# Multimarker Approach Predicts Adverse Cardiovascular Events in Women Evaluated for Suspected Ischemia: Results from the National Heart, Lung, and Blood Institute–Sponsored Women's Ischemia Syndrome Evaluation

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ABSTRACT

*Background:* Inflammatory marker and hemoglobin levels (eg biomarkers) considered separately, predict adverse events in selected populations.

*Hypothesis:* A multiple biomarker approach predicts adverse events in women referred for evaluation of ischemia.

*Methods:* We investigated associations between biomarkers (high sensitivity C-reactive protein, interleukin-6, serum amyloid-A, and hemoglobin levels) with adverse outcomes in women referred for coronary angiography for suspected ischemia in the National Heart, Lung, and Blood Institute (NHLBI)–sponsored Women's Ischemia Syndrome Evaluation (WISE).

*Results:* Among 595 women (mean age 58 years, ejection fraction [EF] 65%, majority without coronary stenosis $\geq$50%) followed for 3.6 $\pm$ 1.8 years (mean $\pm$ SD), those without abnormal markers had fewer events (11.6%) compared to those with 1 (18.4%), 2 (20.9%), or 3 (37%) abnormal markers ($p<0.001$ for trend). Women without abnormal markers had fewer deaths (1.6%) than women with 1 (6.1%), 2 (9.1%), or 3 (17%) abnormal markers ($p<0.001$ for trend). Adding low hemoglobin was associated with higher adverse event and all-cause mortality rates. In multivariate analysis, as the number of abnormal biomarkers increased risk increased. Women with 3 or 4 abnormal biomarkers were approximately 10–20 times more likely to die ($p<0.05$). Biomarkers added to the predictive information provided by the Framingham Risk Score.

*Conclusions:* Among women undergoing coronary angiography for suspected ischemia, a multibiomarker approach predicted adverse events. Biomarkers added prognostic information beyond that obtained from traditional risk factors.

## Introduction

Diagnosis of coronary artery disease (CAD) and identifying women at high risk is key to improving outcomes. Inflammation plays a key role in atherosclerosis,[1] and biomarkers like high sensitivity C-reactive protein (hs-CRP), interleukin-6 (IL-6), and serum amyloid A (SAA)

This work was supported by contracts from the National Heart, Lung, and Blood Institutes, nos. N01-HV-68161, N01-HV-68162, N01-HV-68163, N01-HV-68164, grants U0164829, U01 HL649141, U01 HL649241, a GCRC grant MO1-RR00425 from the National Center for Research Resources, and grants from the Gustavus and Louis Pfeiffer Research Foundation, Denville, New Jersey, The Women's Guild of Cedars-Sinai Medical Center, Los Angeles, California, The Ladies Hospital Aid Society of Western Pennsylvania, Pittsburgh, Pennsylvania, and The Edythe L. Broad Endowment for Women's Heart Research, Los Angeles, California.


Clin. Cardiol. 32, 5, 244–250 (2009)
Published online in Wiley InterScience. (www.interscience.wiley.com)
DOI:10.1002/clc.20454 

Received: May 6, 2008
Accepted: July 15, 2008

predict adverse cardiovascular (CV) events.[2,3] Recently, the combination of inflammatory markers with cardiac troponin and brain natriuretic peptide predicted adverse events in acute coronary syndrome (ACS) patients.[4] In acute plaque rupture/erosion syndromes, a multimarker approach presumably improves assessment of the complex pathobiology over a single marker.

Low hemoglobin (Hgb) level is also associated with adverse outcomes.[5–7] In the Women's Ischemia Syndrome Evaluation (WISE), Hgb levels were inversely associated with inflammatory marker levels, and predicted adverse events independent of traditional risk factors.[8]

Using a multibiomarker approach we investigated associations between inflammatory markers and Hgb levels with adverse outcomes in women undergoing angiography for suspected ischemia.

