Clinical Efficacy of an Automated High-Sensitivity C-Reactive Protein Assay

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Background: **Prospective studies have shown that C-reactive protein (CRP) can be used to predict risk of future cardiovascular events. High-sensitivity methods for CRP (hs-CRP) measurement are needed for this purpose.**

Methods: **We compared the clinical efficacy of an automated and commercially available latex-enhanced assay (Latex) for hs-CRP (Dade Behring) to a validated in-house ELISA, previously shown to predict future peripheral arterial disease (PAD) in asymptomatic populations. Using a prospective, nested, case-control design, we measured baseline hs-CRP concentrations in 144 apparently healthy men who subsequently developed symptomatic PAD and 144 age- and smoking habit-matched controls who remained free of vascular disease over the follow-up period of 60 months.**

Results: **The two hs-CRP assays correlated highly (r = 0.95; P <0.001), and all but two participants were classified into concordant quartiles or varied by only one quartile. The median hs-CRP of the case group was significantly higher than that of controls when measured by either the ELISA (1.34 vs 0.99 mg/L; P = 0.034) or the Latex method (1.80 vs 1.20 mg/L; P = 0.042). Furthermore, for both ELISA and the Latex method, the calculated relative risks of developing PAD increased significantly with each increasing quartile of hs-CRP. The calculated interquartile increase in relative risk of PAD was 31% (95% confidence interval, 5.2–62.2%; P = 0.01) for ELISA and 34% (95% confidence interval, 8.2–66.1%; P = 0.007) for the Latex method.**

Conclusions: **Our findings indicate that the Latex method is equally as efficacious as the validated ELISA in classifying patients into cutoff points established by prospective studies for risk stratification for coronary and cerebrovascular disease.**

Evidence from laboratory, clinical, and epidemiological studies is rapidly accumulating regarding the role of inflammation in atherogenesis. These findings suggest that atherosclerosis is a chronic low-grade inflammatory condition that evolves as a result of a combination of biochemical, physical, and possibly infectious processes. Biochemical markers, such as C-reactive protein (CRP),[4] have been used to detect and assess the severity of systemic inflammation. Several prospective studies have shown that plasma CRP concentrations are increased many years in advance of first coronary and cerebrovascular events in healthy *(1–4)* and high-risk individuals *(5–7)*. Data from the Physician's Health Study, for example, have demonstrated that those apparently healthy males in the highest quartile of CRP concentrations ($\geq$2.11 mg/L) are at almost three times the risk of future myocardial infarction (MI) and two times the risk of ischemic stroke or peripheral arterial disease (PAD) compared with those in the lowest quartile ($\leq$0.55 mg/L) when followed up to 5 years *(1, 2)*.

CRP usually is measured in clinical laboratories by either immunonephelometric or immunoturbidimetric assays. The current methods are generally reproducible, fully automated, and capable of measuring CRP with a detection limit of 3–5 mg/L. Although this detection limit is adequate for the traditional clinical utility of CRP in monitoring infection, it renders most of the current assays useless in assessing and predicting risk of coronary and cerebrovascular disease in apparently healthy populations. In contrast, most of the original studies that examined the clinical utility of CRP in predicting future MI and stroke have used a high-sensitivity (hs-CRP) in-house

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[4] Nonstandard abbreviations: CRP, C-reactive protein; MI, myocardial infarction; PAD, peripheral arterial disease; and hs-CRP, high-sensitivity CRP.

ELISA (8). This ultrasensitive assay is capable of measuring hs-CRP at a concentration of 0.007 mg/L. Recently, an ultrasensitive latex-enhanced immunoassay (Latex) for hs-CRP measurement on the BN II nephelometer (Dade Behring, Newark, DE) has become available (9). The analytical performance of the method has been validated previously (9). In this report, we evaluate the clinical utility of the newly introduced method and compare its performance to that of the in-house ELISA, which we previously showed to predict risk of future PAD among otherwise healthy middle-aged men (2).

Materials and Methods

SUBJECTS

The study population consisted of apparently healthy men participating in the Physician's Health Study, a randomized, double-blind, placebo-controlled trial of aspirin and β-carotene in the primary prevention of heart disease and cancer conducted among 22 071 US male physicians 40–84 years of age (10). Participants had no prior history of cardiovascular disease or cancer and were randomly assigned to one of four treatments: 325 mg of aspirin on alternate days, 50 mg of β-carotene on alternate days, both, or neither. Before randomization, participants were asked to provide baseline blood samples; the procedures used to collect, process, and store these EDTA-anticoagulated baseline blood samples are described elsewhere (1). Overall, 14 916 (68%) of the cohort provided baseline samples.

