dL, the cumulative life mortality increased progressively over 96 months of follow-up (8). One of the first published studies to clearly document the relation between the levels of serum creatinine and disease complications was by Shulman in the Hypertension Detection and Follow-Up Program (9). Subsequent studies evaluating the association between cardiovascular event rates and serum creatinine concentration have reported mixed results. A baseline serum creatinine of 1.5 mg/dL or higher compared with creatinine values lower than 1.5 mg/dL was associated with a twofold higher adjusted risk of cardiovascular events in the Hypertension Optimal Treatment trial (10). Wannamethee examined the relationship between serum creatinine and the subsequent risk of major ischemic heart disease and stroke and all-cause mortality in a prospective population-based study of middle-age British men (11). In this study, a baseline creatinine level in the top 3% of the creatinine distribution ( $\geq 1.3$  mg/dL) was associated with a significantly increased risk of major stroke in both normotensive and hypertensive persons (11). However, the relationship between serum creatinine and the risk of stroke appeared to be independent of current blood pressure. No independent association was observed with major ischemic heart disease events (11). The findings in this study were similar to those of the Hypertension Detection and Follow-up Program (9).

In other studies, a significant association between mildly elevated serum creatinine levels and cardiovascular events was not observed. For example, in the Framingham Heart Study serum creatinine values of 1.5 to 3.0 mg/dL in men and 1.4 to 3.0 mg/dL in women were not significantly associated with subsequent cardiovascular events after adjustment for co-morbid conditions such as advanced age and diabetes (12).

Creatinine metabolism is not constant over time or among individuals and as such serum creatinine concentration has limits as a marker of GFR. Serum creatinine alone should not be used to assess GFR or to detect the presence

of renal impairment because it is affected by GFR and by factors independent of GFR, including age, sex, race, body size, diet, certain drugs, and laboratory analytical methods (13,14).

### *Equations to Estimate GFR*

Serum creatinine concentration is not linearly associated with GFR and is an insensitive reflection of glomerular filtration because of influence by confounding factors (13,14). More accurate and precise estimations of GFR can be obtained with serum creatinine-based equations that empirically combine all of the average effects from factors that affect serum creatinine other than GFR (15). Algorithms used to routinely estimate GFR, such as the Cockcroft-Gault formula (16) or the Modification of Diet in Renal Disease (MDRD) equation (17) are an improvement compared to using serum creatinine alone, and has been more widely proposed in practice guidelines (18, 19). The MDRD equation is currently the recommended equation for estimating GFR (19). The National Kidney Foundation published clinical practice guidelines that define CKD as having an estimated GFR lower than 60 ml/min/1.73 m<sup>2</sup> for  $\geq 3$  months or evidence of structural kidney damage as confirmed by markers such as proteinuria (19).

In a recent study from Kaiser Permanente of Northern California, 1,120,295 insured middle-age adults were followed from 1996 to 2000 for longitudinal changes in kidney function using MDRD estimated GFR (20). Subjects with an estimated GFR  $< 60$  mL/min per 1.73m<sup>2</sup> had an increased risk of heart failure, stroke, CHD, and peripheral artery disease (20). The age-adjusted rate of these cardiovascular events increased substantially at an estimated GFR lower than 45 mL/min per 1.73m<sup>2</sup> (20). Manjunath et al evaluated the relationship between the level of kidney function and atherosclerotic CVD in the Atherosclerosis Risk in Communities (ARIC) study (21). GFR was estimated using the MDRD formula. Adjusted hazard ratios for atherosclerotic CVD were markedly higher in subjects with GFR of 15 to 59 mL/min per 1.73m<sup>2</sup> (HR = 1.38) and GFR of 60 to 89 mL/min per 1.73m<sup>2</sup> (HR = 1.16) compared with subjects with GFR of 90 to 150 mL/min per 1.73m<sup>2</sup> (the reference group) (21). When GFR was expressed as a continuous variable, each 10 mL/min per 1.73m<sup>2</sup> lowering of GFR was associated with an adjusted HR of 1.05 (21). Results from the National Health and Nutrition Examination Survey (NHANES) II, a representative sample of the United States general population, indicated that renal insufficiency is independently associated with increased CVD-related and all-cause mortality rates (22). GFRs were estimated using the MDRD equation for the 6,453 NHANES II participants with baseline serum creatinine measurements by adjusting serum creatinine levels for age, race, and sex. Participants with a baseline estimated GFR lower than 70 mL/min per 1.73m<sup>2</sup> (HR = 1.68) had a 51% higher adjusted risk of cardiovascular death compared with those with estimated GFR  $\geq 90$  mL/min per 1.73m<sup>2</sup> (22). In the Hisayama study, researchers reported the findings of a prospective examination of the relationships between CVD

and the incidence of CHD and stroke in a general Japanese population (23). GFR was estimated in 2,634 subjects using the MDRD equation and CKD was defined as a GFR < 60 mL/min per 1.73m<sup>2</sup>. In multivariate analysis, even after adjustments for traditional and nontraditional CVD risk factors, CKD was found to be an independent risk factor for the occurrence of CHD in men (HR, 2.26) and for the occurrence of ischemic stroke in women (HR, 1.91) (23).

The elderly population is increasing, along with a high prevalence of reduced kidney function, so from a public health standpoint it is especially important to evaluate the relationship between kidney insufficiency and CVD in the elderly. In the Cardiovascular Health Study, a cohort of 4,893 subjects with a mean age of 73.4 years and a baseline estimated GFR of 15 to 59 mL/min per 1.73m<sup>2</sup> had an adjusted 38% increased risk of cardiovascular events when compared with subjects that had an estimated GFR of  $\geq$  90 mL/min per 1.73m<sup>2</sup> (24). In a study of 4,484 apparently healthy subjects (mean age, 69.6 years) in the Rotterdam Study impaired renal function was common and associated with an increased risk of myocardial infarction (25). Estimated GFR was estimated by both the Cockcroft-Gault formula and the MDRD equation. During the 8.6 years of follow-up, a 10 mL/min per 1.73m<sup>2</sup> decrease in GFR was associated with a 32% increased risk of myocardial infarction (25). Based on the Cockcroft-Gault formula, the multivariate-adjusted HR for the risk of myocardial infarction was 3.06 in the quartile with the lowest estimated GFR (25). Using the MDRD equation, the risk estimate in the lowest quartile was a hazard ratio of 1.90 (25).

As indicated at the beginning of this section, not all studies found an association of cardiovascular events with renal insufficiency. Results in the NHANES I Epidemiologic Follow-up Study did not support moderate renal insufficiency as an independent risk factor for CVD (26). After adjustment for traditional cardiovascular risk factors, there was no independent association between moderate renal insufficiency, as defined by a serum creatinine of 1.2 to 1.6 mg/dL in women and 1.4 to 2.0 mg/dL in men (approximate GFR of 30 to 60 mL/min per 1.73 m<sup>2</sup>), and cardiovascular mortality (HR, 1.2) (26). Differences in the findings in the NHANES I and Framingham Study (discussed earlier) may be due to the fact that serum creatinine, which was used as the primary measure of renal function in both studies, is known to be less sensitive than estimated GFR in the detection of small differences in the levels of kidney function. As a result of this limitation, an association in low-risk populations may be less detectable when serum creatinine is used alone.