## Methods

### Study Design

The WISE is a National Heart, Lung, and Blood Institute (NHLBI)–sponsored project aiming to improve diagnosis of ischemic heart disease in women.[9] Institutional review boards at each site approved the study and participant consent was obtained. Briefly, women referred for clinically-indicated angiograms to further evaluate suspected ischemia were screened. Exclusion criteria included comorbidity compromising follow-up, pregnancy, contraindications to diagnostic testing, cardiomyopathy, New York Heart Association (NYHA) class III–IV congestive heart failure (CHF), recent myocardial infarction (MI), and significant valvular or congenital heart disease. Baseline evaluation included collection of clinical and laboratory data. Qualitative and quantitative coronary angiographic analyses were done by a core lab masked to clinical data and obstructive CAD was defined as stenosis $\geq$50% in $\geq$1 artery.[9,10]

### Measurement of Hgb and Inflammatory Markers

Hgb levels were analyzed on site. Inflammatory markers were analyzed by the core lab from plasma samples obtained at entry and frozen at $-70\,^{\circ}$C. SAA and hs-CRP were measured on a Hitachi 911 analyzer by high-sensitivity methods using validated techniques.[11] Reagents for measurement of hs-CRP were from Denka Seiken (Niigata, Japan). Interleukin-6 levels were measured from plasma collected at study entry using a commercially-available ELISA kit using validated techniques (Quantikine hs human IL-6, R&D Systems, Minneapolis, Minn., USA).[11]

### Definitions

Abnormal thresholds were prespecified and defined as $<$12 g/dl for Hgb: $\geq$0.85 mg/L for hs-CRP,[2] $\geq$3.09 pg/mL for IL-6,[12] and $\geq$0.59 mg/dL for SAA.[13]

### Ascertainment of Events

Women were queried in person or by telephone interview for occurrence of adverse events by an experienced nurse and/or physician from each site at 6 wks and yearly. When an adverse event was identified, the referring physician was contacted for documentation. In the event of death, a death certificate and/or hospital records were obtained, and an event committee reviewed available information to determine the likelihood of a CV cause. Deaths confirmed as clearly due to CV causes were classed as CV deaths. An adverse event was defined as a composite of all-cause death or hospitalization for nonfatal MI, CHF, stroke, or other vascular event. Other vascular events primarily included peripheral vascular-related events.

### Statistical Analyses

Data are presented as means and standard deviations (SD) for continuous data and frequencies for categorical. Spearman rank correlation coefficients assessed relationships among inflammatory markers and between markers and Hgb. Mantel-Haenszel chi-square tests for trends assessed associations of events with number of inflammatory markers and low Hgb. Univariate and multivariable Cox regression models were used to identify predictors of events. Univariate predictors of all-cause mortality included a history of hypertension and diabetes as well as age, creatinine, CAD, Hgb, and inflammatory markers. Univariate predictors of adverse events included a history of hypertension, diabetes, and dyslipidemia as well as age, CAD, race, creatinine, Hgb, and inflammatory markers. Multivariable Cox regression models were run as a stepwise procedure with variables chosen for entry based on significant univariate associations. As expected the inflammatory markers were correlated with each other so individual models were constructed with each marker entered into the basic model one at a time. The final model for adverse events and all-cause mortality was that containing the risk factors and 4 variables to show the relationship of each marker to no abnormal markers (hazard ratio [HR] = 1.0). Sensitivity analyses were conducted using the upper quartile of each inflammatory marker. The Kaplan-Meier method was used to estimate the cumulative incidence rates of adverse events, with the log rank statistic used to assess differences by strata of Hgb/marker. The incremental value of multiple markers, beyond that of traditional risk factors (Framingham Risk Score [FRS]), was determined by receiver operating characteristics (ROC) analyses and the increment in area under the curve (AUC) determined when the markers were added to the FRS. All tests were 2-sided, and p values $<$0.05 were considered statistically significant. All statistics were analyzed using SAS version 8.2 (SAS Institute, Inc., Cary, NC).


Published online in Wiley InterScience. (www.interscience.wiley.com)
DOI:10.1002/clc.20454 

## Results

### Demographics

Baseline characteristics for the 595 women (mean age 58 years) in this substudy are in Table 1. Approximately one-quarter were diabetic; over half had a history of hypertension or dyslipidemia; approximately 20% were current smokers; nearly two-thirds had a family history of premature heart disease; three-fourths were postmenopausal; approximately two-thirds were taking aspirin; and over one-quarter ACE-inhibitors and/or statins. Despite 8% reporting a history of CHF, the mean ejection fraction (EF) was 65.2 ± 10.8%. The mean creatinine was 0.9 ± 0.5 mg/dl. Angiographically, 65% had no obstructive CAD.

