Plasma Fluorescent Oxidation Products: Independent Predictors of Coronary Heart Disease in Men

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Fluorescent oxidation products in plasma are stable with routine blood collection methods and reflect oxidation in food, animals, and in vitro. Whether plasma fluorescent oxidation products predict future coronary heart disease has not been established. Among US men without cardiovascular disease who provided blood specimens in 1994 in the Health Professionals Follow-up Study, the authors confirmed 266 incident nonfatal myocardial infarction or fatal coronary heart disease endpoints during 6 years of follow-up. Using a nested case-control design, they measured baseline levels of fluorescent oxidation products. Each case was matched with two controls according to age, smoking status, and time of blood draw. The relative risk of coronary heart disease between extreme quintiles was 1.83 (95% confidence interval: 1.07, 3.13; \( p \) for trend = 0.005) in the multivariate analysis controlling for other cardiovascular risk factors and traditional lipid markers. Further adjustment for C-reactive protein and glycated hemoglobin A1c did not materially attenuate this association. The multivariate-adjusted relative risk between extreme quintiles was 3.36 (95% confidence interval: 1.33, 8.48; \( p \) for trend = 0.005) when the analysis was restricted to men who had fasted for more than 10 hours before blood draw. The authors found that plasma fluorescent oxidation products significantly and independently predicted coronary heart disease incidence among men without previous cardiovascular events.

biological markers; coronary disease; fluorescence; myocardial infarction; oxidation-reduction; oxidative stress; plasma; risk factors

Abbreviations: AIC, Akaike’s Information Criterion; CHD, coronary heart disease; CI, confidence interval; CRP, C-reactive protein.

Although traditional risk factors can explain much of the risk for coronary heart disease (CHD) (1), up to 20 percent of patients who have developed CHD lack traditional risk factors (2). Furthermore, the mechanisms by which traditional risk factors cause CHD are not completely understood. Therefore, identification of a novel biochemical marker may enhance our understanding of the underlying pathophysiology of CHD. Additionally, such a biomarker would be a useful clinical diagnostic tool to complement traditional diagnostic methods. A useful clinical predictor should be stable in samples collected by a simple method, relatively easy to measure in clinical and epidemiologic studies, and independently predictive of disease.

Measurement of fluorescent products has not been widely used in human studies but has been used in the food industry (3–6), in animals, and in vitro experiments to detect oxidation (5, 7–12). This fluorescent assay measures the Schiff base products from interactions of lipid oxidation products with proteins, amino acids, and DNA oxidation products (5, 6). We previously found that this marker is stable (12)

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and relatively easy to measure. Furthermore, a small prospective, community-based study of men and women aged 80 years or older found a sevenfold greater risk of subsequent cardiovascular events between subjects in the extreme quartiles of fluorescent oxidation products (13). Thus, we conducted a nested case-control study of initially healthy middle-aged and older men to evaluate the ability of a plasma fluorescent oxidation marker to predict CHD.

MATERIALS AND METHODS

Study population and collection of blood samples

The Health Professionals Follow-up Study is a prospective cohort study of diet, CHD, and cancer among 51,529 US male health professionals aged 40–75 years at baseline in 1986. Cohort members completed mailed questionnaires providing details on dietary intake, risk factors for heart disease, and medical history at baseline. Updated health information, including newly diagnosed heart disease, is assessed biennially. During 1993 and 1994, we sent venipuncture kits to participants, and 18,140 men returned specimens on ice by using an overnight courier. Multiple aliquots of plasma were stored in liquid nitrogen freezers for future nested case-control analyses. We identified 16,453 men who were free of cardiovascular disease and cancer as the “at-risk” group for this analysis. As documented previously, the men who returned blood samples were not substantially different from the remaining men in the cohort except for the slightly younger age and lower prevalence of current smoking among those who returned blood samples (14).

Ascertainment of cases and selection of controls

The assessment of nonfatal myocardial infarction and fatal CHD has been described in detail elsewhere (15). Briefly, to confirm cases of myocardial infarction, we used World Health Organization criteria, which require symptoms plus either diagnostic electrocardiographic changes or elevated levels of cardiac enzymes.