### *Cystatin C*

Although several equations have been developed to improve the accuracy of serum creatinine level as a measure of GFR, difficulty with these equations continues because they are less accurate at higher levels of kidney function and are affected by interlaboratory variation in measuring serum creatinine (27). The difficulties associated with using creatinine-based

equations to estimate GFR has led to a search for other laboratory markers of renal function. Cystatin C has been proposed and investigated as an improved marker of renal function and as a potential alternative to serum creatinine based estimated GFR (28-30). Results of a meta-analysis support serum cystatin C as a promising, easily measured marker for detecting early kidney function impairment (31). Cystatin C was first suggested as a marker of GFR in 1985 by Simonsen and colleagues (32). Cystatin C is a 13 KD cysteine protease inhibitor produced by nearly all cells in the body and excreted into the bloodstream. Cystatin C is freely filtered by the renal glomerulus, reabsorbed, and then metabolized by the proximal tubule (33, 34). Recent studies have shown that cystatin C is more strongly associated with all-cause and cardiovascular mortality as well as with myocardial infarction, stroke, and peripheral arterial disease (35-38).

Serum cystatin C and creatinine levels in a cohort of 4,637 elderly participants (mean age, 75 years) in the Cardiovascular Health Study (median follow-up, 7.4 years) were compared to evaluate their ability to predict mortality from cardiovascular causes and from all causes (35). Low-risk, intermediate-risk, and high-risk levels were defined corresponding to cystatin C levels of < 1.00 mg/L, 1.00 to 1.28 mg/L, and  $\geq$  1.29 mg/L, respectively, with respect to death from all causes and from cardiovascular causes (35). The high-risk subgroup was associated with a significantly elevated risk of death from cardiovascular causes (HR, 2.27), myocardial infarction (HR, 1.48), and stroke (HR, 1.47) after multivariate adjustment (35). In contrast, creatinine and estimated GFR subgroups had no significant association with the risk of death from cardiovascular causes, myocardial infarction, or stroke (35). Thus, the findings from this evaluation appear to indicate that cystatin C provides a stronger estimate of the risk of cardiovascular events and death among elderly persons than either serum creatinine alone or estimated GFR.

Peripheral arterial disease (PAD) is common in the elderly population and a growing number of studies indicate that renal insufficiency is associated with prevalent and incident PAD (39,40). An evaluation of 4,025 participants in the Cardiovascular Health Study who did not have PAD at baseline found that elevated concentrations of cystatin C were independently predictive of incident PAD in the elderly (36). The risk of a PAD event at the highest quintile (>1.27 mg/L) of cystatin C was HR of 2.5 after multivariate adjustment for known risk factors for PAD (36). The association of quintiles of serum creatinine level and estimated GFR with PAD events was not statistically significant.

Sarnak and coworkers compared serum concentrations of cystatin C and creatinine as predictors of incident heart failure for 4,384 participants without previous heart failure (mean age, 75 years) in the Cardiovascular Health Study cohort (37). The association of kidney function and risk for heart failure was compared by using serum cystatin C and creatinine concentrations and GFR estimated by the MDRD equation. After adjustment for demographic factors, traditional and novel factors, CVD status, and medication use, cystatin C was associated

with an increased risk for incident heart failure (> 1.26 mg/L; HR, 2.16) while serum creatinine and MDRD estimated GFR were not associated with risk for heart failure (37). When cystatin C was measured as a continuous variable, it exhibited a significant interaction with ethnicity in terms of predicting heart failure thus prompting Sarnak et al to perform a further adjusted stratified analysis based on ethnicity (37). Results from this additional analysis found that the association of cystatin C concentration with heart failure appeared to be slightly stronger among African-American participants than white participants (HR by quintiles, 1.0 [reference], 1.32 [95% CI, 0.70 to 2.48], 2.16 [95% CI, 1.14 to 4.12], 1.95 [95% CI, 0.96 to 3.95], and 2.08 [95% CI, 1.05 to 4.12] versus 1.0 [reference], 1.26 [95% CI, 0.88 to 1.80], 1.28 [95% CI, 0.90 to 1.82] 1.37 [95% CI, 0.97 to 1.93], and 1.75 [95% CI, 1.23 to 2.49]) (37).

The Prospective Epidemiological Study of Myocardial Infarction (PRIME) study examined the association between cystatin C levels and the incidence of CHD, as defined by non-fatal MI or coronary death and angina (38). After adjustment for traditional risk factors, cystatin C was found to be significantly associated with the occurrence of a first ischemic coronary event (38). However, when hsCRP was included in the model, cystatin C was no longer significantly ( $P = .15$ ) associated with a future event (38). hsCRP remained highly significant ( $P = .003$ ) and replacing IL-6 in the model gave similar results (38).

**Microalbuminuria**

Proteinuria is an abnormally high amount of proteins, such as albumin, in the urine. This results when the glomeruli are damaged and proteins of various sizes pass through them and are excreted in the urine. Excretion of albumin in the urine varies considerably, ranging from minute quantities to even grams of albumin. Microalbuminuria refers to a range of urinary albumin excretion that is above normal levels but below amounts referred to as macroalbuminuria or proteinuria, which indicates overt kidney damage. The term microalbuminuria is confusing, because it does not reflect small albumin molecules but rather little more than normal quantities of the molecule. To illustrate the confusion, Table 8 shows the definition of microalbuminuria based on the test used.

Microalbuminuria is highly prevalent in several disease states. Recent data from large population-based studies (42-47) and surveys (48) show that the prevalence of microalbuminuria is 20% to 30% in diabetes, 11% to 17% in hypertension, 3%

to 8% in persons without diabetes or hypertension, and 5% to 15% in the general population. It has been known for some time now that microalbuminuria increases the risk of cardiovascular events, cardiovascular deaths, and stroke in patients with diabetes (49, 50) and hypertension (51, 52).

Although the role microalbuminuria plays in the general population is less well known, the last few years have seen a marked increase in the evidence relating microalbuminuria to all-cause and cardiovascular mortality and stroke in the general population. In the large population-based PREVENTD (Prevention of Renal and Vascular End Stage Disease) study, Hillege et al examined the relationship between urinary albumin excretion and all-cause and cardiovascular mortality in more than 40,000 inhabitants of the city of Groningen, the Netherlands (53). Urinary albumin excretion was determined in a morning urine sample measured as urinary albumin concentration (UAC). Microalbuminuria was defined in this study as a UAC between 20 and 200 mg/L. The crude incident rate for cardiovascular death from microalbuminuria was 4.7 (95% CI, 3.2 to 6.6) (53). When mutually adjusted for other cardiovascular risk factors, a two-fold increase in UAC (ie, from 20 to 40 mg/L or 40 to 80 mg/L) was associated with a RR of 1.29 (95% CI, 1.18 to 1.40) for cardiovascular mortality (53). In the Third Copenhagen City Heart Study of 2,762 men and women, the lower cutoff level of microalbuminuria which is associated with increased risk of CHD and death was assessed (54). An increased risk of CHD (RR, 2.0; 95% CI, 1.4 to 3.0) and death (RR, 1.9; 95% CI, 1.5 to 2.4) was associated with a urinary albumin excretion above the upper quartile (ie, 4.8 µg/min) (54). The association was mainly unaffected by adjustment for age, creatinine clearance, hypertension, diabetes, and lipids (54). Klausen et al recommended that the definition of microalbuminuria be revised to the level that increases the risk of CHD and death in the general population rather than the traditional definition which is based on the level of urinary albumin excretion in diabetics that predicts the development of clinical diabetic nephropathy (54). In a 4.4-year mortality follow-up of 2,089 apparently healthy men and women without diabetes and treated hypertension selected from the population-based Nord-Trøndelag Health Study, a positive association was found between all-cause mortality and microalbuminuria (55). The lowest urinary albumin-creatinine ratio (UACR) level associated with increased risk for death was the 60th percentile ( $\geq 6.7 \mu\text{g}/\text{mg}; 0.76 \text{ mg}/\text{mmol}$ ; RR, 2.4; 95% CI, 1.1 to 5.2) (55). In a publication from the EPIC-Norfolk prospective cohort study, the relationship between