Table 1. Baseline Characteristics of Women (N=595)

| Characteristics | Mean ± standard deviation |
|---|---|
| Age (y) | 58.0 ± 11.7 |
| Systolic blood pressure (mmHg) | 137 ± 22 |
| Diastolic blood pressure (mmHg) | 76 ± 11 |
| Pulse (beats per minute) | 74 ± 13 |
| BMI (kg/m²) | 29.8 ± 6.9 |
| Waist circumference (in) | 36.5 ± 7.3 |
| Hemoglobin (g/dl) | 12.9 ± 1.4 |
| Creatinine (mg/dl) | 0.9 ± 0.5 |
| Ejection fraction (%) | 65.2 ± 10.8 |
| Characteristics | Median (interquartile range) |
| Total cholesterol (mg/dl) | 187 (161–216) |
| Triglycerides (mg/dl) | 123 (81–189) |
| Fasting blood sugar (mg/dl) | 100 (88–127) |
| HDL-C (mg/dl) | 52 (44–60) |
| LDL-C (mg/dl) | 105 (84–130) |
| hs-CRP (mg/L) | 0.38 (0.17–0.88) |
| IL-6 (pg/ml) | 3.02 (1.82–5.56) |
| SAA (mg/dl) | 0.55 (0.31–1.02) |
| Characteristic | Percentage (%) |
| Non-white race | 18.3 |
| Obese (BMI ≥30) | 41.5 |
| History of Diabetes | 25.7 |
| (of those with diabetes, using insulin) | 43.8 |
| Dyslipidemia | 54.8 |
| Hypertension | 57.7 |
| Current smoking | 19.7 |
| Congestive heart failure | 8.4 |
| Premature CAD in family | 65.6 |
| Postmenopausal | 75.0 |
| Hysterectomy | 53.6 |
| Currently taking | |
| ACE inhibitors | 26.6 |
| Aspirin | 60.3 |
| Beta blockers | 40.9 |
| Statins | 27.6 |
| HRT (postmenopausal only) | 48.4 |
| Thyroid medication | 14.8 |
| Obstructive CAD | 35.5 |

BMI, body mass index; HDL-C, high-density lipoprotein; LDL-C, low-density lipoprotein; hs-CR p, high-sensitivity C-reactive protein; IL-6, interleukin-6; SAA, serum amyloid A; CAD-coronary artery disease; ACE, angiotensin-converting enzyme; HRT, hormone replacement therapy.

### Analysis of Markers and Hgb

At least 1 inflammatory marker was abnormal in 406 (68%): in 152 (26%) hs-CRP; 295 (50%) IL-6, and 269 (45%) SAA were abnormal. Of the women enrolled, 196 (33%), 110 (18%), and 100 (17%) had 1, 2, and 3 abnormal inflammatory markers, respectively. hs-CRP showed correlation with IL-6 ($r = 0.41$, $p<0.001$) and SAA ($r = 0.57$, $p<0.001$), while IL-6 showed modest correlation with SAA ($r = 0.32$, $p<0.001$).

Abnormal Hgb occurred in 130 (22%); mean 12.9 ± 1.4. Among women with low Hgb, 78% had at least 1 abnormal inflammatory marker. High-sensitivity CRP ($r = -0.08$, $p = 0.06$), IL-6 ($r = -0.14$, p$p<0.001$), and SAA ($r = -0.09$, $p = 0.03$) were all inversely correlated with Hgb.