We confirmed 266 incident nonfatal myocardial infarction or fatal CHD endpoints between 1994 and 2000. We used risk set sampling as described by Prentice and Breslow (16) to select two controls per case randomly from the “at-risk” group of participants at the date of diagnosis of the case. Controls were matched by age, smoking status, and date that the blood samples were returned. Matched case-control triplets were handled identically and together and were assayed in the same analytical run. The order within each case-control triplet was determined at random. Quality controls that were indistinguishable from case and control samples were randomly interspersed within each batch.

Measurement of fluorescent oxidation products, other lipids, and inflammatory markers

Measurement of fluorescent oxidation products was developed originally by Dillard and Tappel in the 1970s (11) and was later modified by Shimasaki (9). We used the modified method with procedures described in detail previously (12). Briefly, plasma was extracted with ethanol-ether (3/1, v/v) and was measured by a spectrofluorometer at a wavelength of 360/430 (excitation/emission wavelength). The fluorescence was determined as relative fluorescent intensity units per milliliter of plasma.

Total cholesterol, high density lipoprotein cholesterol, low density lipoprotein cholesterol, and triglycerides were measured by standard methods using reagent from Roche Diagnostics (Indianapolis, Indiana) and Genzyme Corporation (Cambridge, Massachusetts). C-reactive protein (CRP) was measured by using an immunoturbimetric high-sensitivity assay on a Hitachi 911 analyzer (Roche Diagnostics). Oxidized low density lipoprotein was measured with a sandwich enzyme-linked immunosorbent assay kit by using monoclonal antibody 4E6 (Mercodia, Uppsala, Sweden). Soluble tumor necrosis factor-α receptors types 1 and 2 and interleukin-6 were measured by enzyme-linked immunosorbent assays (R&D Systems, Minneapolis, Minnesota). Plasma creatinine was measured via a modified Jaffe method by using an autoanalyzer (Hitachi Ltd., Tokyo, Japan). Hemoglobin A1c was measured by turbidimetric immunoinhibition.

Reproducibility and stability of the fluorescent oxidation products

Reproducibility of fluorescent oxidation products in plasma samples within individuals over a 1-year period. Using methods identical to those for the larger collection, we collected blood specimens in 2000 and 2001 from a subsample of healthy men from the Health Professionals Follow-up Study. In a subset of 20 men, we found no significant change in mean levels of fluorescent oxidation products over 1 year, and the intraclass correlation coefficient was 0.72. Measurements of fluorescent oxidation marker were stable in whole blood stored on ice for up to 36 hours before centrifugation (12).

Stability of plasma samples stored in liquid nitrogen (−140°C) for 12 years. A total of 21 plasma samples from healthy, nonsmoking female volunteers were tested to determine whether there were large differences in values from samples stored for different time periods. The plasma specimens consisted of three groups: 1) eight samples collected in 1989, 2) eight samples collected in 2000 (both of the above from the Nurses’ Health Study I), and 3) five fresh samples collected in 2002 from healthy, nonsmoking female volunteers at the Harvard School of Public Health. The ages of the subjects ranged from 40 years to 60 years in the three groups. The mean values of fluorescent products were 156, 128, and 144 fluorescent intensity units/ml for 0-, 2-, and 12-year specimens, respectively. Although direct evaluation with the same person would be desirable, these results suggest that there are no large changes in measurements of fluorescent oxidation products in plasma stored in liquid nitrogen for many years. The Institutional Review Board at the Harvard School of Public Health approved this research, and all participants provided informed consent for use of the blood samples.
Statistical analysis

For baseline cardiovascular disease risk factors, we present in this paper continuous variables as means or medians and compare cases and controls via two-sample t tests and Wilcoxon signed-rank tests. We used χ² tests for comparisons of proportions. The age-adjusted partial Spearman rank correlation coefficient was calculated to evaluate the association of fluorescent oxidation products with other continuous variables.