Questionnaires were sent to all participants annually to elicit information on risk factors and incident health events, including self-reports of the development of intermittent claudication and hospitalization for peripheral arterial revascularization procedures. For this analysis, case subjects were defined as those apparently healthy participants who provided an adequate baseline plasma sample and who subsequently reported either intermittent claudication or peripheral arterial revascularization during a mean follow-up period of 60 months. Control subjects were apparently healthy participants who provided baseline plasma samples and who remained free of reported cardiovascular disease at the time the matched case patients reported their events. Control subjects were selected randomly from among study participants who met the matching criteria of age, smoking habit, and length of follow-up. Using these methods, we evaluated 144 patients and an equal number of control subjects in a prospective, nested-control study. None of the case subjects reported a history of intermittent claudication at study entry.

For each case and control subject, plasma collected and stored at baseline was thawed and assayed for hs-CRP as described previously (1, 2, 8). Blood specimens were analyzed in blinded pairs with the position of the patient's specimen varied at random to reduce the possibility of systematic bias and decrease interassay variability.

LABORATORY MEASUREMENTS

Plasma CRP concentrations were measured by two immunoassays in the study samples. The first method, which was an in-house competitive ELISA, used polyclonal anti-CRP antibodies (Calbiochem-Novabiochem) and a calibrator that was traceable to WHO Reference Material 85-506. This method has been reported previously (8) and has been shown efficacious in predicting risk of MI, stroke, and PAD (1, 2). In brief, study samples, controls, and calibrators were pipetted into the anti-CRP antibody-coated wells, and a fixed amount of biotinylated CRP was added immediately. After an overnight incubation at 4 °C, peroxidase-labeled avidin-biotin complex was added to the wells to produce a color reaction. The intensity of the generated color was inversely proportional to the concentration of hs-CRP present in the study samples. The run-to-run precision, reflected by the CVs, at hs-CRP concentrations of 1.05, 2.52, and 2.08 mg/L was 5.7%, 6.8%, and 5.5%, respectively. The second method (Latex), which used particle-enhanced technology, was performed on the Behring BN II nephelometer (Dade Behring) (9). This assay used monoclonal anti-CRP antibodies and a calibrator that was also traceable to WHO Reference Material. In this method, the specific antibodies coated to polystyrene particles formed a complex with CRP present in the measured study sample. The amount of scattered light was directly proportional to the size of the antigen-antibody complex and reflected the hs-CRP concentration present in the study sample. The run-to-run CVs, at hs-CRP concentrations of 0.47, 10.5, and 54.9 mg/L, were 6.4%, 3.7%, and 2.9%, respectively. This method has been shown efficacious in predicting risk of recurrent MI (11).

STATISTICAL ANALYSIS

Because hs-CRP values were skewed rightward, median plasma concentrations were computed and the significance of any difference in the distributions and in median values between case and control subjects was assessed by the use of Wilcoxon rank-sum test. In risk prediction models, hs-CRP concentrations were divided into quartiles defined by the distributions of the control group for each respective assay. Adjusted estimates of risk were obtained by use of conditional logistic-regression models that accounted for the matching variables and also controlled for randomized treatment assignment, body mass index, diabetes, history of hypercholesterolemia, history of hypertension, and a family history of coronary heart disease. The hs-CRP concentrations were log transformed to normalize the data, and linear regression analysis was used to compare the two methods. All P values were two-tailed and $P < 0.05$ was deemed statistically significant. Confidence intervals were computed at the 95% level.

Table 1. Baseline characteristics of study subjects.

	Control subjects (n = 144)	Case subjects (n = 144)	P
Age, years	62.9 ± 9.5	62.9 ± 9.5	MC[a]
Smoking habits, %			
Never	30.1	30.1	
Past	42.7	42.7	MC
Current	27.3	27.3	
Body mass index, kg/m^2	24.6 ± 2.8	24.9 ± 3.1	0.3
Blood pressure, mmHg			
Systolic	131.4 ± 12.4	133.7 ± 13.8	0.1
Diastolic	79.9 ± 6.6	80.6 ± 7.2	0.4
History of hyperlipidemia, %	8.5	10.2	0.7
Diabetes, %	4.2	10.4	0.04
Family history of premature atherosclerosis, %	9.2	16.7	0.06

[a] MC, matching criteria.

Results

The baseline characteristics of the study participants are presented in Table 1. The case group consisted of 144 subjects who developed claudication or underwent peripheral arterial revascularization within 60 months after collection of baseline blood samples, whereas the control group consisted of 144 age- and smoking habit-matched subjects who remained free of vascular disease during the same period as reported previously. No statistically significant difference in body mass index, blood pressure, history of hyperlipidemia, or family history of premature atherosclerosis was noted between the case and control groups (2). However, the incidence of diabetes was slightly higher in case subjects.