### Markers and CV Events

After a mean follow-up of 3.6 ± 1.8 years, 118 (20%) women had an initial adverse CV event. Women with no abnormal inflammatory markers had significantly fewer CV events (11.6%) compared to those with 1 (18.4%), 2 (20.9%), and 3 (37%) abnormal inflammatory markers ($p<0.001$ for trend, Figure 1). Addition of low Hgb to each category of abnormal inflammatory markers was associated with an increased frequency of adverse events (Figure 1). For example, 45.5%

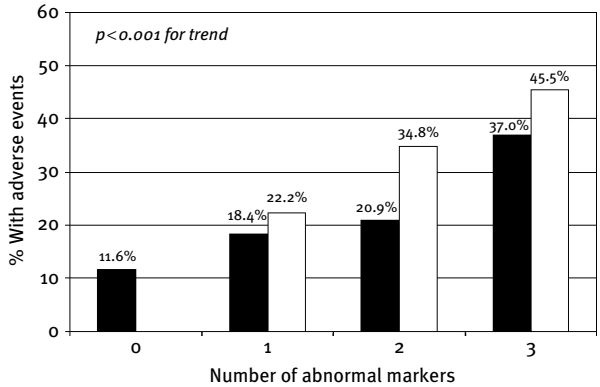


Figure 1. Proportion of women with adverse events by number of abnormal inflammatory markers and hemoglobin level. Adverse event defined by death or hospitalization for myocardial infarction, heart failure, stroke, or other vascular event. Note the significant relationship between increase in events and number of abnormal inflammatory markers (black) and number of abnormal inflammatory markers plus low hemoglobin (Hgb, open).

of women with 3 abnormal inflammatory markers and a low Hgb had an adverse event. Moreover, women with low Hgb and at least 1 abnormal inflammatory marker had significantly less survival free from adverse events compared to those without low Hgb and/or abnormal inflammatory markers ($p<0.001$, Figure 2).

During follow-up, there were 42 (7%) deaths with 48% of definite CV etiology. Women with no abnormal inflammatory markers had fewer deaths (1.6%) than women with 1 (6.1%), 2 (9.1%), or 3 (17%) abnormal inflammatory markers ($p<0.001$ for trend). Addition of low Hgb to each

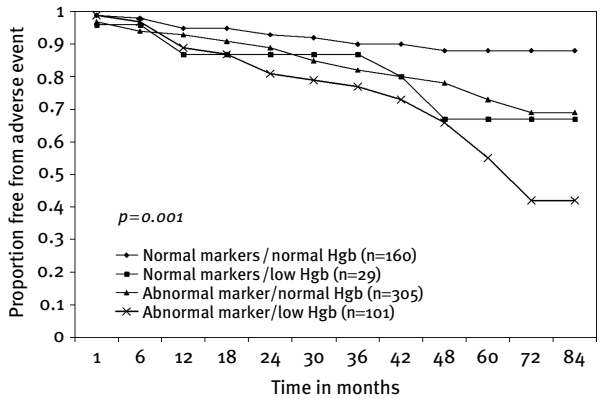


Figure 2. Proportion of women free from adverse events during follow-up by inflammatory marker and hemoglobin level using Kaplan-Meier methods. Adverse events defined by death or hospitalization for myocardial infarction, stroke, congestive heart failure, or other vascular event. Note that adding low hemoglobin (Hgb) level is associated with reduction in the proportion free of events.

category of abnormal markers was associated with an increase in all-cause mortality. For example, 24.2% of women with 3 abnormal inflammatory markers and a low Hgb died during follow-up.

### Unadjusted Predictors of Adverse CV Events and All-Cause Mortality

On univariate analysis using dichotomous variables, abnormal hs-CRP (HR: 2.09, 95% confidence intervals [CI]: 1.44–3.03), abnormal IL-6 (HR: 2.17, 95% CI: 1.48–3.18), abnormal SAA (HR: 1.81, 95% CI: 1.25–2.62), and low Hgb (HR: 2.05, 95% CI: 1.40–3.01) predicted adverse CV events (all $p<0.002$). Moreover, abnormal hs-CRP (HR: 2.80, 95% CI: 1.53–5.13), abnormal IL-6 (HR: 2.61, 95% CI: 1.34–5.10), abnormal SAA (HR: 4.53, 95% CI: 2.17–9.46), and low Hgb (HR: 2.69, 95% CI: 1.45–4.98) predicted all-cause mortality (all $p<0.01$). Using the upper quartile for each inflammatory marker, similar results were seen for hs-CRP (HR: 2.21, 95% CI: 1.52–3.20), IL-6 (HR: 2.52, 95% CI: 1.75–3.64), and SAA (HR: 2.22, 95% CI: 1.54–3.22) in predicting adverse CV events. Similar results were also seen using the upper quartile for hs-CRP (HR: 2.94, 95% CI: 1.61–5.39), IL-6 (HR: 3.55, 95% CI: 1.94–6.51), and SAA (HR: 3.13, 95% CI: 1.71–5.72) in predicting all-cause mortality.