To estimate the relative risk of CHD according to baseline plasma levels of fluorescent oxidation products, we first categorized men into quintiles according to the distribution of plasma levels of oxidation products in the controls. We conducted conditional logistic regression analyses. In our multivariable analysis, we adjusted for traditional risk factors: family history of myocardial infarction, alcohol intake (five categories), body mass index (continuous), physical activity (<4.69, 4.7–40.59, 40.6–88.89, and ≥88.9 metabolic-equivalent task hours/week), and history of diabetes, hypertension, and high cholesterol at the time of blood draw. We also adjusted for creatinine concentration (continuous) because renal function is associated with oxidative stress (17) and CHD (18). Because lipid markers such as low density lipoprotein cholesterol and high density lipoprotein cholesterol play divergent roles in the etiology of CHD and also are susceptible to lipid oxidation, we sequentially adjusted for the potential lipid confounders—low density lipoprotein cholesterol (continuous), high density lipoprotein cholesterol (continuous), and triglycerides (continuous). We also examined the impact of controlling for CRP and other markers of inflammation, as well as for hemoglobin A₁c, as a marker of long-term glycemic control. For all continuous covariates, we first created quintiles based on the distribution among controls and used the median of each quintile to create the continuous variable. To eliminate the potential influence of fasting status on the results, we further limited a secondary analysis to men who were fasting at the time of blood draw.

To characterize renal function, we calculated the glomerular filtration rate as an estimate of renal function based on the formula used in the Modification of Diet in Renal Diseases study (19): glomerular filtration rate = 186 × creatinine⁻¹.¹５⁴ × age⁻⁰.²⁰³ × [1.212 if Black]. We also calculated creatinine clearance by using a modification of the Cockcroft-Gault formula: creatinine clearance = (140 – age) × weight/(72 × creatinine) (20).

To further evaluate the predictive value of this fluorescent oxidation marker, we used two general approaches: the likelihood ratio test (21) and Akaike’s Information Criterion (AIC) (22). The likelihood ratio test is a standard approach to compare nested models, and the AIC is used for comparing nonnested models; lower values indicate better fit. As shown in the last table of this paper, model 1 is defined as the basic model and includes traditional risk factors and confounders. Models 2, 3, and 4 include the basic model plus the fluorescent oxidation marker, CRP, and oxidized low density lipoprotein, respectively. We used the likelihood ratio test to compare model 1 with model 2, with model 3, and with model 4. We used the AIC to compare model 2, model 3, and model 4 to evaluate which had the best fit. In addition, we calculated the C index, analogous to the receiving operating characteristic curve (23, 24). Because of the matched case-control design of our study, we matched two important confounders (age and smoking); thus, selection of our controls was not completely random, and C index may be insensitive as a method of determining model fit.

RESULTS

Baseline characteristics of cases and controls are shown in Table 1. As expected, cases had higher low density lipoprotein cholesterol levels and other previously defined risk indicators. Baseline levels of fluorescent oxidation products were significantly higher in cases than in controls.

To determine which cardiovascular risk factors were most strongly associated with fluorescent oxidation products, we calculated partial correlation coefficients adjusted for age. Among the controls, we found only modest associations (ranging from 0.01 for physical activity to 0.23 for creatinine levels) of cardiovascular risk factors with fluorescent oxidation products (Table 2).

Higher levels of fluorescent oxidation products were associated with increased risk of CHD in a matched analysis (p for trend = 0.02; refer to Table 3); the relative risk between extreme quintiles was 1.50 (95 percent confidence interval (CI): 0.94, 2.41). Further adjustment for other cardiovascular risk factors strengthened the association, and additional adjustment for high density lipoprotein cholesterol, low density lipoprotein cholesterol, and triglycerides strengthened the association further (relative risk = 1.83, 95 percent CI: 1.07, 3.13) among men in the top quintile of fluorescent oxidation products versus the bottom quintile. Further adjustment for CRP, other inflammatory markers, and hemoglobin A₁c did not appreciably change these findings. When we restricted our analysis to men who had fasted for more than 10 hours at the time of blood draw, tests of significance for trends were similar but associations were much stronger (Table 3). In the multivariate-adjusted model including high density lipoprotein cholesterol, low density lipoprotein cholesterol, and triglycerides, the relative risk between extreme quintiles was 3.36 (95 percent CI: 1.33, 8.48) for those fasting for more than 10 hours.

