International Journal of Cardiology 167 (2013) 2904–2911

Contents lists available at ScienceDirect

International Journal of Cardiology

journal homepage: www.elsevier.com/locate/ijcard

# Cardiovascular risk prediction in the general population with use of suPAR, CRP, and Framingham Risk Score

Stig Lyngbæk [a,*], Jacob L. Marott [b], Thomas Sehestedt [c], Tine W. Hansen [d,g], Michael H. Olsen [e,i], Ove Andersen [f], Allan Linneberg [g,i], Steen B. Haugaard [f,h], Jesper Eugen-Olsen [f,i], Peter R. Hansen [a], Jørgen Jeppesen [e,i]

[a] Department of Cardiology, Copenhagen University Hospital Gentofte, Denmark
[b] Copenhagen City Heart Study, Copenhagen University Hospital Bispebjerg, Denmark
[c] Department of Cardiology, Copenhagen University Hospital Herlev, Denmark
[d] Department of Clinical Physiology and Nuclear Medicine and PET, Copenhagen University Hospital Rigshospitalet, Denmark
[e] Department of Medicine, Copenhagen University Hospital Glostrup, Denmark
[f] Clinical Research Center, Copenhagen University Hospital Hvidovre, Denmark
[g] Research Centre for Prevention and Health, Copenhagen University Hospital Glostrup, Denmark
[h] Department of Internal Medicine, Copenhagen University Hospital Amager, Denmark
[i] Faculty of Health Science, University of Copenhagen, Denmark

ARTICLE INFO

*Article history:*
Received 12 January 2012
Received in revised form 19 June 2012
Accepted 21 July 2012
Available online 19 August 2012

*Keywords:*
Biomarkers
Cardiovascular risk
C-reactive protein
Framingham Risk Score
Soluble urokinase plasminogen activator receptor

ABSTRACT

*Background:* The inflammatory biomarkers soluble urokinase plasminogen activator receptor (suPAR) and C-reactive protein (CRP) independently predict cardiovascular disease (CVD). The prognostic implications of suPAR and CRP combined with Framingham Risk Score (FRS) have not been determined.
*Methods:* From 1993 to 1994, baseline levels of suPAR and CRP were obtained from 2315 generally healthy Danish individuals (mean [SD] age: 53.9 [10.6] years) who were followed for the composite outcome of ischemic heart disease, stroke and CVD mortality.
*Results:* During a median follow-up of 12.7 years, 302 events were recorded. After adjusting for FRS, women with suPAR levels in the highest tertile had a 1.74-fold (95% confidence interval [CI]: 1.08–2.81, $p = 0.027$) and men a 2.09-fold (95% CI: 1.37–3.18, $p < 0.001$) increase in risk compared to the lowest tertile. Including suPAR and CRP together resulted in stronger risk prediction with a 3.30-fold (95% CI: 1.36–7.99, $p < 0.01$) increase for women and a 3.53-fold (1.78–7.02, $p < 0.001$) increase for men when both biomarkers were in the highest compared to the lowest tertile. The combined extreme tertiles of suPAR and CRP reallocated individuals predicted to an intermediate 10-year risk of CVD of 10–20% based on FRS, to low (<10%) or high (>20%) risk categories, respectively. This was reflected in a significant improvement of C statistics for men ($p = 0.034$) and borderline significant for women ($p = 0.054$), while the integrated discrimination improvement was highly significant ($P \leq 0.001$) for both genders.
*Conclusions:* suPAR provides prognostic information of CVD risk beyond FRS and improves risk prediction substantially when combined with CRP in this setting.



## 1. Introduction

It has become increasingly clear that inflammation plays a pivotal role in the development of atherosclerosis and cardiovascular disease (CVD). The biological processes involved in atherogenesis are highly complex and only partially understood. In addition to local inflammation in the vessel wall, a low level of chronic inflammation in several organs of the body, e.g. adipose tissue and the liver, is believed to contribute to the inflammatory pathology of atherosclerotic plaque formation in the vascular wall [1]. Since low-grade inflammation is reflected by elevated levels of circulating biomarkers, determining the level of inflammatory markers in plasma could provide prognostic information about future CVD risk [2]. C-reactive protein (CRP) measured with a highly sensitive assay is the most frequently used marker of low levels of inflammation and has been associated with increased risk of CVD as well as the presence of CVD risk factors including adiposity [3]. Furthermore, CRP is used as an adjunct for risk assessment in conjunction with the Framingham Risk Score (FRS) [4].

New biomarkers have emerged which predict CVD independently of CRP presumably reflecting other aspects of the atherosclerotic process [5]. We have in a recent study shown that a new inflammatory

* Corresponding author at: Department of Cardiology, Copenhagen University Hospital Gentofte, Niels Andersens Vej 65, 2900 Hellerup, Denmark. Tel.: +45 39 77 22 74; fax: +45 39 77 76 42.
*E-mail address:* stiglyngbaek@gmail.com (S. Lyngbæk).

0167-5273/$ – see front matter © 2012 Elsevier Ireland Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.ijcard.2012.07.018

biomarker soluble urokinase plasminogen activator receptor (suPAR) is associated with risk of CVD, type 2 diabetes, cancer and all-cause mortality independently of CRP in a generally healthy population [6]. After adjustment for FRS variables, suPAR remained associated with incident CVD. Interestingly, suPAR was not related to CVD risk factors like overweight and abdominal obesity (measured as body mass index and waist circumference) as opposed to CRP [7]. Instead, suPAR seemed to be linked to endothelial dysfunction and presence of atherosclerotic plaques thus potentially representing later occurring processes in the development of CVD [7]. Consequently, it is plausible that the two biomarkers suPAR and CRP reflect different aspects of the pathophysiological mechanisms leading to atherosclerosis and CVD. The present study is an extension of our first study aimed at CVD prediction [6], and here we examine if suPAR in combination with CRP supplements FRS for CVD risk prediction.

## 2. Patients and methods

### 2.1. Study population

In the period from 1982 to 1984, 4581 randomly sampled women and men from Copenhagen County, Denmark, were invited to take part in the monitoring of trends and determinants in cardiovascular disease (MONICA) health survey [8]. In accordance with the MONICA protocol, participants represented an equal number of women and men aged 30, 40, 50 and 60 years. In total, 3785 (83.0%) individuals participated. In 1993 and 1994, the original sample was re-invited to take part in a follow-up examination, which served as the baseline examination for this prospective study. Since the first examination, 428 subjects had died and 23 had moved or could not be reached. Of the remaining 4130 subjects from the original invited sample, 2656 (64.3%) were willing to participate. All participants underwent a brief physical examination, had blood samples drawn, completed a questionnaire regarding current and previous diseases to ascertain baseline comorbidities, use of medication, and presence and absence of CVD risk factors. Three-hundred and forty-one subjects were excluded from the analyses due to a previous diagnosis of myocardial infarction or stroke, medical treatment with digoxin or nitrates (as a proxy for prevalent heart disease), or no serum CRP or plasma suPAR measurements available, leaving 2315 women and men to be included in the study. The study was conducted in accordance with the Second Helsinki Declaration and was approved by the ethics committee for Copenhagen County. Written informed consent was obtained from all participants. The authors of this manuscript have certified that they comply with the principles of ethical publishing in the International Journal of Cardiology.