### Adjusted Predictors of Adverse CV Events

On multivariate analysis in separate Cox regression models, using dichotomous variables, hs-CRP (HR: 1.86, 95% CI: 1.28–2.71; $p = 0.001$), IL-6 (HR: 1.84, 95% CI: 1.25–2.70; $p = 0.002$), and SAA (HR: 1.64, 95% CI: 1.13–2.38; $p = 0.01$) predicted adverse CV events. Again, using the upper quartile for each inflammatory marker in a multivariate analysis, similar results were found (data not shown). Since Hgb was associated with a higher rate of adverse events when added to abnormal inflammatory markers, we included Hgb as a fourth biomarker in addition to hs-CRP, IL-6, and SAA. On multivariate analysis (Figure 3), women with 1 (HR: 1.90, 95% CI: 1.04–3.46; $p = 0.04$), 2 (HR: 1.92, 95% CI: 1.02–3.60; $p = 0.04$), 3 (HR: 3.68, 95% CI: 2.00–6.77; $p = 0.001$), or 4 (HR: 5.50, 95% CI: 2.72–11.14; $p = 0.001$) abnormal biomarkers independently predicted adverse CV events. Using a multibiomarker approach was associated with higher adverse CV events than any single marker alone or other traditional CV risk factors including diabetes (HR: 1.79, 95% CI: 1.21–2.65; $p = 0.01$) and obstructive CAD (HR: 1.65, 95% CI: 1.12–2.42; $p = 0.01$).

### Adjusted Predictors of All-Cause Mortality

On multivariate analysis using dichotomous variables, hs-CRP (HR: 2.67, 95% CI: 1.44–4.96; $p = 0.002$), IL-6 (HR: 2.07, 95% CI: 1.05–4.08; $p = 0.04$), and SAA (HR: 3.75, 95% CI: 1.78–7.87; $p<0.001$) independently predicted all-cause mortality. Using the upper quartile for each inflammatory marker in a multivariate analysis, similar results were found


Published online in Wiley InterScience. (www.interscience.wiley.com)
DOI:10.1002/clc.20454 

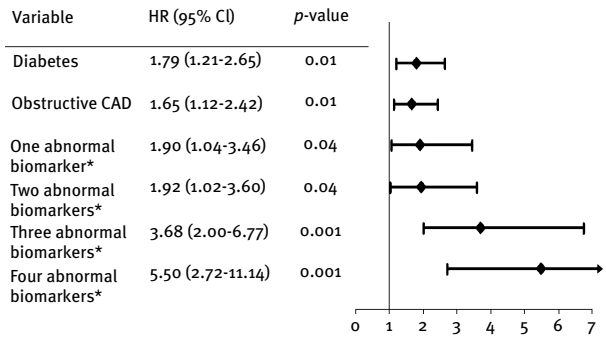


Figure 3. Significant independent predictors of adverse events. Shown are hazards ratio (HR), 95% confidence intervals (95% CI), and *p* values. *Abnormal biomarkers include hs-CRP, IL-6, SAA, and Hgb. Number of women with 1, 2, 3, and 4 abnormal biomarkers is 181, 131, 90, and 33, respectively compared to women with no abnormal markers (n=160).

(data not shown). When low Hgb was included as a fourth biomarker, women with 3 (HR: 11.13, 95% CI: 2.50–49.56; $p = 0.002$) or 4 (HR: 19.23, 95% CI: 4.08–90.70; $p<0.001$) abnormal biomarkers had significantly higher risk of all-cause mortality in comparison to women with no abnormal biomarkers (Figure 4). Using a multibiomarker approach, women with 3 or 4 abnormal biomarkers had a higher all-cause mortality rate than women with a single abnormal biomarker or obstructive CAD (HR: 3.23, 95% CI: 1.69–6.17; $p<0.001$) and creatinine (HR: 1.36, 95% CI: 1.06–1.74; $p = 0.02$).

