Plasma Neutrophil Gelatinase-Associated Lipocalcin in the General Population

Association With Inflammation and Prognosis

Søren Lindberg, Jan S. Jensen, Rasmus Mogelvang, Sune H. Pedersen, Søren Galatius, Allan Flyvbjerg, Nils E. Magnusson

Objective—Neutrophil gelatinase-associated lipocalin (NGAL) is a glycoprotein stored in granules of neutrophil leukocytes participating in inflammatory and atherosclerotic processes and possibly plaque rupture. Despite the putative role of NGAL in atherosclerosis and acute myocardial infarction, human studies of plasma NGAL are still limited.

Approach and Results—We prospectively followed 5599 randomly selected men and women from the community in the fourth Copenhagen Heart Study. Plasma NGAL was measured at study entry. Participants were followed for 10 years. During follow-up, 20% died (n=1120) and 15% (n=884) developed a major adverse cardiovascular event. Plasma NGAL associated strongly with all inflammatory markers (high-sensitivity C-reactive protein, total leukocyte count, neutrophil count) and inversely with estimated glomerular filtration rate (all, \( P<0.001 \)). Multivariate analysis identified neutrophil leukocyte count as the main determinant of plasma NGAL. During follow-up, participants with increasing NGAL had increased risk of all-cause mortality and major adverse cardiovascular event (both, \( P<0.001 \)). Even after adjustment for confounding risk factors by Cox regression analysis, NGAL remained an independent predictor of both all-cause mortality and major adverse cardiovascular event. When added to the Framingham risk score, NGAL improved c-statistics and correctly reclassified \( \approx 15\% \) into more appropriate risk groups. In comparison with high-sensitivity C-reactive protein, when both markers were added to the Framingham risk score, NGAL conferred 3× to 4× the risk.

Conclusions—Plasma NGAL is strongly associated with inflammation in the general population. NGAL independently associated with 10-year outcome, and when added to the Framingham risk score, NGAL both improves c-statistics and correctly reclassifies participants into more accurate risk categories. (Arterioscler Thromb Vasc Biol. 2014;34:2135-2142.)

Key Word: inflammation

Neutrophil gelatinase-associated lipocalin (NGAL) is a glycoprotein stored in granules of neutrophil leukocytes. In response to cellular stress, NGAL is rapidly released by hepatocytes, endothelial and smooth muscle cells, renal tubular cells, and activated neutrophils.

Clinical use of NGAL has until recently focused on its role as a marker of acute kidney injury (AKI) because especially urinary NGAL is a sensitive and early marker of AKI preceding serum creatinine elevation with \( \approx 48 \) to \( 72 \) hours. However, lately attention has focused on NGAL in cardiovascular disease (CVD).

NGAL was originally identified as part of the innate immune system, mediating inflammatory activity through binding to chemotactic peptides, leukotrienes, and platelet-activating factor. NGAL is upregulated in endothelial dysfunction and inflammatory vascular damage. Furthermore NGAL is highly expressed in atheromatous plaques and correlated with characteristics of unstable plaques: infiltrating inflammatory cells, thrombus formation, plaque hemorrhage, and central necrosis. Through formation of a complex with metalloproteinase-9 (MMP-9), an important mediator of vascular remodeling and plaque instability, NGAL may also play an important role in plaque rupture.

Plasma NGAL is elevated in the presence of coronary artery disease, correlates with its severity, is even further increased in patients with acute myocardial infarction, and a strong predictor of outcome in patients with ST-segment–elevation myocardial infarction.

Despite this putative role of NGAL in atherosclerosis and CVD, human studies of plasma NGAL are still limited. Accordingly, we measured plasma NGAL in a large community-based cohort to elucidate the relationship with inflammation, kidney function, pro brain natriuretic peptide (proBNP), and prognosis.

Materials and Methods

Materials and Methods are available in the online-only Supplement.

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2135
Results

The median plasma level of NGAL was 202 μg/L (interquartile range: 158–265) and similar across sex (203 μg/L in men versus 201 in women; \( P=0.10 \)). The 95% range of NGAL concentrations was from 100 to 498 μg/L. During the 10 years follow-up, 1120 participants died (20%) and 864 (15%) reached the combined end point of major adverse cardiovascular event (MACE). Of interest, we tested for a possible interaction between NGAL and eGFR <60; however, the interaction term were nonsignificant for all-cause mortality (\( P=0.08 \)) and MACE (\( P=0.25 \)), meaning that NGAL predicted outcome similarly in patients with normal and reduced eGFR.