**Table 8. Definitions of Microalbuminuria (41)**

	Urinary Albumin Excretion Rate		Urinary Albumin-Creatinine Ratio	
	Urinary albumin excretion	Urinary albumin excretion rate	Urinary albumin concentration	Urinary albumin-to-creatinine ratio
Specimen definition	24-hour urine collection 30 to 300 mg/24 hours	Timed urine collection 20 to 200 µg/min	Spot urine collection 20 to 200 mg/L	Spot urine collection 2.5 to 25 mg/mmol in men or 3.5 to 25 mg/mmol in women

microalbuminuria and incident CHD was investigated in 22,368 men and women without prevalent baseline CHD (56). Microalbuminuria was defined as an UACR of 2.5 to 25 mg/mmol. The prevalence of microalbuminuria in the total study population was 11.5% (56). There was an approximate 40% increased RR of incident primary CHD for microalbuminuria (HR, 1.36; CI, 1.12 to 1.64) (56).

An association between microalbuminuria and cerebrovascular disease has been suggested based on results from previous cross-sectional studies (57, 58). It is only recently that evidence of the association between microalbuminuria and cerebrovascular disease has been examined in a large population-based prospective study. Yuyun and colleagues examined the relationship between microalbuminuria and incident stroke in 23,630 individuals age 40 to 79 years recruited for the EPIC-Norfolk Study (mean follow-up, 7.2 years) (59). Microalbuminuria was defined as an UACR of 2.5 to 25 mg/mmol. Stroke endpoints were subdivided into stroke subtypes (ischemic, hemorrhagic, and unspecified). Microalbuminuria was found to be independently associated with an approximate 50% increased risk of stroke (59). The multivariate adjusted HR for stroke associated with microalbuminuria in all men and women was 1.49 (95% CI, 1.13 to 2.14) (59). After stratifying by stroke subtype, microalbuminuria was only independently predictive of ischemic stroke, HR 2.01 (95% CI, 1.29 to 3.31) (59).

## Analytical Considerations for Measurement of Renal Markers

### *Serum Creatinine*

Serum creatinine measurement is a key component to reliable estimation of impaired renal function. The NKDEP Laboratory Working Group, in collaboration with international professional organizations, has developed a plan with recommendations that will enable the standardization and improvement of serum creatinine measurements (27). The reader is also referred to the NKDEP web site for continuous updated information for laboratory professionals and clinicians ([www.NKDEP.NIH.gov](http://www.NKDEP.NIH.gov)).

### *Cystatin C*

The first immunoassay to quantify cystatin C, an enzyme amplified single radial immunodiffusion method, was first described by Lofberg and Grubb in 1979 (60). Today cystatin C is measured by automated homogeneous immunoassays utilizing latex or polystyrene particles coated with cystatin C-specific antibodies. Particle-enhanced turbidimetric immunoassays (PETIA) (61) and particle-enhanced nephelometric immunoassays (PENIA) (62) are two versions of the fully automated assays currently available. In general, these assays are more precise, rapid, and convenient for routine use than earlier methods. The CV for the PETIA assays is 2% to 8% and for the PENIA assays 3% to 6% (63). The Dade Behring Nephelometric II system is currently the only clinical method approved by the FDA for routine use. The College of American

Pathologists started a proficiency testing survey for cystatin C in 2006 and CVs seen are in the 6% to 9% range (64). Cystatin C exhibits good stability in serum and can be stored up to at least 7 days at room temperature, in a refrigerator, or in a freezer (-20° C) and for at least 6 months when stored at -80° C (65). There is not much data available concerning biological variation of cystatin C. Keevil et al found within subject variation of 13.3% and between subject variance of 8.1% (66). Uhlmann et al (67) and Finney et al (68) both studies using the Dade Behring BNII system, found comparable reference intervals for cystatin C of 0.51 to 0.92 mg/L and 0.51 to 0.98 mg/L, respectively,

### *Microalbuminuria*

Urinary excretion of albumin is traditionally determined by immunochemical methods, such as immunonephelometry, immunoturbidimetry, and radioimmunoassay. Unfortunately these laboratory methods have shown considerable variation when evaluated in comparison studies (69). Another limitation of these immunochemical methods is they can only determine albumin which is immunoreactive. Researchers have recently discovered that albumin is also excreted in the urine as immunochemically nonreactive intact albumin (70). Thus by not measuring all of the intact albumin in the urine, the potential for reporting false-negative levels for microalbuminuria exists. Size-exclusion high-performance liquid chromatography (HPLC) methods are capable of measuring both immunoreactive and immunounreactive intact urinary albumin substantially decreasing the potential for misdetection and mismeasurement of microalbuminuria (69). Studies using HPLC found that this technique may be more sensitive in its ability as an indicator of CVD risk because it picks up microalbuminuria in earlier stages of the disease (71,72).

In the point-of-care setting, various semiquantitative dipstick tests, which involve wetting a chemically impregnated test with a urine sample, are used to detect microalbuminuria. A positive result for microalbuminuria can be confirmed and quantified by one of the laboratory methods described earlier.

Specimen storage can have an impact on the reliability of albumin determination. Some study results have indicated no effect of freezing on urinary albumin concentration (73) while other studies have found erroneously low values when samples were frozen at -20°C (74). In a recent study, Brinkman, et al reported that urine samples can be stored at -20° C for 5 months without great changes in mean albumin concentration when samples are mixed adequately after thawing (75).

## DISCUSSION

In general, the results from studies investigating the association of renal insufficiency and the risk for incident CVD events and stroke support a relationship between impaired renal function and risk for CVD and stroke in the general population. It is unclear whether these associations reflect a causal association or whether impaired renal function is a marker for underlying

CVD. The question then is why would kidney dysfunction lead to a higher risk of CVD morbidity and mortality? The exact causes have not been fully delineated but multiple mechanisms that are possible have been proposed (76). Although the current recommended approach to evaluate impaired renal function is estimated GFR based on serum creatinine, cystatin C shows great promise as a potential alternative to this approach. However, cystatin C has not yet been fully and adequately evaluated as an index of GFR nor how it relates to cardiovascular risk.

For microalbuminuria, results from population-based studies indicate that it is an independent and significant predictor of CVD events and all-cause mortality in the general population. Although the pathophysiological mechanism of the association between albuminuria and CVD and stroke remains unclear, there are several hypotheses (77,78). There is also evidence that the conventional cutpoints for defining microalbuminuria based on the risk of nephropathy in patients with diabetes need to be revised because there is growing evidence showing that urinary albumin levels below the microalbuminuria cutpoint predict cardiovascular events whether in diabetes, hypertension, nondiabetes, nonhypertension, or in the general population (54). However, until adequate evidence is available, the definition of microalbuminuria should remain unchanged. In the future, microalbuminuria may become a modifiable CVD/renal disease risk factor similar to cholesterol that will support its testing in the general population. However, this will depend on the availability of more supporting data from prospective, randomized intervention trials targeting microalbuminuria for cardiovascular protection.