### 2.2. Measurements and data collection

All laboratory measures were done in centralized laboratories. Venous blood samples were collected after overnight fasting and analyzed by standard laboratory methods for levels of lipids, glucose, and CRP, as previously described [9]. In brief, serum CRP concentrations were determined using a particle-enhanced immunoturbidimetry assay with a lower detection limit of 0.03 mg/L. CRP was analyzed from frozen samples, in which CRP has previously been shown to be stable [9].

Plasma levels of suPAR were measured from frozen samples using the commercially available suPARnostic® kit, according to the manufactures instructions (ViroGates, Copenhagen, Denmark). The technician was blinded to the identity of the patient. The intra-assay variation was 2.75% and inter-assay variation was 9.17%. The standard curve of the assay was validated to measure suPAR levels between 0.6 and 22.0 ng/mL. The time of sample freezing did not appear to influence the plasma level of suPAR as evidenced by lack of correlation between suPAR levels and the date of plasma sampling from 14th of June 1993 to 2nd of December 1994 (Spearman $r = 0.001$; $p = 0.96$).

A brief physical examination was performed by trained staff and included anthropometric measurements (weight, height, waist and hip circumference), and blood pressure measurement in the sitting position after 5 min of rest using a random zero mercury sphygmomanometer. The mean of two measurements was reported. Information about lifestyle risk factors including smoking habits was obtained from the questionnaire.

### 2.3. Outcome

Complete follow-up with respect to death was obtained from the Civil Registration System. Information on cardiovascular mortality was obtained from the National Death Register. Information on hospitalizations was obtained from the National Patient Register. The pre-specified outcome in this study was the combination of cardiovascular mortality, ischemic heart disease (ICD-8 code 410 to 414 or ICD-10 codes I20 to I25), and stroke (ICD-8 codes 431, 433, or 434 or ICD-10 codes I61 or I63).

### 2.4. Definitions of clinical conditions

Presence of diabetes mellitus at baseline was defined as having a diagnosis of diabetes, based on national register information or made by the participants' own physician, or reported use of anti-diabetic drugs in the questionnaire and/or having a fasting plasma glucose $\geq 7.0$ mmol/L.

With respect to FRS, we used the risk score profile developed for 10-year risk of coronary heart disease (CHD) [10] and not the risk score profile developed for general CVD, as the CHD algorithm better predicted our composite outcome (hazard ratio [HR] 1.27 (95% confidence interval [CI] 1.23–1.32) vs. HR 1.21 (95% CI 1.18–1.24)) per FRS point ($p = 0.044$).

### 2.5. Statistical analyses

Calculation of differences between baseline characteristics is presented as means ± standard deviations (SDs), medians with 5 and 95 percentiles, or percentage frequencies. Normally distributed values were compared with Student's t-tests, and skewedly distributed continuous variables by non-parametric rank sum tests. Chi-square tests or Fisher's exact test were used for categorical variables. The relationship between suPAR and CRP at baseline was explored using Spearman correlation analysis. Tertiles of suPAR and CRP were defined for each gender separately, and overall we present gender-specific data. Cox proportional hazard models were used for the analyses of the relationship between the composite outcome and tertiles (categorical) of suPAR, CRP and their combination (both biomarkers in the lowest tertile [1. combined tertile], both in the highest tertile [3. combined tertile], and all other combinations of tertiles [2. combined tertile]) adjusted for FRS points. Survival curves, based on Cox models adjusted for FRS and stratified by tertiles of suPAR and CRP, were generated for the composite outcome [11]. P values for trend were based on the linear inclusion of tertiles of biomarkers into the Cox proportional hazard models. The assumption of proportionality in the Cox regression models was assessed with the score process test [12]. If participants experienced multiple events, only the first event was considered. The risk of CVD for the combined event within 10 years was estimated for tertiles of suPAR, CRP, and their combination based on three risk categories of FRS (<10%, 10–20%, and >20%), as described previously [13]. The functional form of the FRS points was tested to check model assumptions. Interaction between biomarkers (in tertiles), Framingham risk category or FRS was tested with the Wald test. Linear trends were tested as mentioned previously.

To determine the absolute risk of CVD in 10 years, new risk models were constructed based on Framingham risk category (low, intermediate or high risk) or FRS and the addition of biomarker levels according to tertiles of suPAR, CRP, and their combination as described above. The predictive performance of these models were summarized using Harrell's C statistics, which is conceptually analogous to the area under curve (AUC) estimated from logistic models, but allows for right-censored data and variable follow-up [14]. Model fit was then tested using the Grønnesby–Borgan statistic, where significant P values suggest poor model calibration [15]. In our MONICA study, the global risk was generally slightly lower than that in the Framingham study, meaning that for the same number of FRS points the CVD risk was lower (data not shown). However, the model based exclusively on FRS points and the expanded model, including biomarkers, were found to be very well calibrated (all $p > 0.05$ by the goodness-of-fit test by Grønnesby–Borgan statistics), and the risk of CVD (based on FRS) within 10 years in our cohort corresponded to the risk of the FRS categories (Fig. 2, white columns). Thus, no recalibration of FRS points was needed for men or women. Furthermore, we calculated category-free net reclassification improvement (NRI) when tertiles of suPAR and CRP was added to FRS [16]. NRI is a measure of the capability of the expanded model to correctly reclassify individuals with and without events during follow-up into higher or lower risk, respectively. The expected number of events and non-events were used in the estimation of NRI to account for censored data and calculated by multiplying the total number of people by the Kaplan–Meier rates at end of follow-up. This approach was found optimal for assessing calibration of survival models [17]. We estimated the bias-corrected confidence intervals for NRI by bootstrap resampling (1000 replicates). Finally, we estimated the integrated discrimination improvement (IDI) [18], which is the difference in the proportion of variance explained by the expanded and the traditional model. All statistical tests were two-sided and significance was concluded for values of $p < 0.05$.

All analyses were performed with the Statistical Analysis System (SAS), version 9.1 (SAS Institute, Cary, North Carolina, USA) or R version 2.15.0 (R Foundation for Statistical Computing, Vienna, Austria).