### Traditional Risk Predictors to Predict CV events

Incremental value beyond the FRS, a traditional risk prediction tool that integrates most CV risk factors was 14.2 for the entire cohort corresponding to an overall 10 y risk of 4.6%. The FRS increased with increasing severity of CAD as would be expected for women with no, mild, and severe CAD having a corresponding FRS of 2.9%, 4.8%, and 6.4%, respectively. Higher FRS was associated with a more abnormal inflammatory marker level. Women with 0, 1, 2, 3, and 4 abnormal inflammatory markers had a corresponding FRS of 3.9%, 4.3%, 4.7%, 5.4%, and 6.9%, respectively. The number of abnormal biomarkers increased with increasing BMI, waist circumference, and waist/hip ratio (data not shown). However, neither BMI nor measures of abdominal obesity (waist circumference or waist hip ratio) were significant univariate predictors of adverse events and mortality.

On univariate analysis, FRS was a predictor of adverse events (HR = 1.08, $p = 0.0002$) and all-cause mortality, however on multivariate analysis, the FRS was not selected. When FRS was forced into the model with the biomarkers, the multimarker approach independently predicted adverse events and all-cause mortality. With FRS forced into the model, women with 3 (HR: 3.26, 95% CI: 1.75–6.06, $p = 0.0002$) or 4 (HR: 4.86, 95% CI: 2.38–9.92, $p<0.0001$) abnormal inflammatory markers were 3 to 5 times more likely to have an adverse CV event during follow-up. Furthermore, women with 3 (HR: 10.42, 95% CI: 2.33–46.52, $p = 0.002$) or 4 (HR: 16.47, 95% CI: 3.44–78.70, $p = 0.0004$) were >10 times more likely to die. Using ROC analysis, the FRS was associated with an overall accuracy predicting adverse events of 0.66 AUC, (Figure 5). However adding the combination of multiple biomarkers to the FRS improved the accuracy of predicting adverse events (AUC = 0.71, $p<0.0001$). The combination of biomarkers plus FRS was also associated with higher predictive accuracy for all-cause mortality (AUC 0.78 versus 0.72, $p<0.0001$).

## Discussion

A combination of multiple biomarkers, including hs-CRP, IL-6, SAA, and Hgb, is incrementally and independently

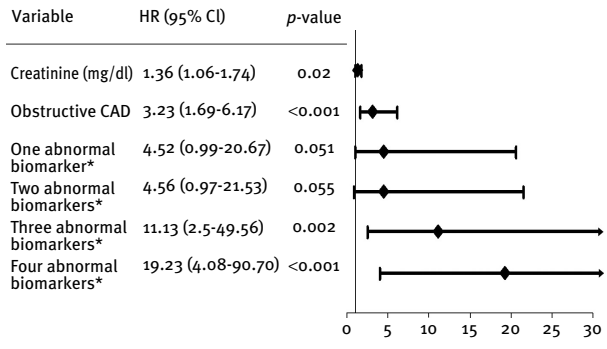


Figure 4. Significant independent predictors of all-cause mortality. Shown are hazards ratio (HR), 95% confidence intervals (95% CI), and *p* values. *Abnormal biomarkers include hs-CRP, IL-6, SAA, and Hgb. Number of women with 1, 2, 3 and 4 abnormal biomarkers was 181, 131, 90, and 33, respectively compared to women with no abnormal markers (n=160).

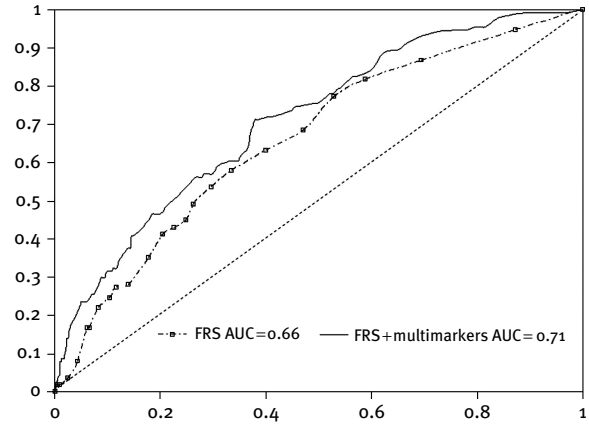


Figure 5. Receiver operating characteristics (ROC) analysis for Framingham Risk Score (FRS) alone and FRS plus multimarkers for prediction of adverse cardiovascular events.