Baseline Characteristics

Table 1 describes baseline characteristics according to quartiles of plasma NGAL. Associations between NGAL, inflammatory biomarkers, and estimated glomerular filtration rate (eGFR) are shown in Figure 1. As seen, plasma NGAL associated strongly with all 3 markers of inflammation and inversely with eGFR (all, \( P<0.001 \)).

NGAL levels were increased slightly in participants with a history of CVD, median (interquartile range) 218 (210–226) versus 205 (200–211), \( P<0.001 \). By contrast, NGAL levels were similar in patients with prevalence of traditional cardiovascular risk factors (diabetes mellitus, hypertension, hypercholesterolemia, and smoking; Table 2).

In a multivariate model including all variables, NGAL associated positively with age, male sex, alcohol consumption, high-sensitivity C-reactive protein (hsCRP), and neutrophil leukocyte count and inversely with eGFR (all, \( P<0.001 \)). Neutrophil leukocyte count was the main predictor of plasma NGAL; a doubling in neutrophil leukocyte count associated with an increase in NGAL of 34% (29%–39%). eGFR appeared to associate closer with NGAL when eGFR was reduced (≤60 mL/min; Figure 1). This was confirmed using cubic regression splines (\( P<0.001 \)). Importantly this nonlinear association between NGAL and eGFR significantly affected the association between eGFR and NGAL; when eGFR was reduced (≥60 mL/min), a decrease of 10 in eGFR associated with an increase in NGAL of 13% (9%–18%). By comparison, a decrease of 10 in eGFR only associated with an NGAL increase of 3% (2%–4%) when eGFR was in the normal range. Weaker associations were seen with a history of CVD and body mass index. Of interest, NGAL did not associate with plasma proBNP (\( P=0.23 \)).

NGAL and Outcome

Kaplan–Meier curves demonstrated a significant association between quartiles of plasma NGAL and all-cause mortality and MACE (Figure 2). The association between log-transformed NGAL and both end points was examined in both univariable and several multivariable Cox regression analyses (Table 3). When added to the Framingham risk score, NGAL remained a significantly independent predictor of all-cause mortality and MACE; each doubling in NGAL associated with a hazard ratio (HR) of 1.45 (95% confidence interval, 1.30–1.62; \( P<0.001 \)) and 1.71 (95% confidence interval, 1.46–2.01; \( P<0.001 \)), respectively. By contrast, eGFR did not predict all-cause mortality (\( P=0.82 \)) or MACE (\( P=0.18 \)).

To compare NGAL with neutrophil leukocyte count, both were added to the Framingham risk score (model 5). In this model, NGAL remained highly significantly associated with all-cause mortality and MACE (both \( P<0.001 \)). By comparison, each doubling in neutrophil leukocyte count associated with a HR of 1.24 (95% confidence interval, 1.06–1.44; \( P=0.006 \)) for all-cause mortality and 1.25 (95% confidence interval, 1.06–1.48; \( P=0.008 \)) for MACE.

We found no interactions between NGAL and other variables. Of specific interest, we tested for a possible interaction between NGAL and eGFR <60; however, the interaction term were nonsignificant for all-cause mortality (\( P=0.08 \)) and MACE (\( P=0.25 \)), meaning that NGAL predicted outcome similarly in participants with normal and reduced eGFR.

To compare the discrimination performance of NGAL, we calculated the c-statistic before and after the addition of NGAL. When NGAL was added to the Framingham risk score, the c-statistic improved for all-cause mortality (0.74 [0.72–0.75] versus 0.73 [0.71–0.74]; \( P=0.002 \)) and MACE (0.73 [0.71–0.74] versus 0.71 [0.70–0.73]; \( P<0.001 \)). The addition of NGAL to model 2 also improved c-statistic (0.83 [0.82–0.84] versus 0.82 [0.81–0.84]; \( P=0.048 \) and 0.81 [0.79–0.82] versus 0.80 [0.79–0.82]; \( P=0.013 \)), respectively. When added to model 3, c-statistic did not improve (\( P=0.64 \) for all-cause mortality and \( P=0.46 \) for MACE).