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# Homocysteine and Cardiovascular Disease Risk

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## RECOMMENDATIONS FOR HOMOCYSTEINE

The clinical application of Hcy measurement for risk assessment of primary prevention of CVD is uncertain given that several trials investigating folic acid and B vitamin supplementation published after our literature review indicated no benefit or lowering of CVD risk. Based on a thorough review of the published literature, the following are recommendations for the clinical use and measurement of Hcy in assessing risk for CHD and stroke.

### **Recommendation 1**

Hcy concentrations ( $\mu\text{mol/L}$ ) derived from standardized assays categorize patients as follows:

- Desirable  $\leq 10$
- Intermediate (low to high)  $> 10$  to  $< 15$
- High  $\geq 15$  to  $< 30$
- Very high  $\geq 30$

**Classification of recommendation:** IIa

**Level of evidence** C

### **Recommendation 2**

The analytical performance goal for clinical usefulness for measurement of Hcy should be  $< 10\%$  for bias,  $< 5\%$  for precision, and  $< 18\%$  for total error. Manufacturers of diagnostic assays for Hcy should follow approved value transfer protocols to assure that standardized assays are used for vascular risk assessment.

**Classification of recommendation:** IIa

**Level of evidence,** C

contains average concentrations of 1% free Hcy thiol, 5% to 10% homocystine disulfide, 5% to 10% Hcy cysteine, and 80% to 90% Hcy protein. All compounds of Hcy must be hydrolyzed and reduced to Hcy before analytical measurement. Hcy is involved in transmethylation mechanisms in protein metabolism and varies in concentration with serum levels of B vitamins, folate, B12, riboflavin, and pyridoxine. Elevated urine concentrations of Hcy are an indicator of homocystinuria.

Hcy gained attention as a potential risk factor for CHD and stroke between 1995 and 2000, mainly because of early reports from observational studies showing strong associations between Hcy concentrations and CVD risk. Between 2000 and 2008, however, prospective population studies and B-vitamin intervention trials have suggested a weaker or even tenuous relationship. Currently Hcy is regarded as a modest independent CVD risk factor and Hcy screening for primary prevention and assessment of CVD risk in healthy individuals is not warranted.

## CLINICAL RATIONALE/EVIDENCE

In 1995, Boushey et al conducted a meta-analysis of early cross-sectional and retrospective case-control studies to determine the risk for CHD and stroke associated with elevated concentrations of total Hcy (1). The authors found an OR of 1.6 (95% CI, 1.4 to 1.7) for men and 1.8 (95% CI, 1.3 to 1.9) for women for a  $5\text{-}\mu\text{mol/L}$  Hcy increment, suggesting that about 10% of the population's CHD risk appears attributable to Hcy. This stimulated clinical investigations of Hcy as a risk factor for CHD and its use in clinical patient care. Over the following 10 years, results from several meta-analyses and most prospective cohort studies provided evidence that Hcy is at most a weak risk factor for CHD, that findings are probably confounded by lifestyle risk factors, and that Hcy is much less strongly related to CHD and stroke than what was reported in earlier publications from 1995 to 1999.

## SUPPORTING EVIDENCE

### Introduction

Hcy is a sulfur-containing amino acid formed by demethylation of the essential amino acid methionine that occurs naturally in small concentrations in blood. In healthy individuals, blood

### Meta-Analyses and Review Articles of Hcy as a Risk Factor for CVD

Meta-analyses published from 2000 and 2002 predominantly concluded that there was significant heterogeneity between studies, that elevated Hcy was at most a modest independent

predictor of CHD and stroke risk in healthy populations, and that Hcy may be an indicator of an unhealthy lifestyle (2-4).

Cleophas et al conducted a meta-analysis on the risk of CAD associated with elevated plasma Hcy concentrations using 22 previously published case-control and 11 cohort studies (2). Five of 11 cohort studies could not demonstrate a significant association between elevated Hcy concentrations and CAD and the authors concluded that Hcy may indeed not be as harmful for the heart as previously believed. At the same time, however, Hcy may be an indicator for unhealthy lifestyles, and therefore, an important variable for cardiologists to take into account when assessing patients for CAD.

A meta-analysis by the Homocysteine Studies Collaboration determined in multivariate analysis of 12 prospective studies that a 25% lower than usual Hcy level was associated with an OR of 0.89 (95% CI, 0.83 to 0.96) for ischemic heart disease risk and 0.81 (95% CI, 0.69 to 0.95) for stroke risk (3). This meta-analysis also suggested that elevated Hcy is at most a modest independent predictor of risk in healthy populations, in this case for ischemic heart disease and stroke.

Bautista et al conducted a meta-analysis of 14 prospective cohort studies assessing the risk of CVD and Hcy (4). Using a fixed and random effects model, they calculated the average RR to be 1.49 (95% CI, 1.31 to 1.70) for cardiac events and 1.37 (95% CI, 0.99 to 1.99) for ischemic stroke. They concluded that hyperhomocysteinemia moderately increases the risk for a first cardiovascular event, regardless of age and follow-up duration.

Mixed results have been reported from meta-analyses assessing the risk of the MTHFR 677C/T polymorphism and CHD and ischemic stroke, possibly due to an interaction between the MTHFR 677C/T polymorphism and folate status (5-10). Wald et al conducted a meta-analysis of 72 studies in which the prevalence of a mutation in the *MTHFR* gene was determined in cases and controls, and 20 prospective studies of serum Hcy and CVD risk (5). The authors concluded that both the genetic and the prospective studies yielded similar evidence that the association between Hcy and CVD is causal. Klerk et al conducted a meta-analysis of 40 case-control studies from Europe, North America, and other continents with data on the MTHFR 677C/T polymorphism and CHD risk (6). They found that individuals with MTHFR T/T genotype had a 16% (OR 1.16; 95% CI, 1.05 to 1.28) higher risk for CHD, particularly in the setting of low folate status. Regional effects were also found by Lewis et al in a meta-analysis of case-control and prospective studies on the association of MTHFR polymorphism and myocardial infarction or coronary artery occlusion (OR 1.14; 95% CI, 1.05 to 1.24; for T/T versus C/C genotype) (7) and by den Heijer et al on the risk of venous thrombosis (8). The 677T/T genotype had no effect in North America, due probably to the higher intake of folate and riboflavin, but resulted in a 15% increased risk (OR 1.15; 95% CI, 1.02 to 1.30) in Europe and a 60% increased risk (OR 1.60; 95% CI, 1.27 to 2.02) in other countries (8). The effect of MTHFR T/T genotype on ischemic stroke was also studied in meta-analyses. Kelly et al determined that this genotype may have a small

influence in determining susceptibility to ischemic stroke (9). Cronin et al found a graded increase in ischemic stroke risk with increasing MTHFR 677T, consistent with the view that the polymorphism is a genetic risk factor and supports a causal relationship between elevated Hcy and stroke (10).

Most review articles agree that Hcy is regarded as a risk factor for CVD; however, it remains unclear as to whether hyperhomocysteinemia is a direct cause of CVD (11-21). They point to the randomized folate intervention trials to address this question. Moat et al provide a useful review of the various types of studies: case-control and prospective studies, and various types of clinical trials (17).