Because renal function may be better represented by glomerular filtration rate and creatinine clearance than by creatinine levels, we also replaced creatinine levels with these two variables in the multivariate model adjusted for high density lipoprotein cholesterol, low density lipoprotein cholesterol, and triglycerides, and the relative risk did not change materially. When we replaced creatinine with glomerular filtration rate, for men who had fasted and not fasted before blood draw, the relative risks were 0.86 (95 percent CI: 0.50, 1.48), 1.16 (95 percent CI: 0.68, 1.98), 1.41 (95 percent CI: 0.82, 2.44), and 1.76 (95 percent CI: 1.02, 3.01) for quintiles 2, 3, 4, and 5, respectively, compared with quintile 1 (p for trend = 0.0067). Among those fasting for more than 10 hours, the respective relative risks were 0.94 (95 percent CI: 0.40, 2.18), 1.18 (95 percent CI: 0.46, 3.01), 1.85 (95 percent CI: 0.73, 4.69), and 3.14 (95 percent CI: 1.22, 8.05) (p for trend = 0.0069). Results were similar.
when we replaced creatinine with creatinine clearance in the multivariate model.

We also entered fluorescent oxidation marker as a continuous variable, and the relative risks for an increase of 140 fluorescent intensity units/ml of fluorescent oxidation products (interquintile range) were 2.00 (95 percent CI: 1.23, 3.24) for the set that included data for the nonfasting and fasting men and 3.55 (95 percent CI: 1.46, 8.62) for the set that included data for only those men who had fasted for more than 10 hours.

Table 4 compares the relative contribution of each novel biomarker (fluorescent oxidation marker, CRP, and oxidized low density lipoprotein) with the basic model that includes the traditional risk factors and confounders. The likelihood ratio test (LRT) suggests that the basic model with fluorescent oxidation marker was marginally or significantly different from the basic model without the fluorescent oxidation marker ($p_{LRT} = 0.07$ for the entire data set and $p_{LRT} = 0.05$ for the fasting data set). For CRP, the basic model with CRP was significantly different from the basic model without CRP in the entire data set but not in the fasting data set, in which we had less power to distinguish those risk factors that were only modestly predictive.

The AIC results show similar trends. In the whole data set, when fluorescent oxidation marker was added to the basic model, the AIC decreased; when oxidized low density lipoprotein was added to the basic model, the AIC increased. Among men who had fasted for more than 10 hours before blood draw, the AIC was the lowest when fluorescent oxidation marker was added to the basic model compared...
TABLE 2. Age-adjusted Spearman rank correlation coefficients between fluorescent oxidation products and selected cardiovascular risk factors among 532 healthy US male controls in the Health Professionals Follow-up Study (present study conclusion date, 2006)

<table>
<thead>
<tr>
<th>Fluorescent oxidation marker (r)</th>
<th>Demographic and behavioral variables</th>
<th>Plasma biomarkers</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Body mass index</td>
<td>Physical activity</td>
</tr>
<tr>
<td></td>
<td>0.02</td>
<td>0.01</td>
</tr>
</tbody>
</table>

* p < 0.05; **p < 0.01; ***p < 0.001.

with when CRP or oxidized low density lipoprotein was added to the basic model.

Although the C index may be insensitive in a matched case-control study, it was still able to rank the relative contributions of fluorescent oxidation marker, CRP, and oxidized low density lipoprotein in the fasting data set. It indicated that the fluorescent oxidation marker improved model fit more than did CRP or oxidized low density lipoprotein.

Collectively, the likelihood ratio test, the AIC, and the C index all suggested that the fluorescent marker provides additional information beyond the basic model. In the fasting data set, all three tests demonstrated that, of the three novel biomarkers, the fluorescent oxidation marker provides the strongest improvement as a risk predictor.