NGAL and hsCRP

To compare the prognostic value of NGAL to hsCRP, we divided patients into groups based on hsCRP and NGAL using the standard cutoffs for hsCRP (1 and 2 mg/L) and >75th percentile for NGAL (264.9 μg/mL). Participants were then sorted by high/low hsCRP and high/low NGAL. Table 4 shows a Cox regression model estimating the risk between the 4 groups. As seen, the risk of both all-cause mortality and MACE doubled in patients with high NGAL and low hsCRP. In comparison, elevated hsCRP conferred slightly less increased risk. When both markers were high, the risk for both outcomes about tripled. Figure 3 depicts Kaplan–Meier curves for risk of MACE for the 4 groups. As shown, high hsCRP/high NGAL had the worst outcomes, whereas participants with low levels of both markers had the best prognosis.

When adding both markers to the Framingham risk score, a doubling in NGAL associated with increased risk of all-cause mortality (HR: 1.35 [1.20–1.51]; \( P<0.001 \)) and MACE (HR: 1.39 [1.22–1.59]; \( P<0.001 \)). By comparison, a doubling in hsCRP increased the risk much less (HR: 1.11 [1.07–1.17]; \( P<0.001 \) and HR: 1.12 [1.06–1.18]; \( P<0.001 \)), respectively. Thus, NGAL conferred ≈3 to 4 the risk as hsCRP.

Reclassification

To address the clinical application of NGAL, we calculated how many patients were reclassified after the addition of
NGAL to one of the currently used risk scores for both end points (all-cause mortality and MACE). Table 5 presents the proportion of patients initially classified as having a 10-year risk of <5%, 5% to 10%, 10% to 20%, 20% to 30%, and >30% based on the Framingham risk score, who would be reclassified to either higher or lower risk categories by the addition of NGAL.

As shown for all-cause mortality (Table 5), the proportion of patients reclassified was relatively low for participants with a 10-year risk of <10% (9%). However, 19% and 25% of all

![Figure 1. Associations between plasma neutrophil gelatinase-associated lipocalin (NGAL), markers of inflammation, and estimated kidney function, adjusted for age, sex, and traditional cardiovascular risk factors (hypertension, diabetes mellitus, hypercholesterolemia, and smoking). Values are displayed as mean and 95% confidence interval. eGFR indicates estimated glomerular filtration rate.](image-url)
patients estimated to be at 10% to 20% or at 20% to 30% using the Framingham risk score were reclassified into higher or lower risk categories. Table 4 also shows that actual event rates matched well with the predicted in almost all risk categories; a total of 665 (13%) patients were reclassified, of which 525 (79%) were placed into more accurate risk categories.

Table 6 presents similar analyses for the end point MACE. As shown, 34% of all patients with an estimated 10-year risk of MACE of 20% to 30% according to the Framingham risk score were reclassified after the addition of NGAL. There was almost perfect matching of observed and predicted risks after the addition of NGAL; of the 771 (16%) patients reclassified, all but 27 were placed into more accurate risk categories.

Discussion

In brief, the present study shows for the first time that the main determinant of plasma NGAL in the general population is neutrophil leukocyte count, whereas kidney function contributes less. The main finding is that during 10-year follow-up, NGAL independently associates with both all-cause mortality

![Figure 2](image-url)
Recently, Hemdahl et al. demonstrated increased NGAL in atherosclerotic plaques. NGAL inhibits MMP-9 inactivation, an endopeptidase responsible for cellular degradation. The central part of NGAL in atherosclerosis seems centered on thrombus formation, neutrophils released twice the amount of NGAL in myocardial remodeling. Our finding of an incremental association between NGAL and outcome in 597 older adults when adjusting for CRP. However, this may be because of a nonstandardized handling of blood samples, resulting in increased variability in plasma NGAL.

NGAL has been demonstrated efficient in predicting AKI 24 to 72 hours before creatinine increase in critically ill patients and patients undergoing cardiac surgery. However, there are several indications that urinary NGAL may be a more specific marker of AKI compared with plasma NGAL. During ischemic and toxic renal injury, both synthesis and secretion of NGAL are upregulated in the proximal tubules of the distal nephron, resulting in a massive increase in urinary NGAL. By contrast, the contribution to plasma NGAL from the kidney has been questioned, and the main source of plasma NGAL is suggested to come from activated neutrophils. Our results clearly support this theory; however, AKI is not supposed to be present in our study because we investigated participants from the community. In fact, eGFR was high (median 80 mL/min [70–91 mL/min]), and only 11% (n=605) of the participants had a reduced eGFR (<60 mL/min). It is possible, that the association between plasma NGAL and eGFR could be stronger in a population with a lower kidney function. Also, we did not have measurements of association between plasma NGAL and outcome in 597 older adults when adjusting for CRP. However, this may be because of a nonstandardized handling of blood samples, resulting in increased variability in plasma NGAL.