## 3. Results

### 3.1. Study duration and the composite outcome

The median study duration, from baseline until follow-up on 31st December 2006, was 12.7 years (5 and 95 percentiles: 4.0–13.4 years). During follow-up, 302 combined events were recorded, consisting of 81 CVD deaths, 167 cases with nonfatal ischemic heart disease, including 69 acute myocardial infarctions, and 54 nonfatal strokes. In total 229 non-CVD deaths were observed.

### 3.2. Baseline characteristics and biomarkers

Baseline characteristics stratified by gender are summarized in Table 1. Of note, FRS starts with negative values, because young healthy women are assigned negative points (http://www.framinghamheartstudy.org/index.html). The Spearman correlation

**Table 1**
Baseline characteristics stratified by gender.

| Variables | Women (n = 1168) | Men (n = 1147) | P* |
|---|---|---|---|
| Age, years | 53.8 ± 10.6 | 54.1 ± 10.6 | 0.57 |
| Total cholesterol, mmol/L | 6.15 ± 1.12 | 6.16 ± 1.10 | 0.85 |
| HDL cholesterol, mmol/L | 1.54 (1.00–2.37) | 1.26 (0.85–1.92) | <0.0001 |
| Systolic blood pressure, mm Hg | 127.1 ± 19.0 | 131.0 ± 18.7 | <0.0001 |
| Diabetes, % | 2.4 | 5.0 | 0.001 |
| Smoking, % | 43.1 | 49.1 | 0.004 |
| Framingham Risk Score, points | 7.0 (−3.0 to 12.0) | 6.0 (2.0–12.0) | 0.005 |
| C-reactive protein, mg/L | 1.73 (0.30–12.8) | 1.74 (0.35–10.9) | 0.71 |
| suPAR, ng/mL | 4.46 ± 1.35 | 4.06 ± 1.36 | <0.0001 |

Data are presented as means ± standard deviations for continuous variables with normal distribution, medians (5%–95% percentiles) for continuous variables with skewed distribution, and proportions in percent for categorical variables. HDL = high-density lipoprotein; suPAR = soluble urokinase plasminogen activator receptor.
* P-value for difference between groups.

coefficient between suPAR and CRP concentrations were 0.30 ($p<0.0001$) in women and 0.36 in men ($p<0.0001$). To discern baseline characteristics related to CRP from those related to suPAR, we also stratified our study population by the following combinations of biomarker levels: lowest tertile of CRP and highest tertile of suPAR, highest tertile of CRP and lowest tertile of suPAR, and all other combinations. Table 2 shows that high CRP was associated with metabolic factors, such as higher body mass index, increased waist circumference, and higher triglyceride levels, whereas high suPAR was associated with a higher prevalence of smoking. Finally, baseline characteristics stratified by event status are shown in Table 3. Participants with events had higher FRS and higher levels of suPAR and CRP at baseline.

### 3.3. Biomarkers and outcome

Survival curves adjusted for FRS based on Cox proportional hazards models are shown for tertiles of suPAR, CRP and their combination (Fig. 1). From these curves, it is apparent that even after adjustment for FRS, tertiles of both biomarkers and especially their combination notably distinguished individuals at increased risk. It is clear, however, that women had less risk of the combined event and demonstrated less separation of risk with increasing tertiles of biomarker levels compared to men (all p for interaction $<0.0001$). This was particularly evident with increasing tertiles of CRP in women, which showed no significant relationship with the composite outcome ($p = 0.14$).

In Table 4, we present the results of the Cox proportional hazard models with tertiles of suPAR, CRP, and their combination adjusted for FRS. The results of the Cox analyses demonstrated that the addition of biomarkers, except for CRP in women, significantly increased risk prediction beyond FRS, and that the combination of suPAR and CRP was associated with an approximately 3.5-fold increase in risk for both genders.

**Table 3**
Baseline characteristics stratified by event status.

| Variables | No event (n = 2013) | Event (n = 302) | P* |
|---|---|---|---|
| Female sex, % | 52.6 | 36.4 | <0.0001 |
| Age, years | 53.0 ± 10.4 | 60.4 ± 9.8 | <0.0001 |
| Total cholesterol, mmol/L | 6.10 ± 1.09 | 6.50 ± 1.19 | <0.0001 |
| HDL cholesterol, mmol/L | 1.46 ± 0.41 | 1.37 ± 0.40 | <0.001 |
| Systolic blood pressure, mm Hg | 127.5 ± 18.3 | 139.0 ± 20.1 | <0.0001 |
| Diabetes, % | 2.8 | 9.6 | <0.0001 |
| Smoking, % | 44.7 | 55.3 | <0.001 |
| Framingham Risk Score, points | 6.0 (−1.0 to 11.0) | 9.0 (4.0–13.0) | <0.0001 |
| C-reactive protein, mg/L | 1.61 (0.31–11.1) | 2.63 (0.40–13.8) | <0.0001 |
| suPAR, ng/mL | 3.93 (2.61–6.48) | 4.53 (2.86–7.86) | <0.0001 |

Data are presented as mean ± standard deviation for continuous variables with normal distribution, median (5%–95% percentile) for continuous variables with skewed distribution, and proportions in percent for categorical variables. HDL = high density lipoprotein; suPAR = soluble urokinase plasminogen activator receptor.
* P-value for difference between groups.