DISCUSSION

We found that high levels of fluorescent oxidation products were significantly associated with incidence of CHD among men without previous cardiovascular events, especially those who provided fasting samples at the time of blood draw. This relation persisted after controlling for traditional risk factors, common lipid markers, CRP, other inflammatory markers, and hemoglobin A1c.

Several studies have linked oxidative stress to congestive heart failure, CHD, or coronary artery calcification (25–28). However, these studies were small and also cross-sectional, making it difficult to determine whether oxidative stress was the outcome or the cause of the CHD. Many of these studies assessed oxidation markers such as malondialdehyde or F2-isoprostanes, which are not sufficiently stable to be assessed in stored plasma samples typically collected in epidemiologic studies (14). This is one reason that few large, prospective studies have demonstrated that oxidative stress plays an important role in CHD.

Until now, only one small prospective study from Italy (102 men and women at baseline and 32 CHD events) is known to have used a fluorescent oxidation marker; it noted a sevenfold (95 percent CI: 2.23, 25.96) increased risk of

TABLE 3. Relative risk of coronary heart disease across quintiles of fluorescent oxidation products using conditional logistic regression, Health Professionals Follow-up Study, United States (present study conclusion date, 2006)

<table>
<thead>
<tr>
<th>Quintile of fluorescent marker (median Fl*nl/ml (no. of cases))</th>
<th>1 (110 (49))</th>
<th>2 (135 (40))</th>
<th>3 (155 (52))</th>
<th>4 (190 (55))</th>
<th>5 (250 (70))</th>
<th>p for trend</th>
</tr>
</thead>
<tbody>
<tr>
<td>RR*</td>
<td>RR 95% CI*</td>
<td>RR 95% CI</td>
<td>RR 95% CI</td>
<td>RR 95% CI</td>
<td>RR 95% CI</td>
<td></td>
</tr>
<tr>
<td>Model 1 (matching factors)†</td>
<td>1</td>
<td>0.83 0.51, 1.35</td>
<td>1.05 0.65, 1.71</td>
<td>1.16 0.71, 1.89</td>
<td>1.50 0.94, 2.41</td>
<td>0.02</td>
</tr>
<tr>
<td>Model 2 (multivariable)‡</td>
<td>1</td>
<td>0.89 0.53, 1.48</td>
<td>1.08 0.64, 1.79</td>
<td>1.25 0.75, 2.10</td>
<td>1.57 0.95, 2.62</td>
<td>0.02</td>
</tr>
<tr>
<td>Model 3 (model 2 + high density lipoprotein cholesterol, low density lipoprotein cholesterol, and triglycerides)</td>
<td>1</td>
<td>0.92 0.54, 1.56</td>
<td>1.12 0.66, 1.89</td>
<td>1.38 0.80, 2.36</td>
<td>1.83 1.07, 3.13</td>
<td>0.005</td>
</tr>
<tr>
<td>Model 4 (model 3 + C-reactive protein)</td>
<td>1</td>
<td>0.94 0.55, 1.59</td>
<td>1.15 0.67, 1.96</td>
<td>1.37 0.80, 2.35</td>
<td>1.84 1.07, 3.16</td>
<td>0.006</td>
</tr>
<tr>
<td>Among men who fasted for more than 10 hours before blood draw (n = 128 cases)</td>
<td>1</td>
<td>1.03 0.45, 2.36</td>
<td>1.02 0.42, 2.47</td>
<td>1.74 0.71, 4.29</td>
<td>3.36 1.33, 8.48</td>
<td>0.005</td>
</tr>
</tbody>
</table>

* Fl, fluorescent intensity (unit); RR, relative risk; CI, confidence interval.
† Matched on age, smoking status, and time of blood draw.
‡ Model 2 was adjusted for fasting status; body mass index; physical activity; alcohol consumption; history of high blood pressure, high cholesterol, and diabetes; family history of coronary heart disease; and creatinine.