Table 3. Cox Regression Proportional Hazards Models Estimating the Risk of All-Cause Mortality and MACE for a Doubling in Plasma NGAL Adjusted for Various Baseline Variables

<table>
<thead>
<tr>
<th>Models</th>
<th>HR 95% CI</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model 1: NGAL (univariate); model 2: NGAL+age, sex, and traditional cardiovascular risk factors (smoking, hypertension, diabetes mellitus, and hypercholesterolemia); model 3: model 2+symptomatic and diastolic blood pressure, alcohol consumption, cardiovascular disease, BMI, total cholesterol, LDL, HDL, triglyceride, hemoglobin A1c, eGFR, hsCRP, and plasma proBNP; model 4: NGAL and Framingham risk score; and model 5: NGAL, neutrophil leukocyte count, and Framingham risk score. BMI indicates body mass index; CI, confidence interval; eGFR, estimated glomerular filtration rate; HDL, high-density lipoprotein; HR, hazard ratio; LDL, low-density lipoprotein; MACE, major adverse cardiovascular events; and NGAL, neutrophil gelatinase-associated lipocalin.</td>
<td></td>
<td></td>
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</table>

and MACE. Compared with the established inflammatory biomarker hsCRP, NGAL conferred more risk. NGAL is a small, 25-kDa glycoprotein belonging to the lipocalin superfamily and located in neutrophilic granules. It acts as a scavenger in inflamed areas and modulates inflammation through binding to chemotaxic peptides. However, the central part of NGAL in atherosclerosis seems centered on MMP-9, an endopeptidase responsible for cellular degradation. NGAL inhibits MMP-9 inactivation by forming a complex with MMP-9, thereby prolonging the proteolytic activity leading to decreased collagen content, weakening the plaque structure, and making it more prone to rupture, which is the main cause of myocardial infarction. Hemdahl et al. demonstrated increased NGAL expression in atherosclerotic plaques and colocalization with MMP-9 especially in areas with high proteolytic activity. In patients with abdominal aortic aneurism and thus intraluminal thrombus formation, neutrophils released twice the amount of NGAL as in healthy controls. In heart failure (HF), NGAL expression increased in cardiomyocytes in the failing myocardium, and through MMP-9, NGAL may possibly be involved in myocardial remodeling. Our finding of an incremental association between NGAL and cardiovascular outcome further supports a role of NGAL in atherosclerosis. We were able to show that in the general population neutrophil leukocytes were the main determinant of plasma NGAL. Although eGFR was still independently associated with NGAL, the contribution to NGAL levels was relatively small. These results confirm that plasma NGAL in a healthy population mainly reflects inflammation and not chronic kidney disease as opposed to urinary NGAL.

Although NGAL was strongly associated with other markers of inflammation, it still added prognostic value beyond the established inflammatory marker hsCRP, indicating that NGAL complements information on vascular inflammation. By contrast, Helmersson-Karlqvist et al. did not find an

Table 4. Risk of All-Cause Mortality and MACE Based on Groups Based on NGAL and hsCRP

<table>
<thead>
<tr>
<th>Groups Based on NGAL and hsCRP</th>
<th>HR 95% CI</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>NGAL&lt;264.9, hsCRP&lt;2 (n=2704)</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>NGAL&lt;264.9, hsCRP≥2 (n=1429)</td>
<td>1.93</td>
<td>1.65–2.25</td>
</tr>
<tr>
<td>NGAL≥264.9, hsCRP&lt;2 (n=576)</td>
<td>2.32</td>
<td>1.92–2.81</td>
</tr>
<tr>
<td>NGAL≥264.9, hsCRP≥2 (n=769)</td>
<td>3.38</td>
<td>2.88–3.97</td>
</tr>
</tbody>
</table>

CI indicates confidence interval; HR, hazard ratio; MACE major adverse cardiovascular events; and NGAL, neutrophil gelatinase-associated lipocalin.
urinary NGAL, which would have been interesting to compare with plasma NGAL as done by Helmersson-Karlqvist et al. Still, from our results, it is tempting to hypothesize that in critically ill patients with an increased inflammatory response (such as sepsis, cancer, myocardial infarction), plasma NGAL may reflect both the systemic inflammation, as well as possible AKI. However, more research is needed before firm conclusions can be drawn.