associated with increased adverse events in the WISE cohort. Compared to women with no abnormal inflammatory markers, those with 4 abnormal biomarkers were >5 times more likely to have an adverse event and 19 times more likely to die during follow-up. Over 40% of women with 4 abnormal biomarkers had an adverse event, and a quarter died within 4 y. Moreover, women with low Hgb and at least 1 abnormal inflammatory marker had significantly worse event-free survival.

The FRS, a commonly used traditional risk factor predicting tool, markedly underestimated risk in this population. When added to the FRS, the biomarkers provided incremental prognostic information over that ascertained from traditional risk factors.

Elevated inflammatory marker levels and anemia appear to predict adverse events in a variety of conditions.[2,3,5–8] In patients presenting with acute myocardial infarction (AMI) and decompensated heart failure, adverse events are typically related to atherothrombotic coronary occlusion and impaired left ventricular function. However, in this cohort of clinically stable women only 35% had a flow limiting coronary stenosis ≥50%, and mean ejection fraction was 65%. In ACS, women's risk of events and response to invasive management are better predicted by inflammatory markers and brain natriuretic peptides than by traditional markers of myocardial necrosis (cardiac troponin or CK-MB).[14] One proposed explanation for this finding is that women may have more inflammation and microvascular coronary dysfunction underlying ACS, as opposed to atherothrombotic occlusion. Support for this notion has also been provided in a preliminary report from Burke et al. showing that women dying suddenly have more area of microfibrosis and microvascular embolic than observed in men.[15] In previous WISE analyses, women evaluated for suspected ischemia with nonobstructive CAD frequently had microvascular dysfunction.[16] Adverse effects of inflammation on vascular function may contribute to microvascular dysfunction and the high event rate seen in the WISE cohort. Mediators of inflammation are under complex regulation and are produced at various sites including endothelial cells, macrophages, adipocytes, and atherosclerotic plaques.[17,18] Each marker likely provides only a glimpse of the immunopathology underlying vascular disease so a multimarker approach likely provides more information about inflammation.

Furthermore, when added to traditional inflammatory markers, low Hgb levels added predictive value for risk. The cause for the low Hgb was not ascertained in the WISE cohort, it is likely that a high proportion of these cases were secondary to chronic disease. However, the additional prognostic information associated with low Hgb level, both alone and in a multimarker approach, suggests that Hgb mediates CV effects independent of the other markers.

### Study Limitations

This prospective observational study has inherent limitations of such a design and case selection. Thresholds for abnormal biomarker levels were from previous studies in different cohorts, so the optimal thresholds for risk stratification in this population may be different. In addition, this was a relatively small population of women with markers measured only at baseline and the influence on outcomes of changes in biomarkers over time is unknown. Also, these markers were measured after approximately 300 women were enrolled in the WISE, so women enrolled prior to ascertainment of marker levels may have died or been lost to follow-up as result of an adverse event (e.g., survival bias); therefore an underestimation of the event rate is possible. Finally, before these markers can be used in practice, the results must be confirmed in independent populations particularly where the risk is lower.

## Conclusion

In women undergoing coronary angiography for suspected ischemia, the cumulative number of abnormal biomarkers was associated with an incrementally increased risk of adverse events independent of traditional risk factors including diabetes and angiographic CAD. The multimarker approach provided more prognostic information than traditional risk factors. These markers relate to mediators of inflammation involved in development and progression of atherosclerosis and this information should stimulate interest in novel anti-inflammatory approaches to reducing residual risk.

### Acknowledgements

The authors thank Tjendimin Tjandrawan, BS for his laboratory assistance.

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Published online in Wiley InterScience. (www.interscience.wiley.com)
DOI:10.1002/clc.20454 

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Published online in Wiley InterScience. (www.interscience.wiley.com)
DOI:10.1002/clc.20454 