The hs-CRP concentrations of the study population ranged from 0.12 to 25.7 mg/L, as assessed with ELISA, and from 0.20 to 34.8 mg/L, as assessed with the Latex method. When either the actual or the log transformed values were used, hs-CRP concentrations measured by ELISA correlated highly with those determined by the Latex method ($r = 0.95$ and $r = 0.93$, respectively; $P < 0.001$; Fig. 1; Table 2). The relatively small biases seen in slope and intercept indicate that both assays are similarly standardized. In addition, the frequency distributions of hs-CRP concentrations measured by the two assays were very comparable, further demonstrating the similarity and consistency between the two methods (Fig. 2). Finally, in an analysis that divided the study population into quartiles, 78.3% of subjects were classified concordantly and 20.6% of subjects varied by one quartile; only two subjects varied by two quartiles.

The median hs-CRP concentration of the case group at baseline was significantly higher than that of controls when measured by either the ELISA (1.34 vs 0.99 mg/L; $P = 0.034$) or the Latex method (1.80 vs 1.20 mg/L; $P = 0.042$). The quartile cutoff points for hs-CRP measured by both methods are presented in Table 3. These cutoff points are based on the distribution of hs-CRP among control subjects. The calculated relative risks of developing PAD increased significantly with each increasing quartile of hs-CRP (Fig. 3). This increase was similarly significant when hs-CRP was measured by either ELISA or the Latex method; the calculated interquartile increase in relative risk of developing PAD was 31% (95% confidence interval, 5.2–62.2%; $P = 0.01$) for ELISA and 34% (95% confidence interval, 8.2–66.1%; $P = 0.007$) for the Latex
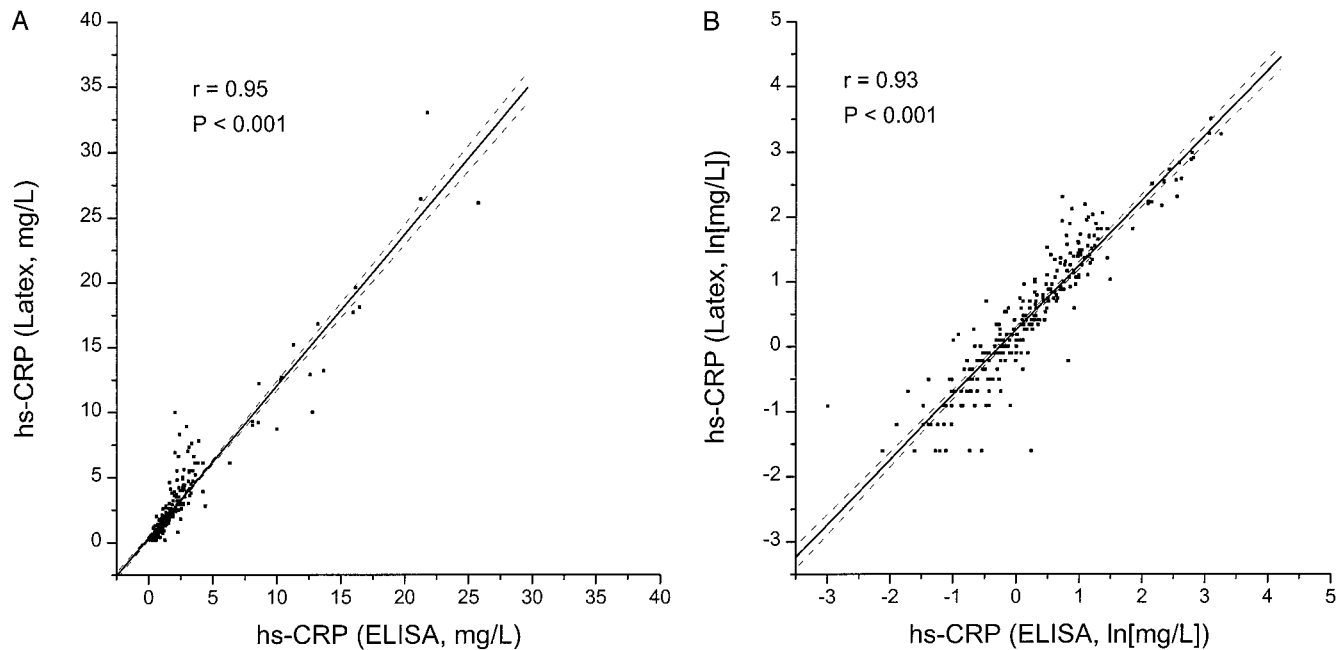

Fig. 1. Correlation between the in-house ELISA and the Latex method using samples of both case and control groups.