**Table 2**
Baseline characteristics stratified by combinations of biomarkers.

| Women | | | | |
|---|---|---|---|---|
| Variables | Lowest CRP and highest suPAR tertile (n = 76) | All other combinations (n = 1004) | Highest CRP and lowest suPAR tertile (n = 88) | P* |
| Age, years | 53.6 ± 11.5 | 53.9 ± 10.6 | 52.3 ± 9.9 | 0.15 |
| Total cholesterol, mmol/L | 6.27 ± 1.17 | 6.16 ± 1.12 | 5.98 ± 1.07 | 0.10 |
| HDL cholesterol, mmol/L | 1.61 ± 0.40 | 1.60 ± 0.42 | 1.53 ± 0.36 | 0.15 |
| Triglycerides, mmol/L | 0.96 (0.64–1.92) | 1.09 (0.58–2.55) | 1.25 (0.65–2.52) | 0.005 |
| Systolic blood pressure, mm Hg | 124.8 ± 22.1 | 127.0 ± 18.9 | 130.4 ± 17.6 | 0.059 |
| Body mass index, kg/m$^2$ | 23.0 (18.3–28.2) | 24.6 (19.7–34.3) | 25.2 (21.2–36.6) | <0.0001 |
| Waist circumference, cm | 75.5 ± 8.0 | 81.6 ± 11.3 | 83.4 ± 10.3 | <0.0001 |
| Diabetes, % | 1.3 | 2.6 | 1.1 | 0.57 |
| Smoking, % | 68.4 | 42.7 | 25.0 | <0.0001 |
| Framingham Risk Score, points | 7.0 (−1.0 to 12.0) | 7.0 (−3.0 to 12.0) | 6.0 (−3.00 to 12.0) | 0.92 |
| C-reactive protein, mg/L | 0.61 (0.23–0.93) | 1.69 (0.30.11.7) | 4.78 (3.29–36.3) | <0.0001 |
| suPAR, ng/mL | 5.36 (4.74–7.91) | 4.25 (2.90–6.90) | 3.37 (2.73–3.73) | <0.0001 |
| Men | | | | |
| Variables | Lowest CRP and highest suPAR tertile (n = 83) | All other combinations (n = 991) | Highest CRP and lowest suPAR tertile (n = 73) | P* |
| Age, years | 54.3 ± 11.3 | 54.3 ± 10.5 | 51.0 ± 10.3 | 0.011 |
| Total cholesterol, mmol/L | 5.99 ± 1.03 | 6.17 ± 1.10 | 6.16 ± 1.23 | 0.14 |
| HDL cholesterol, mmol/L | 1.43 (0.94–2.05) | 1.26 (0.85–1.90) | 1.14 (0.83–1.88) | 0.0001 |
| Triglycerides, mmol/L | 1.15 (0.63–4.60) | 1.30 (0.65–3.31) | 1.49 (0.59–3.66) | 0.045 |
| Systolic blood pressure, mm Hg | 128.3 ± 19.7 | 131.3 ± 18.7 | 130.7 ± 17.1 | 0.16 |
| Body mass index, kg/m$^2$ | 24.1 ± 2.9 | 26.6 ± 3.7 | 28.7 ± 4.4 | <0.0001 |
| Waist circumference, cm | 88.9 ± 8.6 | 94.0 ± 10.4 | 98.7 ± 12.6 | <0.0001 |
| Diabetes, % | 7.2 | 4.7 | 5.5 | 0.59 |
| Smoking, % | 66.3 | 48.6 | 35.6 | <0.001 |
| Framingham Risk Score, points | 7.0 (4.0–11.0) | 7.0 (2.0–12.0) | 6.0 (2.0–11.0) | 0.047 |
| C-reactive protein, mg/L | 0.66 (0.35–1.02) | 1.77 (0.34–11.1) | 4.41 (2.87–13.4) | <0.0001 |
| suPAR, ng/mL | 4.74 (4.25–6.49) | 3.79 (2.52–6.53) | 2.89 (2.43–3.30) | <0.0001 |

Data are presented as mean ± standard deviation for continuous variables with normal distribution, median (5 and 95 percentile) for continuous variables with skewed distribution, and proportions in percent for categorical variables. CRP = C-reactive protein; suPAR = soluble urokinase plasminogen activator receptor; HDL = high density lipoprotein.
* P-value for difference between groups.

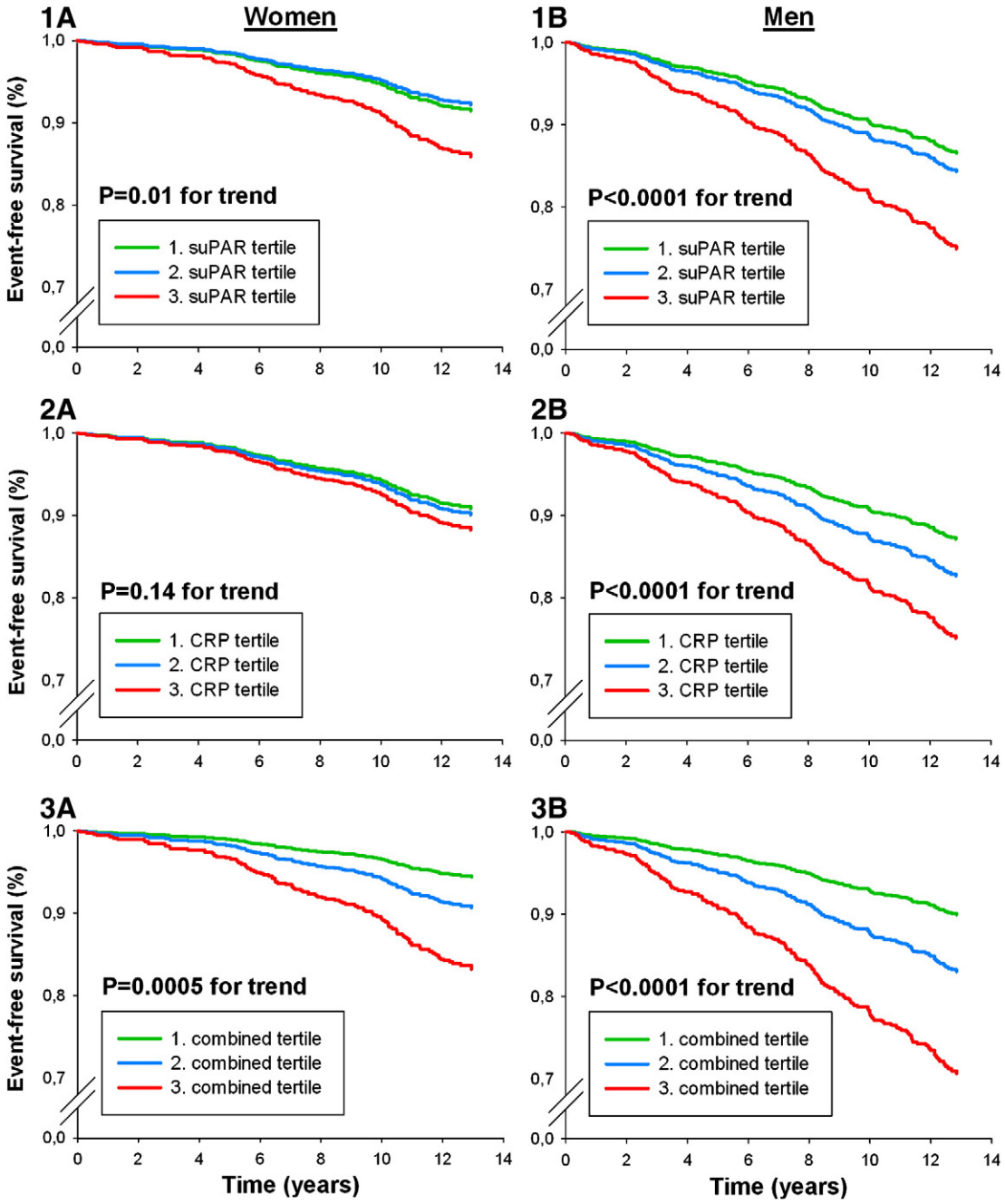


**Fig. 1.** Gender specific survival curves for the composite outcome adjusted for FRS (Framingham Risk Score) by Cox proportional hazard models for suPAR, CRP, and combined tertiles. Survival curves for the composite outcome stratified by tertiles of suPAR (1A and 1B), CRP (2A and 2B) and suPAR/CRP (3A and 3B) for women (left column) and men (right column) adjusted for FRS by Cox proportion hazard models. CRP = C-reactive protein; suPAR = soluble urokinase plasminogen activator receptor.