### Randomized Controlled Trials to Assess the Benefit of Hcy-Lowering Therapies on Cardiovascular Events

It has been shown extensively that dietary supplementation with B vitamins lowers plasma Hcy concentrations. In a meta-analysis of 25 randomized controlled trials, the Homocysteine Lowering Trialists' Collaboration assessed the effect of different doses of folic acid on reduction of plasma total Hcy concentrations in approximately 2,500 subjects and found that daily doses of 200 µg are associated with 60% of the maximal reduction achieved with doses of 800 µg and more (22). Several large-scale trials of B-vitamin supplementation in people with prior CHD, stroke, or renal disease were designed to test whether Hcy lowering also results in a lower risk of CVD (23). Some of these trials have been completed and results have been published and were included in our literature review: the Cambridge Heart Antioxidant Study (CHAOS2) (24), the Vitamin Intervention for Stroke Prevention (VISP) trial (25-26), the Heart Outcomes Prevention Evaluation (HOPE2) study (27), and the Norwegian Vitamin (NORVIT) trial (28). Generally, there was no reduction in vascular outcomes or clinical endpoints, even though most trials showed at least a moderate reduction in Hcy concentrations. Some limitations might have been the short trial durations, the fact that some trials were underpowered, and that Hcy concentrations might have been too low to start with.

A few important meta-analyses of randomized controlled trials have emerged since the completion of our literature review (29-31). To evaluate the effects of folic acid supplementation on risk of CVD and all-cause mortality among persons with pre-existing CVD or renal disease, Bazzano et al conducted a meta-analysis of 12 randomized controlled trials (29). They reported overall RRs of outcomes for patients treated with folic acid supplementation compared with controls of 0.95 for CVD (95% CI, 0.88 to 1.03), 1.04 for CHD (95% CI, 0.92 to 1.17), 0.86 for stroke (95% CI, 0.71 to 1.04), and 0.96 for all-cause mortality (95% CI, 0.88 to 1.04). They concluded that folic acid supplementation has not been shown to reduce risk of CVD or all-cause mortality among participants with prior history of vascular disease, however, data from several ongoing trials with larger sample sizes might have to be included in a future meta-analysis to provide a definitive answer to this important clinical and public health question.

Bleys et al studied the effect of vitamin-mineral supplementation on atherosclerosis progression in a meta-analysis of randomized controlled trials (30). They found no evidence of a protective effect of antioxidant or B-vitamin supplements on the progression of atherosclerosis, possibly providing a mechanistic explanation for their lack of effect on clinical cardiovascular events.

Wang et al conducted a meta-analysis of eight randomized trials of folic acid that had stroke reported as one of the endpoints (31). People who regularly took a supplement of folic acid reduced their RR of stroke by an average of 18%. An even greater risk reduction of 30% was seen when the treatment lasted more than 36 months. If the individual had no past history of stroke, folic acid supplementation reduced their risk by 25%.

This meta-analysis might have led to some cautious optimism that B vitamins may at least protect against stroke. However, results from recent randomized controlled trials do not seem to support that (32-34). The Women's Antioxidant and Folic Acid Cardiovascular Study (WAFACS) by Albert et al found no benefit for B-vitamin supplements on CHD or stroke in high-risk women with and without CVD, even though follow-up was extended for 7.3 years (32). The Western Norway B Vitamin Intervention Trial (WENBIT) by Ebbing et al was terminated early and had only a 38 months follow-up, however, the authors did not find an effect of treatment with B-vitamin supplements on total mortality or cardiovascular events even though Hcy concentrations were lowered by 30% after 1 year of treatment (33). And finally, first results from the Study of the Effectiveness of Additional Reductions in Cholesterol and Homocysteine (SEARCH) were reported at the American Heart Association 2008 Scientific Sessions—a null effect of the Hcy lowering arm.

### **Effects of Folic Acid Fortification on Hcy Concentrations and Cardiovascular Events**

After the introduction of folic acid fortification in 1998, Hcy concentrations have decreased by approximately 10% in the US population, as monitored through the National Health and Nutrition Examination Survey (NHANES) (34). It remains to be seen whether lowering Hcy concentrations in the US population will have any effects on CVD primary and/or secondary prevention. Yang et al reported recently on what might be a first effect of fortification on stroke mortality the ongoing decline in stroke mortality observed in the US between 1990 and 1997 accelerated in 1998 to 2002 in nearly all population strata (35). Similar effects were seen in Canada, while the decline in stroke mortality in England and Wales did not change significantly between 1990 and 2002 (35).

### **Relationship Between Elevated Hcy and Other CVD Risk Factors**

A report from the Nurses' Health Study found that among women none of the lipid biomarkers were significantly related to Hcy concentrations, but extreme concentration quartiles of

Hcy indicated a positive association between Hcy and CHD risk when lipids and CRP were incorporated into the model (36). In a German and a Syrian population, the CHD risk increased markedly in subjects with elevated concentrations of Hcy and CRP or with elevated concentrations of Hcy and LDL-C (37). The Kuopio Ischemic Heart Disease Risk Factor Study concluded that high Hcy concentrations may increase the risk of CVD mortality in middle-age men and especially increase the risk when present with other CVD risk factors (38-39). An electron-beam computed tomography study found the presence of elevated Hcy strongly and independently predicted progression of coronary plaque burden (40). The ATTICA study evaluated the association between lifestyle-related CHD risk factors and Hcy concentrations (41). The authors reported that Hcy concentrations were higher in men than in women, and were higher in smokers and people with sedentary physical activity, but they found no associations between Hcy concentrations and most foods (41). Data from two independent German study populations showed that the combination of a high intake of whole-grain bread, fresh fruit, olive oil, mushrooms, cruciferous vegetables, wine, and nuts with a low intake of fried potatoes was associated with a favorable biomarker profile of Hcy metabolism and reduced risk of CHD (42). A recent report from the Hordaland Homocysteine Study on the association of dietary fat intake and plasma Hcy concentrations showed that Hcy appears to be a good marker of "adherence to dietary guidelines"; high intakes of saturated fatty acids were associated with high plasma Hcy concentrations; an inverse association between dietary intakes of very long chain n-3 fatty acids and plasma Hcy concentrations was apparent at high B-vitamin intakes only (43). Finally, data from the 10-year follow-up of the Women's Health Study showed that the initial association of Hcy concentrations with CVD was almost completely attenuated after adjustment for established CVD risk factors, including socioeconomic status (44).

### **Vascular Diseases Related to CHD (peripheral arterial disease, hypertension, and retinal occlusion disease)**

Studies on cardiovascular-related diseases have predominantly observed that elevated Hcy concentrations are significantly associated with many other vascular diseases. The Physicians' Health Study found that the RR for PAD by Hcy quartiles did not show any discernible risk gradient (45). No evidence was found to warrant Hcy screening for PAD in the population (45). The ATTICA study found that systolic and diastolic blood pressures were positively correlated with Hcy serum concentrations and that a positive association was found between prehypertension status and hypercholesterolemia (46). The Australian and Singapore Centers for Eye Research found a positive association of elevated Hcy with retinal vein occlusion vascular disease in the general population and reported that the association between elevated Hcy retinal vein occlusion vascular disease was independent of other risk factors (47). These findings are in agreement with an earlier meta-analysis of case-control studies (48).