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myocardial infarction between extreme quartiles in this elderly population (13). It is difficult to compare our results directly with those of the Italian study because the sample sizes, the underlying at-risk populations, and the clinical endpoints were different. The Italian study followed both men and women but was restricted to those 80 years of age or older at baseline. This elderly population would likely have a much higher baseline risk. The 32 cardiovascular events in their study (13) included 12 myocardial infarctions, 16 strokes, and four cases of congestive heart failure—each of which may have a different association with oxidation. To remove extraneous heterogeneity, we focused on men only, aged 47–83 years at baseline and followed for risk of myocardial infarction only. The authors of the Italian study readily admitted (13) that they could not rule out the possibility of confounding due to subclinical CHD events because they did not exclude the cases from the first 2 years or extensively investigate the presence of subclinical CHD. When we removed the first 2 years of cases from the analysis of myocardial infarction, the relative risks between extreme quintiles became stronger by 15 percent in the whole data set and 18 percent in the fasting data set (data not shown). A final difference between the two studies relates to the basic laboratory methodology. Mezzetti et al. (13) used chloroform/methanol to extract the samples and, for technical reasons, we used ethanol/ether. Whether this makes a substantial difference needs to be evaluated in future studies.

In a separate cross-sectional analysis, we found that several traditional risk factors such as smoking, hypertension, reduced renal function, age, and elevated cholesterol levels were associated with fluorescent oxidation products (data not shown). However, the independent associations between fluorescent marker and CHD remained or were strengthened after controlling for these and other standard risk factors, suggesting that the fluorescent marker provides additional predictive value.

Furthermore, results from the models using the likelihood ratio test, the AIC, and the C index suggest that fluorescent oxidation products add additional predictive value beyond that provided by traditional risk factors and other confounders. We previously showed that oxidized low density lipoprotein as measured by 4E6 antibody was not an independent predictor of CHD after adjustment for lipid markers (14); however, the fluorescent oxidation marker remained significant after a similar adjustment.

The importance of using fasting samples to measure oxidation markers as a predictor of CHD has not been thoroughly explored. We found that number of hours of fasting was inversely associated with levels of fluorescent oxidation products (table 2) and that the fluorescent marker was more strongly related to CHD among men who had fasted before the blood draw. Determining the relation between oxidative stress and CHD is challenging because oxidation occurs in vivo under many circumstances, including physiologic and pathologic processes, and can be influenced by extraneous factors. For example, oxidative stress occurs during normal metabolism, with hypertension and in renal failure, and can be increased by smoking and potentially by long-term and postprandial dietary factors. Postprandial oxidation may more likely be influenced by the most recent diet (29–31), whereas, under fasting conditions, postprandial oxidation is minimized and the levels of oxidation products may be more closely related to pathologic oxidative stress in vivo.

Our study has several strengths and limitations. In our pilot study, the within-person reproducibility for fluorescent oxidation products was good, with an intraclass correlation...
coefficient of 0.72, which is comparable to that of commonly used clinical markers such as total cholesterol. We indirectly assessed the long-term stability in liquid nitrogen, which also provided reassuring results. We did not find a deterioration of fluorescent oxidation products, but even if a slight deterioration did occur, it would affect specimens from cases and controls similarly. Second, we collected our blood samples before the CHD events occurred, which should reduce the impact of CHD on the level of fluorescent oxidation products. Third, although residual confounding might still exist in observational studies, we assessed many lifestyle factors and measured multiple markers of lipids and inflammation typically used in clinical predictor scores.

Although we had only one time point of exposure assessment, this limitation could lead to only an underestimation of the true effect. Another limitation of this study is that the fluorescent oxidation measure was not fully characterized and could reflect products in addition to oxidative stress. However, a complex measure of oxidative damage to lipids, proteins, and other molecules may be a better cumulative marker predicted future risk of CHD independently of that lipid oxidation. In summary, we found that a plasma fluorescent oxidation marker predicted future risk of CHD independently of that predicted by lipid parameters, inflammatory markers, and other risk factors. The association was stronger with measurements from fasting blood specimens. Further validations of this fluorescent oxidation marker in other populations are needed before its clinical utility can be evaluated.

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Conflict of interest: none declared.

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