The strength of univariate associations between suPAR and the composite outcome for each gender was similar to the relation of suPAR with the individual endpoints (cardiovascular mortality, ischemic heart disease and stroke – data not shown).

### 3.4. Risk of composite outcome in different Framingham Risk Categories

To characterize the predictive capabilities of suPAR and CRP within different risk strata, the participants were grouped according to Framingham risk categories (risk of composite outcome in 10 years of <10%, 10–20%, and >20%), as depicted in Fig. 2. For the Framingham risk categories, the risk increased significantly with increasing tertiles of suPAR, CRP, and their combination during 10 years of follow-up. Of note, with increasing risk category individuals with both biomarkers in the lowest tertile had substantial absolute risk reductions compared to individuals with coincident levels of suPAR and CRP both in the highest tertile, e.g., in the Framingham risk category with a 10-year risk of 10–20% for the composite outcome, the combination of suPAR and CRP in their lowest tertiles persistently defined individuals with risk of CVD less than 10% within 10 years compared to a risk of >20% when both biomarkers were in their highest tertiles.

### 3.5. Risk discrimination and reclassification

The C statistics for men improved significantly from 0.722 (95% CI 0.686–0.757) when tertile levels of suPAR (0.733 [95% CI 0.699–

**Table 4**
Framingham Risk Score combined event.

| Men (range of tertiles of biomarker) | Hazard ratio (95% CI) | P* |
|---|---|---|
| suPAR 1st tertile (1.34–3.34 ng/mL) | 1 | – |
| suPAR 2nd tertile (3.35–4.35 ng/mL) | 1.19 (0.76–1.86) | 0.44 |
| suPAR 3rd tertile (4.36–17.80 ng/mL) | 2.09 (1.37–3.18) | <0.001 |
| CRP 1st tertile (0.12–1.12 mg/L) | 1 | – |
| CRP 2nd tertile (1.13–2.81 mg/L) | 1.41 (0.93–2.14) | 0.11 |
| CRP 3rd tertile (2.82–92.55 mg/L) | 2.19 (1.47–3.25) | 0.0001 |
| suPAR/CRP both 1st tertiles | 1 | – |
| suPAR/CRP other tertile combinations | 1.80 (0.93–3.46) | 0.08 |
| suPAR/CRP both 3rd tertiles | 3.53 (1.78–7.02) | <0.001 |
| **Women (range of tertiles of biomarker)** | **Hazard ratio (95% CI)** | **P*** |
| suPAR 1st tertile (1.91–3.77 ng/mL) | 1 | – |
| suPAR 2nd tertile (3.77–4.72 ng/mL) | 0.91 (0.53–1.57) | 0.73 |
| suPAR 3rd tertile (4.73–19.95 ng/mL) | 1.74 (1.08–2.81) | 0.027 |
| CRP 1st tertile (0.13–1.00 mg/L) | 1 | – |
| CRP 2nd tertile (1.01–2.97 mg/L) | 1.24 (0.74–2.10) | 0.41 |
| CRP 3rd tertile (2.98–98.45 mg/L) | 1.46 (0.88–2.41) | 0.14 |
| suPAR/CRP both 1st tertiles | 1 | – |
| suPAR/CRP other tertile combinations | 1.72 (0.74–3.99) | 0.21 |
| suPAR/CRP both 3rd tertiles | 3.30 (1.36–7.99) | <0.01 |

All variables are adjusted for Framingham Risk Score points. CI = confidence interval; suPAR = soluble urokinase plasminogen activator receptor; CRP = C-reactive protein; all tertiles are gender-specific tertiles.
* P-value compared to reference group.

0.769], p = 0.038), CRP (0.734 [95% CI 0.699–0.769], p = 0.037) and their combination (0.737 [95% CI 0.703–0.772], p = 0.034) were added to FRS. A similar trend was seen for women, but did not reach statistical significance (C statistics for model with FRS 0.717 [95% CI 0.674–0.759]; suPAR 0.730 [95% CI 0.687–0.774], p = 0.114; CRP 0.724 [95% CI 0.679–0.769], p = 0.262; and their combination 0.734 [95% CI 0.691–0.778], p = 0.054).

Reclassification by adding the biomarkers to FRS was assessed by improvement in category-free NRI and IDI. Category-free assessment of NRI and IDI resulted in a robust improvement of reclassification for suPAR for men (NRI 0.506 [95% CI 0.240, 0.772], p<0.001; IDI 0.016 [95% CI 0.005–0.026], p = 0.003) and for women (NRI 0.571 [95% CI 0.253–0.889], p<0.001; IDI 0.012 [95% CI 0.003–0.021] p<0.01). Similar results were obtained for CRP in men (NRI 0.308 [95% CI 0.081–0.534], p<0.01; IDI 0.018 [95% CI 0.008–0.028], p<0.001) but not in women (NRI −0.083 [95% CI −0.354, 0.189], p = 0.55; IDI 0.004 [95% CI −0.001–0.008]; p = 0.058). Finally, the combination of tertiles of suPAR and CRP in men did not improve NRI but led to a highly significant improvement for IDI (NRI 0.010 [95% CI −0.077–0.010], p = 0.82; IDI 0.022 [95% CI 0.009–0.035], p<0.001), whereas the combination of biomarkers improved both NRI and IDI significantly in women (NRI 0.136 [95% CI 0.003–0.269], p = 0.045; IDI 0.019 [95% CI 0.007–0.030], p = 0.001).

### 3.6. Additional analyses

When new biomarkers are tested against established risk scoring systems such as FRS, which was validated on another cohort, this might introduce a potential advantage for the new risk markers because these are adjusted for the specific population under investigation. The purpose of the current study was, however, to enable clinicians to appreciate the change in risk prediction offered by the two inflammatory biomarkers when added to FRS points. Nevertheless, we tested if a model with the individual FRS covariates (and thus adjusted for our cohort) instead of prespecified FRS points improved the parameter risk estimates for the composite outcome. For male subjects the parameter estimates (standard error) for the combination of suPAR and CRP were similar whether individual FRS variables or FRS points were included in the models, e.g., the parameter estimate was 1.29 (0.35) for both biomarkers being in their highest tertile when individual FRS variables were included, as opposed to 1.28 (0.35) when FRS points were included. In women, the corresponding values using FRS were 0.87 (0.46) versus 1.19 (0.45), suggesting less accuracy of FRS in women compared to men. In order to test if a risk score developed for a European population would be more suitable for risk prediction in our cohort, the European Heart Score (SCORE) and FRS (points) were included in the same model, but only FRS significantly predicted outcome (data not shown).