## ANALYTICAL CONSIDERATIONS

In 2004, a group of European clinical laboratory and scientific experts on Hcy, methylmalonic acid, and B vitamins published an expert opinion on facts and recommendations about total Hcy determinations (49). The authors wrote that there is a need for standardization of Hcy assays, mainly because of the lack of certified reference materials at the time, but also because different types of calibrator materials often yield different values, calibrators in water often have greater imprecision than plasma-based calibrators, and inclusion of an internal standard does not always improve performance. They covered a variety of topics, such as methods and their performance, sample collection and handling, biological determinants, reference intervals, within-person variability, and the methionine loading test. They also discussed facts and recommendations for the use of Hcy in diagnosis and risk assessment. For each topic, the authors provided separate recommendations for the routine clinical setting and for the research setting. Some important recommendations for the routine clinical setting relative to Hcy determinations were:

- Inaccuracy (bias) should preferably be  $< 10\%$ , and imprecision (CV) preferably  $< 5\%$ .
- The analytical range should cover the 0.5th to 99.5th percentiles in the general population (approximately 3 to 40  $\mu\text{mol/L}$ ).
- Because the accuracy of Hcy measurements differs among methods and laboratories, caution should be used in comparing values obtained in different laboratories.
- Participation in an external quality-control program is strongly recommended.
- One type of collection tube should be recommended. EDTA tubes are most widely used, but use of serum or citrated or heparinized rather than EDTA plasma will not materially influence the results.
- Blood samples should be centrifuged within 1 h or kept cold until centrifugation ( $< 8$  hours).
- Each laboratory should establish reference limits for its region.
- Separate reference limits for children, adults, the elderly, and pregnant women should be used.
- A single Hcy measurement usually reflects the mean Hcy concentration and is adequate in most settings.
- A Hcy change  $> 25\%$  to  $30\%$  between samples collected on two occasions is likely to be significant.
- The methionine loading test is not recommended since it is cumbersome and its clinical value is uncertain.

A variety of Hcy methods are used in clinical and research laboratories (50). HPLC with fluorimetric detection is the most widely used chromatographic procedure and is characterized by low assay variability (approximately 1% within-assay and 3% among-assay variability). The high clinical interest during the 1990s stimulated the development of rapid automated immunoassays. The fluorescence polarization immunoassay (FPIA) appears to be the method of choice for clinical laboratories, in part because of its low assay variability ( $< 5\%$  CV).

The French Society for Clinical Biology Working Group on Homocysteine (SFBC) has recommended the following Hcy reference system (50):

- Candidate definitive method: liquid chromatography/mass spectrometry with isotope dilution, using deuterated homocysteine as the internal standard.
- Reference method: gas chromatography/mass spectrometry with isotope dilution.
- Field methods: HPLC, capillary electrophoresis, immunoassays (including FPIA, chemiluminescence immunoassay, and enzyme-linked immunoassay).

In fall 2005, the National Institute of Standards & Technology (NIST) issued a certificate for the first matrix-based reference material (standard reference material 1955) with certified values for Hcy in frozen human serum (51). This is a three-level material with total Hcy values (means and uncertainties) of  $3.98 \pm 0.18$ ,  $8.85 \pm 0.60$ , and  $17.7 \pm 1.1 \mu\text{mol/L}$ . Within a limited concentration range (normal to borderline high), this material was found to be commutable with selected Hcy immunoassays and enzymatic assays (52).

## DISCUSSION

The strong positive association of Hcy with CHD and stroke found in early case-control studies does not agree with the less impressive results of more recent nested case-control studies (14). Selection bias, publication bias, and reverse causality bias are three of several possible reasons for the discrepant results of case-control and nested case-control studies (14). Cohort and genetic polymorphism studies show a quantitatively similar association between decreased Hcy concentrations and risk of heart disease and stroke, but there is heterogeneity between the results from different studies, possibly due to differences in folate and B-vitamin status among populations (21). Among the genetic polymorphism studies, those with the greatest difference in Hcy concentrations between the MTHFR T/T and C/C homozygotes showed the greatest difference in CVD risk (21). A number of randomized controlled trials of the effect of reducing Hcy concentrations on CHD and stroke are still underway, but the ones that have been completed showed no or very modest effects. Since only modest reductions are expected and the number of adverse events recorded is relatively small, most trials lack statistical power, and it has been suggested that a meta-analysis of all trials will be necessary to come to a conclusion (23). Since short trial duration is another point of criticism, extending the duration of treatment in these trials would allow any effects associated with prolonged differences in Hcy concentrations to emerge (23).

During the late 1990s, different heart associations in the US, Canada, and Europe all agreed that the evidence was not strong enough to recommend routine population screening for elevated Hcy concentrations (53-57). While a vast amount of data was created during the last 10 years, there is still no convincing evidence to recommend routine Hcy measurements for primary population screening. The 2004 European Expert

Opinion recommendations for the use of Hcy in CVD risk assessment echo this sentiment, but recommend using Hcy concentrations as a prognostic factor for CVD events and mortality in patients with CVD or persons with high risk of CVD events (49). In a 2006 report from the series of the Atherosclerosis Risk in Communities (ARIC) study, Folsom et al evaluated the association of 19 novel risk markers with incident CHD in almost 16,000 adults followed since 1987 to 1989 (58). Most of the novel risk markers, including Hcy, did not add significantly to the area under the ROC curve generated by traditional risk factors. Therefore, we conclude that the clinical application of Hcy measurement for risk assessment of primary prevention of CVD is currently uncertain.

The classification of plasma Hcy concentrations has slightly shifted over time. Traditionally, plasma Hcy concentrations above 15  $\mu\text{mol/L}$  were considered elevated: 15 to 30  $\mu\text{mol/L}$  was classified as mild, 31 to 100  $\mu\text{mol/L}$  was classified as intermediate, and  $>100$   $\mu\text{mol/L}$  was classified as severe hyperhomocysteinemia (59). More recently, Hcy concentrations between 12 to 30  $\mu\text{mol/L}$  were considered to be moderate hyperhomocysteinemia, values between 10 to 12  $\mu\text{mol/L}$  were considered to be tolerable, and values  $<10$   $\mu\text{mol/L}$  were considered to be safe (15). While the upper reference limit may be 15 to 20  $\mu\text{mol/L}$  or even higher in adults who do not eat food fortified with folic acid, in adults with good vitamin status or a healthy lifestyle, the upper reference limit is approximately 12  $\mu\text{mol/L}$  (50). Using a mathematical model to calculate the expected distribution of Hcy in a population sample supplemented with daily vitamins, Ubbink predicted that Hcy has a normal frequency distribution with a 95% reference interval of 4.9 to 11  $\mu\text{mol/L}$  (60). The studies he reviewed indicated that the lowest risk to CVD was found in people with Hcy concentrations below the 25th percentile, equal to 9  $\mu\text{mol/L}$ . Ubbink therefore recommended  $<9$   $\mu\text{mol/L}$  as a desirable Hcy concentration. As a result of these considerations, our group recommended a practical level of  $<10$   $\mu\text{mol/L}$  as a desirable Hcy concentration.

As pointed out by the European Expert Opinion article, there is a need for standardization of Hcy assays (50). This becomes even more relevant as new clinical assays for Hcy are added to the test menu. A multi-level serum-based certified reference material is now available and is also generally commutable for selected immunoassays and enzymatic assays. Manufacturers should use this material to make their assays traceable to high-order reference methods. Furthermore, analytical performance goals based on biological variation should be followed to ensure clinical usefulness of Hcy assays. In addition to requirements for bias ( $<10\%$ ) and precision ( $<5\%$ ), total error is becoming more widely used in the interpretation of analytical results in clinical laboratories as well as in research investigations and clinical trials. The total error recommended by Westgard should be  $<18\%$  (61, 62). These measures in addition to maintaining a continuous quality control and assurance system in the laboratory and to participating in external quality assurance programs will help to generate high-quality Hcy data for clinical and research needs.

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# Natriuretic Peptides (BNP and NT-proBNP) and Cardiovascular Disease Risk

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## RECOMMENDATIONS FOR BNP AND NTproBNP

Based on a thorough review of the published literature, the following are recommendations for the clinical use and measurement of BNP and NTproBNP in assessing risk for CHD and stroke in primary prevention.