(A), correlation based on original data; (B), correlation based on log-normalized data.

Table 2. Correlation study between the in-house ELISA and the Latex method for the measurement of hs-CRP.

	r	Slope	Intercept	β	$S_{y|x}$	P
Original data	0.95	0.99	0.36	0.782	0.14	<0.001
Log-normalized data	0.93	1.17	0.26	0.868	0.02	<0.001

Table 3. Quartile cutoff points of hs-CRP measured by the in-house ELISA and the Latex method.

	Quartile			
	1	2	3	4
ELISA, mg/L	<0.55	0.55–0.99	1.0–2.1	>2.1
Latex, mg/L	<0.65	0.65–1.2	1.3–3.1	>3.1

method (Fig. 3). In ROC curve analyses, no statistically significant difference was observed between assays in terms of risk prediction. The adjustment of this analysis for body mass index, diabetes, blood pressure, history of hypercholesterolemia, and a family history of coronary heart disease did not significantly alter these findings.

Discussion

These data demonstrate that a commercially available method (Latex) for the measurement of hs-CRP is as efficacious as a previously validated ELISA for the prediction of future PAD. The calculated relative risks of developing PAD increased significantly with each increasing quartile of baseline hs-CRP measured by either immunoassay. Furthermore, our data show that hs-CRP concentrations measured by ELISA and the Latex method are increased at baseline in subjects who subsequently developed symptomatic PAD compared with those who did not. The high correlation seen between the two examined assays and their comparable clinical perfor-
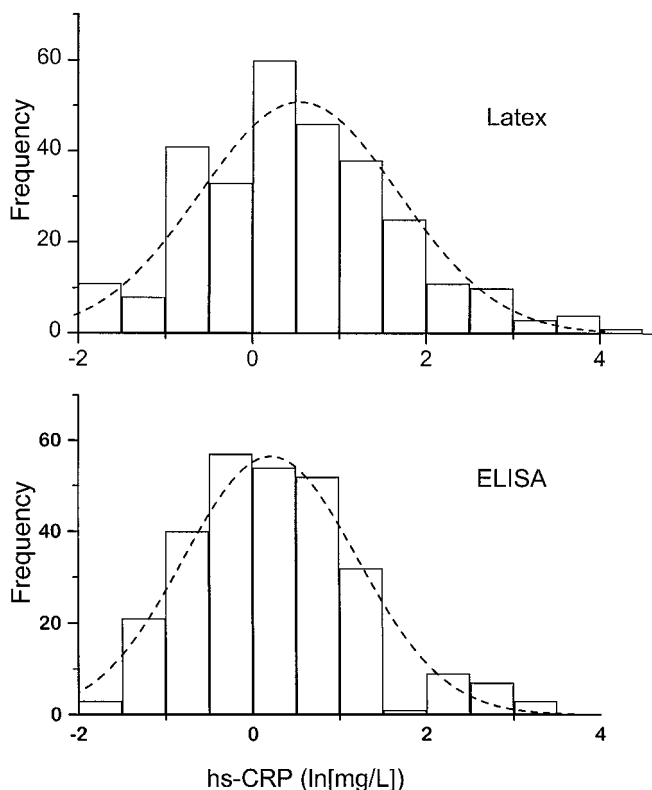
mance suggest that the Latex method provides the needed sensitivity and reliability to predict future risk of cardiovascular events in apparently healthy populations.

Acute phase reactants in general and hs-CRP in particular have been the subjects of intense investigations in both symptomatic and asymptomatic populations to further elucidate the contribution of inflammation to atherogenesis. Because its de novo hepatic synthesis is triggered by the pleiotropic cytokine interleukin-6 (12), CRP appears to act as a reliable surrogate marker for interleukin-6 and other inflammatory mediators such as tumor necrosis factor. The concentration of CRP is increased in patients with unstable angina and MI compared with patients with chronic stable angina (13, 14). Recently, it has been shown that CRP has a useful prognostic utility in patients with unstable angina or non-Q-wave MI (15, 16). Furthermore, CRP appears to be a strong predictor of recurrent coronary events in patients who suffered acute MI (11). Several prospective studies have shown that hs-CRP is a predictor of increased risk for future MI, stroke, or PAD in asymptomatic individuals with no known coronary heart disease (1–7).