## 4. Discussion

suPAR and CRP are two inflammatory biomarkers that seem to represent different biological processes in atherogenesis and both independently predict CVD after adjusting for FRS. In this pathophysiological perspective, the principal finding of this study is that the combined use of suPAR and CRP was able to further improve risk stratification based on FRS in the general population. Both biomarkers had a graded relationship with the incidence of CVD and this effect was evident for individuals at low, intermediate, and high risk of CVD within 10 years. The combined use of suPAR and CRP enabled a significant improvement in risk prediction on top of FRS. The C statistic, a measure of the discriminatory capability of the prediction models, improved significantly with the addition of suPAR, CRP and their combination to a model based on FRS in men, whereas only the combination of biomarkers reached borderline significance in women.

In agreement with our data, suPAR has recently been shown to be a strong predictor of CVD and mortality in healthy men participating in a prostate cancer-screening program [19] and in the elderly general population [20]. As previously mentioned, suPAR is also a marker of cancer, diabetes, as well as all-cause mortality in the general population [6]. Moreover, suPAR predicts outcome in patients in intensive care [21] and in patients with bacteriemia [22]. Thus, suPAR is an unspecific but important predictor of adverse events and seems to be more potent in this regard in patients with severe underlying diseases. In view of our current results, it is therefore possible, that suPAR may be even better for prediction of future CVD and death in a population at increased risk of CVD, e.g. patients with known ischemic heart disease.

The origin of circulating suPAR is unknown but varying tissues and cell types, such as T-cells, macrophages and endothelial cells are likely to be contributory depending on the underlying pathology [23–27]. The source of circulating suPAR is thus likely to differ between healthy stable individuals [6,19] and seriously ill patients [21,22]. In the general population, we have shown that suPAR and CRP are very differently related to anthropometric and subclinical cardiovascular damage, while both independently associated with CVD risk [7]. Indeed, circulating suPAR levels are associated with subclinical organ damage manifested as carotid atherosclerotic plaques and increased urine albumine/creatine ratio [28]. We therefore speculate that suPAR may be an integral biomarker of vascular inflammation while CRP mainly reflects inflammation associated with metabolic disturbances. Whether suPAR might be directly implicated in disease progression, i.e. a maker and not merely a marker of disease, is unclear at present. However, new data have linked suPAR to focal segmental glomerulosclerosis in the kidneys by activation of integrin beta-3 on podocytes [29]. Although similar activation by suPAR of integrin beta-3 on endothelial cells and thrombocytes could potentially contribute to vascular inflammation and thrombosis, the role of suPAR in CVD clearly requires further study.

### 4.1. Clinical implications

The treatment goals for lipid-lowering in primary prevention depend on the Framingham risk category and pharmacological lipid-lowering treatment is usually recommended for individuals with a 10-year risk of CVD > 20% [30]. With respect to hypertension a particular rigorous blood pressure-lowering treatment to a target level of 130/80 mm Hg is recommended for individuals with a predicted 10-year risk of CVD > 10% [31]. Thus, the clinical implications of our findings