### *Recommendation 1*

Increased B-type natriuretic peptide (BNP) or N-terminal proBNP (NT-proBNP) concentrations are associated with increased mortality in the next 2 to 7 years in community-based populations. However, the benefits of therapy based on these measurements are uncertain. Measurement for CVD risk assessment in the primary prevention setting is unwarranted.

**Classification of recommendation:** III (against measurement);

**Level of evidence:** B.

### *Recommendation 2*

More research should be performed to determine if BNP and NT-proBNP measurements are useful in identifying individuals who are at increased risk of developing heart failure and might benefit from therapies for prevention of heart failure and CVD.

**Classification of recommendation:** I

**Level of evidence:** C

### *Recommendation 3*

Manufacturers of reagents and kits for measurement of the BNP and NT-proBNP should be in compliance with current specifications developed by government and professional organizations, such as the IFCC.

**Classification of recommendation:** I

**Level of evidence:** C

### *Recommendation 4*

Laboratorians, clinicians, and manufacturers involved in utilizing and/or producing natriuretic peptide assays must work together to assure that all stakeholders are properly educated regarding preanalytical (eg, biological variation, specimen stability), analytical (the impact of various proBNP-derived peptides forms on assays, methodological variation of BNP results), and postanalytical (appropriate reference intervals, decision limits, and confounding clinical conditions) issues.

**Classification of recommendation:** I

**Level of evidence:** C

## BACKGROUND

The heart must be viewed as both a biological pump that cycles approximately 100,000 times per day and as an important hormone producing organ that produces biochemical signals that are antagonists for the sympathetic nervous system and the renin angiotensin aldosterone axis (1-3). Physiologically, there are a number of structurally and functionally related heart hormones manufactured by human cardiocytes. These include atrial natriuretic peptide (ANP), brain (or B-type) natriuretic peptide (BNP) and their respective N-terminal (NT) metabolic peptides termed NT-proANP and NT-proBNP. Release of the natriuretic peptides is stimulated by hemodynamic stress; these hormones have powerful diuretic, natriuretic, and vascular smooth muscle relaxing actions (1-3). Because of their central pathophysiological role in the cardiovascular system, the natriuretic peptides are elevated in conditions characterized by wall stretch, ventricular dilation, and/or increased pressures resulting from excess fluid retention (2). Activation of the natriuretic system normally functions to reduce fluid blood volume and blood pressure.

BNP and its associated metabolite NT-proBNP have emerged as biomarkers of hemodynamic stress in acutely ill patients. Compared to ANP, BNP has a 2- to 3-fold more powerful natriuretic and blood pressure lowering effect on a molar basis (4). Under physiologic conditions, BNP concentrations are lower than ANP; however as the severity of hemodynamic stress

increases, plasma BNP levels increase more than corresponding ANP values (5). There is a positive correlation between blood BNP concentrations and left ventricular end diastolic pressure and inverse correlation to left ventricular function (6). In a study by Cowie et al (7), BNP showed the greatest predictive power as an indicator of heart failure when compared with either ANP or NT-proANP. Further, in the assessment of ventricular dysfunction and prediction of mortality in patients with severe heart failure, BNP was advocated as a better biomarker than either NT-proANP or ANP (8). BNP and NT-proBNP have emerged as the preferred biomarkers for assessing heart-related stress.

Regulation of BNP synthesis and secretion occurs mainly at the gene level; under physiologic conditions, BNP is preferentially produced and secreted in the ventricles of the heart without storage in granules (9,10). However, both ANP and BNP can be synthesized in either the atrium or ventricles, or both, under pathologic conditions; chronic fluid overload may cause rapid BNP production in both heart chambers, and production in the atrium may exceed the amount of ANP (11,12). According to the conventional model, human BNP is synthesized within the myocytes from the 134-aa precursor preproBNP. Upon stimulation for release, a 26-aa signal peptide sequence is cleaved from the N-terminus of preproBNP to form proBNP<sub>1-108</sub>. During release into circulation, the proBNP<sub>1-108</sub> prohormone is further cleaved by corin, a membrane-bound serine protease, into an N-terminal pro-BNP<sub>1-76</sub> fragment termed NT-proBNP and the active 32-peptide, C-terminal pro-BNP<sub>77-108</sub> hormone-termed BNP. Although this model of BNP and NT-proBNP release is widely accepted, there is a body of evidence that the forms of BNP and NT-proBNP released into circulation are not as well understood as previously thought. A recent study utilizing innovative liquid chromatographic and mass spectrometry technology conclusively demonstrated that cross-reactive species contribute substantially to BNP measurements in patients with severe heart failure (13). It is important to note, however, that this finding does not mitigate clinical trials showing the diagnostic and prognostic utility of BNP and NT-proBNP measurements by immunoassay (13).

Table 9 shows the performance of BNP and NT-proBNP in the acute setting for use in diagnosing decompensated heart failure (14). This good performance and the basic physiology of the biomarkers also prompted interest in utilizing these biomarkers as tools for detecting left ventricular systolic dysfunction (Table 9) (14). Although BNP is more thoroughly studied, there is no evidence that for clinical purposes the accuracy of

BNP differs from that of NT-proBNP for the cross-sectional applications of decompensated heart failure diagnosis or assessment of left ventricular systolic dysfunction (14).

Concerns regarding the equivalency of BNP and NT-proBNP in the setting of renal insufficiency have been raised due to the notion that NT-proBNP clearance is more dependant on renal function than BNP (15). This is of significant concern since 33% to 57% of patients with heart failure have impairment of renal function (16-18). Recent data have shown that renal extraction of NT-proBNP and BNP is comparable across a broad range of renal function (19,20). Also, direct comparison of NT-proBNP and BNP in a large observational cohort of patients showed that the biomarker assays examined appeared to be equivalent tools for diagnosis of decompensated heart failure in patients with renal insufficiency as well as in patients with GFR > 60 mL/min (21).

Patients having decompensated heart failure diagnosis and/or left ventricular systolic dysfunction are clearly at high risk for adverse short- and long-term outcomes. Also, these patients are likely to have ischemic heart disease as this is the most common cause of heart failure. For this reason, the committee investigated evidence to examine if BNP and NT-proBNP measurements can add information to traditional risk factors for primary prevention assessment.

## DISCUSSION OF EVIDENCE

The committee identified several longitudinal studies (22-25) which adjusted for appropriate risk factors such as age, total cholesterol, smoking habit. Table 10 presents a summary of studies which led to the recommendations for BNP and NTproBNP. Evidence indicates that measurement of BNP and NTproBNP provide prognostic information of mortality and first cardiovascular events beyond traditional risk factors (22,23). Also, NT-proBNP was a stronger risk biomarker in nonhospitalized individuals 50 to 89 years old for predicting CVD and death than CRP (22). Excess risk was apparent at levels well below current thresholds used to diagnose decompensated heart failure (22, 23). Also, the association of BNP and NT-proBNP with increased risk of cardiac morbidity and mortality also held for very elderly patients (24). As in the cross-sectional studies included in a National Health Service technology assessment report (14), there are indications that NT-proBNP performs equivalently with BNP for detecting left

**Table 9. BNP and NT-proBNP Performance for Diagnosis of DHF and Detecting LVSD**

	Sensitivity (95% CI)	Specificity (95% CI)	DOR (95% CI)
DHF Diagnosis			
BNP	0.91 (0.90-0.93)	0.73 (0.71-0.75)	35.66 (17.11-74.30)
NT-proBNP	0.91 (0.88-0.93)	0.76 (0.75-0.77)	39.86 (18.13-87.64)
LVSD Detection			
BNP	0.88 (0.84-0.91)	0.62 (0.60-0.63)	10.74 (6.51-17.72)
NT-proBNP	0.84 (0.80-0.88)	0.65 (0.64-0.67)	14.96 (10.69-20.94)
Abbreviations: BNP, NT-proBNP, DHF, decompensated heart failure; LVSD, left ventricular systolic dysfunction; DOR, diagnostic odds ratio. NOTE. Data from NHS Health Technology Assessment (14).			