Most currently available immunoassays are capable of measuring CRP concentrations for the purpose of stratifying risk in patients with acute coronary syndromes. However, they are not adequate for use in predicting future coronary events in asymptomatic populations because they lack the desired sensitivity. Several of the original studies (Physician's Health Study, Cardiovascular Health Study, and Rural Health Promotion Project)

Fig. 2. Frequency distribution of CRP in the study population presented for the in-house ELISA and the Latex method using log-normalized data.
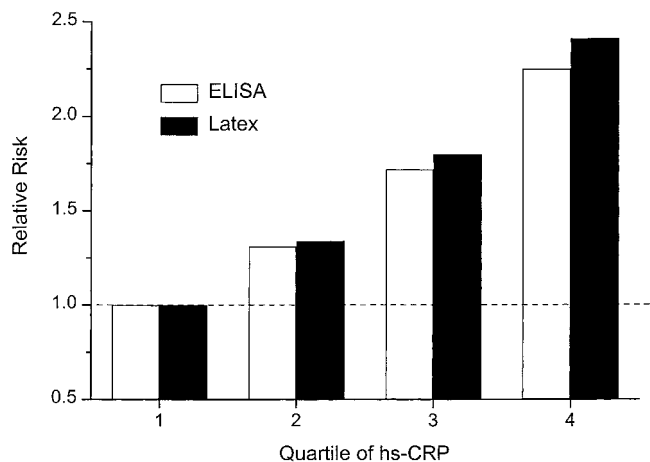
Fig. 3. Calculated relative risks of PAD according to baseline CRP concentration measured by the in-house ELISA and the Latex method.

that demonstrated the utility of hs-CRP as a risk indicator of future MI or stroke used an in-house ELISA (1, 2, 5). Although this assay is accurate and precise, it cannot be widely used clinically because it is time-consuming, technically demanding, not fully automated, and not available commercially. Recently, a high-sensitivity method (Latex) for the determination of hs-CRP has become commercially available. The assay is fully automated, highly precise, and capable of measuring large numbers of samples relatively quickly. The patient correlation study performed here revealed that ELISA and the Latex method correlated highly and showed relatively small biases in slope and intercept. This finding is not unexpected considering both methods used calibrators that are traceable to WHO Reference Materials. Discrepancies between methods that claim the use of calibrators that are traceable to the same reference source are not that uncommon (9). Manufacturers and investigators often fail to follow the recommended protocol for value transfer from the primary to their secondary calibrator, which can lead to inaccurate value assignment. Such practices may have serious clinical consequences. In the case of hs-CRP, for example, apparently healthy subjects will be classified into quartiles, depending on their hs-CRP concentrations, that reflect their risk of developing future coronary or cerebrovascular events. If the assay used is not properly standardized, subjects may be placed in the wrong quartile and their risk incorrectly assigned; in this study, of the 288 individuals, only 2 varied by more than one quartile. To take advantage of the database established by these prospective studies, ultrasensitive CRP assays must be well standardized. Earlier standardization efforts have led to reliable measurement of CRP at relatively high concentrations (5–200 mg/L). Several new assays for the ultrasensitive measurement of CRP are under development worldwide and are expected to be commercially available in the near future. Similar standardization activities should be undertaken to ensure the reliable measurement of this protein at low concentrations by these newer methods.

Claudication is a common consequence of PAD that affects 2–5% of subjects in the US >50 years of age. The chronic lower extremity ischemia, seen in those with severe claudication, can lead to recurrent infection, the need for surgical revascularization, and limb loss. Although the risk factors for PAD are similar to those for coronary heart disease, many patients who develop the clinical symptoms of claudication do not have these factors. In this study, we assessed whether CRP concentrations measured by ELISA and the Latex method are equally useful predictors of PAD in a cohort of men participating in the Physician's Health Study. The clinical utility of hs-CRP, using ELISA, in the risk stratification for PAD in this population has been documented previously (2). The data show that the median baseline hs-CRP concentration among those who subsequently developed PAD is higher than among those who did not, and the

calculated relative risks of developing PAD increased significantly with each increasing quartile of hs-CRP regardless of the method used to measure this protein. These findings remained unchanged after the analyses were corrected for body mass index, diabetes, blood pressure, history of hypercholesterolemia, and a family history of coronary heart disease.

In conclusion, in this prospective, nested, case-control study, hs-CRP values measured by ELISA and the Latex method were similarly increased in baseline samples of subjects who subsequently developed PAD compared with controls, and the calculated relative risks for PAD increased significantly with each increasing quartile of measured hs-CRP by either method. Our findings indicate that the Latex method is comparable to ELISA and, therefore, can be used clinically in classifying patients into cutoff points established by prospective studies for risk stratification for coronary and cerebrovascular disease.

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