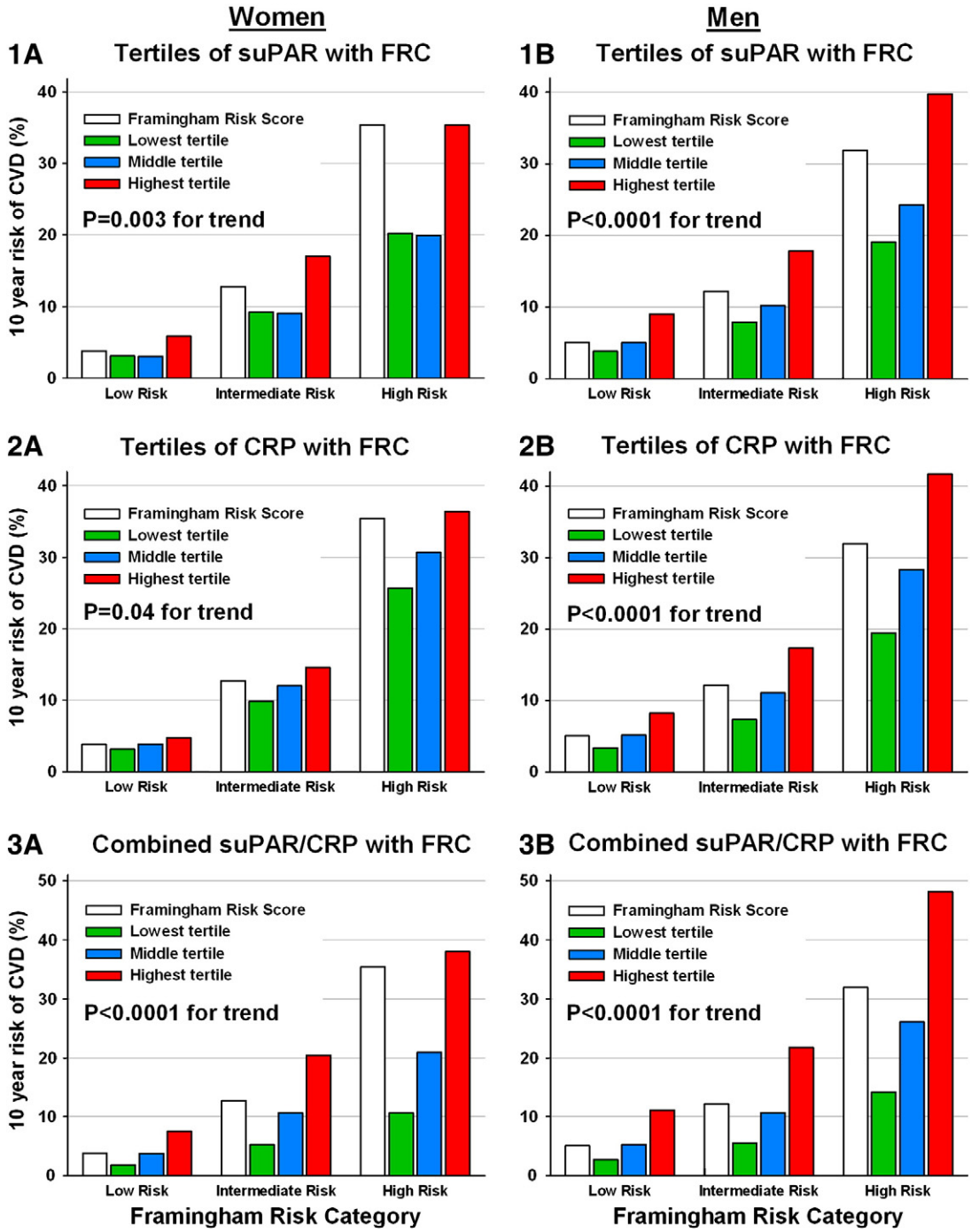


**Fig. 2.** Gender specific cumulative 10-year cardiovascular disease risk for Framingham Risk Score risk categories (FRCs) stratified by suPAR, CRP, and combined tertiles. 10-year risk of CVD with increasing tertiles (lowest tertile, green bar; middle tertile, blue bar; and highest tertile, red bar) of suPAR (1A and 1B), CRP (2A and 2B), and suPAR/CRP (3A and 3B) depending on FRC (low risk 0–10%, intermediate risk 10–20%, and high risk > 20%) for women (left) and men (right). Framingham Risk Score (FRS) is shown for comparison (white bar). CRP = C-reactive protein; suPAR = soluble urokinase plasminogen activator receptor.

are particularly evident for individuals with a predicted 10-year risk of CVD of 10–20% based on FRS and values of both suPAR and CRP in either the highest or lowest tertile. Individuals in this intermediate risk category will reallocate to the high-risk category (resulting in primary prevention with lipid-lowering drugs and rigorous antihypertensive treatment) when both suPAR and CRP are in their highest tertiles, and to the low-risk category when both biomarkers are in their lowest tertiles (Fig. 2). These reallocations are only partially reflected by a modest but significantly improved NRI in women for the combined use of suPAR and CRP, whereas NRI for men did not reach statistical significance even though both suPAR and CRP each markedly improved NRI. This probably reflected that the combined use of tertiles as applied in our study left 2/3 of the subjects in the second combined tertile, i.e. all other combinations of suPAR and CRP different from the combined lowest or highest tertiles, from where patients are likely to be incorrectly assigned lower or higher risk of suffering the outcome. This interpretation is supported by the highly significant improvement in IDI with the combined use of suPAR and CRP in addition to a model based on FRS, which reflected that the expanded model explained a significantly higher proportion of the model variance compared to the model consisting of FRS only. The combination of suPAR and CRP therefore enabled an improved absolute prognostication for patients at intermediate

and high risk of CVD within 10 years. The combination of these two biomarkers could prove useful for future enhanced prediction of CVD.

### 4.2. Use of FRS in an European population

To aid clinicians estimate the CVD risk of patients, several risk prediction scores have been developed [10,32]. FRS is probably the most widely known, but the SCORE risk chart algorithm is preferred in Europe [32]. The appropriateness of using a risk score program in other populations than the population in which the particular score program was originally developed has been discussed, because the CVD risk in a community sample may differ due to differences in presence of underlying predisposing (genetic and nongenetic) risk factors. Nevertheless, it is not possible for every community to develop its own risk prediction score and some degree of extrapolation has to be accepted. As an example of how different risk scoring systems allocate individuals to different risk categories, Manzoli et al. [33] recently reported that in an extensive Italian survey, the agreement between the SCORE and the CUORE risk chart (a risk score method based on a risk function derived from several Italian cohorts) was only moderate (kappa = 0.51). In the present study, we used FRS for coronary heart disease, because this risk prediction score is used worldwide and, in addition, it predicted CVD risk better than the European based SCORE risk chart in our cohort. In order not to overestimate the predictive potential of a new risk factor, it should ideally be tested when used on top of the risk score system with the best prediction capability as done in the present study. Moreover, FRS was well calibrated in our cohort and the reasonable agreement between parameter estimates, whether based on FRS covariates adjusted for the present cohort or on the original FRS points, supports the notion that use of the easily applicable FRS in our European population was a methodologically sound approach.

### 4.3. Study limitations

The relative uniformity in ethnicity and socioeconomic status of the participants in this study might make these results less valid for other populations. The statistical power was limited by the relatively few number of CVD events in a relatively young and otherwise healthy population. The follow-up of the study patients relied on the accuracy of the diagnoses listed in the Danish national registries, and the diagnosis of MI in this setting has been validated previously [34]. Moreover, use of national registers for follow-up did not allow for detailed assessment of the causes of death. We had only one blood sample from each participant in the study and are therefore not able to investigate how temporary changes in biomarkers blood levels might impact outcome. Furthermore, both suPAR and CRP are unspecific inflammatory biomarkers and might momentarily increase in response to transient diseases that eventually do not portend increased risk of CVD. Finally, we have chosen to adjust the statistical models for FRS. We are thus not able to determine if other established risk factors for CVD, e.g. kidney function and physical inactivity, impact the predictive capabilities of the biomarkers.

## 5. Conclusion

The inflammatory biomarkers suPAR and CRP and their combination improve absolute risk prediction in a general healthy population beyond that of Framingham Risk Score.

## Sources of funding

This study was funded by grants from the Danish Heart Foundation, the Danish Medical Association Research Fund/Volten, the Danish Pharmaceutical Association, the Lundbeck Foundation, and the Novo Nordisk Foundation. The suPARnostic® kits were kindly donated by ViroGates A/S, Copenhagen, Denmark. ViroGates had neither any role in the design, data collection, analysis, or interpretation of study finding or in the decision to submit the manuscript for publication.

## Conflict of interest

None of the authors have any conflicts of interests or disclosures with respect to the content of this manuscript, except for Dr Eugen-Olsen who is a founder, board member and shareholder of ViroGates, Denmark, and Copenhagen University Hospital Hvidovre that holds patents on the use of suPAR in prognostics with Drs Eugen-Olsen, Andersen, and Haugaard mentioned as inventors.