Reference	Study Design	Follow up	Endpoints	Adjusted hazard ratio or OR (95% CI)	Assay Used	Comparison Biomarker(s)
22 (Wang et al)*	Community based cohort	Mean: 5.2 years	Death Firstst major CV event Heart failure Htrial fibrillation Stroke or TIA CHD events	1.62 (1.08-2.42) 1.76(1.06-2.92) 3.07 (1.51-6.26) 1.91 (1.13-3.25) 1.99(1.09-3.62) 1.30 (0.79-2.15)	Shiono RIA BNP assay	NT-ANP
24 (Ueda et al) **	Cohort of elderly patients (mean 85 years)	2 years	Mortality Cardiac hospitalization	Each 50 pg/mL increased rate of mortality 1.4-fold (1.2-1.6) Each 50 pg/mL increased rate of cardiac events 1.6-fold (1.2-2.1)	Shiono RIA BNP assay	None
23 (Kistorp et al)***	Population based cohort	Mean: 5- years	All cause mortality First major cardiac event without CVD at baseline	HR 1.96 (1.21-3.19)  HR 3.24 (1.80- 5.79) (Top quartile of NT-proBNP results)	NT- proBNP Roche	hsCRP, 1.17 (0.95- 1.43), 1.46 (0.89- 2.24); Alb/creat, 1.38 (1.16-1.65), 1.88 (1.18-2.98); hsCRP, 1.15 (0.88- 1.51), 1.02 (0.56- 1.85); Alb/creat, 1.57 1.26-1.95), 2.32 ((1.33-4.05)
25 (McKie et al)****	Community based cohort	5.6 years	All cause mortality	Adjusted: NT- proBNP 1.63 (1.25-2.13);	BNP Biosite and  Biosite: 1.50 (1.15-1.95); 1.39 ) (1.10-1.74)	Two BNP assays and 1 NT-proBNP assay Shionogi, NT- proBNP Roche

\*Age, sex, presence or absence of hypertension, ratio of total to HDL cholesterol, smoking status, presence or absence of diabetes mellitus, BMI, serum creatinine.  
\*\*Age, sex, BMI, Blood pressure, heart rate, total protein, creatinine, Hb A1c, total cholesterol, ischemic heart disease, EKG abnormality, history of stroke, ADL score  
\*\*\*Age, sex, current smoking, diabetes mellitus, hypertension, and ischemic heart disease, total cholesterol, and serum creatinine.  
\*\*\*\*Age and sex adjusted; Model 1: age, sex, total cholesterol, and serum creatinine, presence of diabetes mellitus, hypertension, and coronary artery disease.  
Abbreviations: BNP, brain (B-type) natriuretic peptide; NT proBNP, N-terminal pro B-type natriuretic peptide; OR, odds ratio; TIA, transient ischemic attack; NT-ANP, N-terminal atrial natriuretic peptide; CV, cardiovascular; CHD, coronary heart disease; hsCRP, Alb, albumin; creat, creatinine.

ventricular dysfunction (25). There are data suggesting that NT-proBNP and BNP may be potentially useful for predicting future events and that these measurements may reveal underlying cardiac remodeling secondary to diverse CVD entities (25). However, there is currently no evidence that treatment or intervention based on the increased risk implied by these biomarkers improves patient outcomes. Thus, the committee advises against routine measurement in the primary prevention population. The potential benefit of therapies may be substantial and should be considered an important area of future research.

Regarding possible cutpoints to utilize for identifying high risk patients, it must be noted that reference intervals for BNP and particularly NT-proBNP show a dependence on age and sex. Of interest, a nested case-control study was performed in a large cohort of men (> 10,000), age 35 to 59 years, with a median follow-up of 2.66 years. A highly significant difference in NT-proBNP values ( $P < .0001$ ) was found between cases having coronary events (median, 48.5 pg/mL; interquartile range, 26.4 to 116.6 pg/mL) and controls with no events (median, 30.0 pg/mL; 1 interquartile range, 9.5 to 47.6 pg/mL) (26). Thus, the major finding of this study was that NT-pro-BNP is a strong pre-

dictor of coronary events in working men after adjustment for conventional risk factors (26). Another study of community based older individuals between 50 to 89 years found that a NT-proBNP value exceeding the 80th percentile value of 655 ng/L corresponding to an adjusted HR for mortality of 1.96 (95% CI, 1.21 to 3.19) (23). Based on this information, the committee suggests that the 80th percentile of the control reference population may be useful as a cutpoint, but requires further validation.

As with any biomarker, analytical specifications for BNP and NT-proBNP must be driven by a balance between physiology and clinical utilization. A recent report aimed at improving the quality of immunochemical measurements of BNP and NT-proBNP was recently published by the IFCC Committee on Standardization of Markers of Cardiac Damage (26). The recommendations proposed were intended for use by manufacturers of commercial assays, by clinical laboratories using those assays, by clinical trial groups and research investigators, as well as by regulatory agencies such as the United States Food and Drug Administration (27). This document was developed by experts who reviewed and abstracted the scientific literature pertaining to the needed quality specifications for BNP and NT-proBNP assays. These evidence-based recommendations encourage manufacturers of BNP and NT-proBNP diagnostics to include information in their package inserts that includes assay design, preanalytical performance characteristics, analytical performance characteristics, and clinical performance. In addition, regulatory agencies are encouraged to adopt a minimal and uniform set of criteria to help guide manufacturers seeking clearance for new and/or improved assays.

Knowledge in the context of the natriuretic is evolving rapidly. Utilization of BNP and NT-proBNP measurements is complicated by preanalytical issues. Stability at room temperature facilitates handling of specimens in routine laboratories in the preanalytical phase, and NT-proBNP is more stable in vitro than BNP (28). Serum or plasma NT-proBNP measurements are stable for 7 days at room temperature, 10 days at 4° C, and at least several months at -20° C or lower temperatures (29,30). Five freeze-thaw cycles do not diminish NT-proBNP concentrations significantly (29,31). BNP stability is dependent on the specific assay (28,32). At room temperature BNP measurements appear to diminish soon after collection, at 4° C levels are stable for about 4 hours, and at -20° C or colder BNP levels appear to decrease significantly within a few weeks (28). Other preanalytical issues, such as, biological variability, age, and sex differences, analytical issues such as assay performance, as well as post analytical issues such as result reporting and recognizing that different diseases and patient populations must utilize the test differently. Communication, cooperation, and education, both initially and continuous learning, are vital components for appropriate utility of BNP and NT-proBNP measurements in patient care.

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## Chapter **10**

# **Guideline Implementation**

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Adoption of these guidelines is voluntary. The literature continues to grow with published reports providing new information on these and other emerging biomarkers for heart disease and stroke. It is increasingly important that as these candidate biomarkers emerge, their value for possible clinical application along with measurement issues be properly

evaluated. As a result of this continuous expanding body of research, the current NACB guidelines will undoubtedly require continuous review and updating as knowledge and understanding of existing and new biomarkers for primary prevention of heart disease and stroke emerge.