## References

[1] Libby P. Inflammation in atherosclerosis. Nature 2002;420(6917):868-74.
[2] Ridker PM, Danielson E, Fonseca FA, et al. Rosuvastatin to prevent vascular events in men and women with elevated C-reactive protein. N Engl J Med 2008;359(21):2195-207.
[3] Yudkin JS, Stehouwer CDA, Emeis JJ, Coppack SW. C-reactive protein in healthy subjects: associations with obesity, insulin resistance, and endothelial dysfunction: a potential role for cytokines originating from adipose tissue? Arterioscler Thromb Vasc Biol 1999;19(4):972-8.
[4] Ridker PM, Cook N. Clinical usefulness of very high and very low levels of C-reactive protein across the full range of Framingham Risk Scores. Circulation 2004;109(16):1955-9.
[5] Tsimikas S, Willerson JT, Ridker PM. C-reactive protein and other emerging blood biomarkers to optimize risk stratification of vulnerable patients. J Am Coll Cardiol 2006;47(8 Suppl.):C19-31.
[6] Eugen-Olsen J, Andersen O, Linneberg A, et al. Circulating soluble urokinase plasminogen activator receptor predicts cancer, cardiovascular disease, diabetes and mortality in the general population. J Intern Med 2010;268(3):296-308.
[7] Lyngbaek S, Sehestedt T, Marott JL, et al. CRP and suPAR are differently related to anthropometry and subclinical organ damage. Int J Cardiol 2013 Aug 10;167(3):781-5.
[8] Osler M, Linneberg A, Glumer C, Jorgensen T. The cohorts at the Research Centre for Prevention and Health, formerly 'The Glostrup Population Studies'. Int J Epidemiol Jun 2011;40(3):602-10.
[9] Jeppesen J, Hansen TW, Olsen MH, et al. C-reactive protein, insulin resistance and risk of cardiovascular disease: a population-based study. Eur J Cardiovasc Prev Rehabil 2008;15(5):594-8.
[10] Wilson PW, D'Agostino RB, Levy D, Belanger AM, Silbershatz H, Kannel WB. Prediction of coronary heart disease using risk factor categories. Circulation 1998;97(18):1837-47.
[11] Zhang X, Zhang MJ. SAS macros for estimation of direct adjusted cumulative incidence curves under proportional subdistribution hazards models. Comput Methods Programs Biomed 2011;101(1):87-93.
[12] Lin DY, Wei LJ, Ying Z. Checking the Cox model with cumulative sums of martingale-based residuals. Biometrika 1993;80(3):557-72.
[13] Wilson PW, Nam BH, Pencina M, D'Agostino Sr RB, Benjamin EJ, O'Donnell CJ. C-reactive protein and risk of cardiovascular disease in men and women from the Framingham Heart Study. Arch Intern Med 2005;165(21):2473-8.
[14] Uno H, Cai T, Pencina MJ, D'Agostino RB, Wei LJ. On the C-statistics for evaluating overall adequacy of risk prediction procedures with censored survival data. Stat Med 2011;30(10):1105-17.
[15] May S, Hosmer DW. A simplified method of calculating an overall goodness-of-fit test for the Cox proportional hazards model. Lifetime Data Anal 1998;4(2):109-20.
[16] Pencina MJ, D'Agostino Sr RB, Steyerberg EW. Extensions of net reclassification improvement calculations to measure usefulness of new biomarkers. Stat Med 2011;30(1):11-21.
[17] Viallon V, Ragusa S, Clavel-Chapelon F, Benichou J. How to evaluate the calibration of a disease risk prediction tool. Stat Med 2009;28(6):901-16.
[18] Pencina MJ, D'Agostino Sr RB, D'Agostino Jr RB, Vasan RS. Evaluating the added predictive ability of a new marker: from area under the ROC curve to reclassification and beyond. Stat Med 2008;27(2):157-72.
[19] Kjellman A, Akre O, Gustafsson O, et al. Soluble urokinase plasminogen activator receptor as a prognostic marker in men participating in prostate cancer screening. J Intern Med 2011;269(3):299-305.
[20] Persson M, Engstrom G, Bjorkbacka H, Hedblad B. Soluble urokinase plasminogen activator receptor in plasma is associated with incidence of CVD. Results from the Malmo Diet and Cancer Study. Atherosclerosis Feb 2012;220(2):502-5.
[21] Koch A, Voigt S, Kruschinski C, et al. Circulating soluble urokinase plasminogen activator receptor is stably elevated during the first week of treatment in the intensive care unit and predicts mortality in critically ill patients. Crit Care 2011;15(1):R63.
[22] Huttunen R, Syrjanen J, Vuento R, et al. Plasma level of soluble urokinase-type plasminogen activator receptor as a predictor of disease severity and case fatality in patients with bacteraemia: a prospective cohort study. J Intern Med Jul 2011;270(1):32-40.
[23] Chavakis T, Willuweit AK, Lupu F, Preissner KT, Kanse SM. Release of soluble urokinase receptor from vascular cells. Thromb Haemost 2001;86(2):686-93.
[24] Fevang B, Eugen-Olsen J, Yndestad A, et al. Enhanced levels of urokinase plasminogen activator and its soluble receptor in common variable immunodeficiency. Clin Immunol 2009;131(3):438-46.
[25] May AE, Schmidt R, Kanse SM, et al. Urokinase receptor surface expression regulates monocyte adhesion in acute myocardial infarction. Blood 2002;100(10):3611-7.

[26] Mustjoki S, Sidenius N, Vaheri A. Enhanced release of soluble urokinase receptor by endothelial cells in contact with peripheral blood cells. FEBS Lett 2000;486(3): 237-42.
[27] Steins MB, Padro T, Schwaenen C, et al. Overexpression of urokinase receptor and cell surface urokinase-type plasminogen activator in the human vessel wall with different types of atherosclerotic lesions. Blood Coagul Fibrinolysis 2004;15(5):383-91.
[28] Sehestedt T, Lyngbaek S, Eugen-Olsen J, et al. Soluble urokinase plasminogen activator receptor is associated with subclinical organ damage and cardiovascular events. Atherosclerosis 2011;216(1):237-43.
[29] Wei C, El HS, Li J, et al. Circulating urokinase receptor as a cause of focal segmental glomerulosclerosis. Nat Med 2011;17(8):952-60.
[30] Executive summary of the third report of The National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III). JAMA 2001;285(19):2486-97.
[31] Rosendorff C, Black HR, Cannon CP, et al. Treatment of hypertension in the prevention and management of ischemic heart disease: a scientific statement from the American Heart Association Council for High Blood Pressure Research and the Councils on Clinical Cardiology and Epidemiology and Prevention. Circulation 2007;115(21): 2761-88.
[32] Conroy RM, Pyorala K, Fitzgerald AP, et al. Estimation of ten-year risk of fatal cardiovascular disease in Europe: the SCORE project. Eur Heart J 2003;24(11): 987–1003.
[33] Manzoli L, Palumbo W, Ruotolo P, Panella M, Mezzetti A, Di SF. Cardiovascular risk of the general population assessed through SCORE and CUORE charts: an extensive survey by the general practitioners from Abruzzo, Italy. Int J Cardiol 2010;144(1): 47-52.
[34] Madsen M, Davidsen M, Rasmussen S, Abildstrom SZ, Osler M. The validity of the diagnosis of acute myocardial infarction in routine statistics: a comparison of mortality and hospital discharge data with the Danish MONICA registry. J Clin Epidemiol 2003;56(2):124-30.