It is of interest that NGAL did not associate with proBNP, which currently is the golden standard prognostic marker in CVD. ProBNP reflects myocardial wall stress and is strongly correlated with hypertension, whereas NGAL supposedly mediates vascular damage and plaque rupture. Thus the 2 markers reflect distinct pathophysiological mechanisms of CVD. An association between the 2 markers has previously been described in HF. However, our finding that plasma NGAL is not associated with proBNP is in agreement with Daniels et al from the Rancho Bernard Study. In that study, plasma NGAL added prognostic value beyond and above NT-proBNP (N-terminal proBNP) in 1393 community-dwelling older adults.

Table 5. Risk Reclassification After Addition of NGAL to the Framingham Risk Score for the End Point All-Cause Mortality

<table>
<thead>
<tr>
<th>Framingham Risk Score Risk Categories, %</th>
<th>All-Cause Mortality</th>
<th>Framingham Risk Score+NGAL</th>
<th>No. (%) Reclassified</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>&lt;5%</td>
<td>5% to 10%</td>
<td>10% to 20%</td>
</tr>
<tr>
<td>&lt;5%</td>
<td>1532</td>
<td>146</td>
<td>0</td>
</tr>
<tr>
<td>Actual event rate, %</td>
<td>1.8</td>
<td>6.9</td>
<td>0</td>
</tr>
<tr>
<td>5% to 10%</td>
<td>12</td>
<td>392</td>
<td>24</td>
</tr>
<tr>
<td>Actual event rate, %</td>
<td>0</td>
<td>8.4</td>
<td>4.2</td>
</tr>
<tr>
<td>10% to 20%</td>
<td>0</td>
<td>115</td>
<td>765</td>
</tr>
<tr>
<td>Actual event rate, %</td>
<td>0</td>
<td>16.5</td>
<td>15.8</td>
</tr>
<tr>
<td>20% to 30%</td>
<td>0</td>
<td>0</td>
<td>133</td>
</tr>
<tr>
<td>Actual event rate, %</td>
<td>0</td>
<td>0.0</td>
<td>21.8</td>
</tr>
<tr>
<td>≥30%</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Actual event rate, %</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

NGAL indicates neutrophil gelatinase-associated lipocalin.

Table 6. Risk Reclassification After Addition of NGAL to the Framingham Risk Score for the End Point MACE

<table>
<thead>
<tr>
<th>Framingham Risk Score Risk Categories, %</th>
<th>MACE</th>
<th>Framingham Risk Score+NGAL</th>
<th>No. (%) Reclassified</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>&lt;5%</td>
<td>5% to 10%</td>
<td>10% to 20%</td>
</tr>
<tr>
<td>&lt;5%</td>
<td>1716</td>
<td>59</td>
<td>0</td>
</tr>
<tr>
<td>Actual event rate, %</td>
<td>1.6</td>
<td>3.4</td>
<td>0</td>
</tr>
<tr>
<td>5% to 10%</td>
<td>64</td>
<td>523</td>
<td>27</td>
</tr>
<tr>
<td>Actual event rate, %</td>
<td>6.3</td>
<td>10.7</td>
<td>14.8</td>
</tr>
<tr>
<td>10% to 20%</td>
<td>0</td>
<td>124</td>
<td>887</td>
</tr>
<tr>
<td>Actual event rate, %</td>
<td>0</td>
<td>6.5</td>
<td>16.0</td>
</tr>
<tr>
<td>20% to 30%</td>
<td>0</td>
<td>12</td>
<td>231</td>
</tr>
<tr>
<td>Actual event rate, %</td>
<td>0</td>
<td>22.3</td>
<td>21.7</td>
</tr>
<tr>
<td>≥30%</td>
<td>0</td>
<td>0</td>
<td>15</td>
</tr>
<tr>
<td>Actual event rate, %</td>
<td>0</td>
<td>0</td>
<td>27.9</td>
</tr>
</tbody>
</table>

MACE indicates major adverse cardiovascular events; and NGAL, neutrophil gelatinase-associated lipocalin.
Recent studies suggest that NGAL is a strong marker of outcome in patients with acute myocardial infarction and HF.11,32 We have also shown this in 584 patients with ST-segment-elevation myocardial infarction.14 In 186 patients with acute HF, Maisel et al.31 demonstrated that NGAL added to the predictive value of proBNP, with the worst outcome occurring in the group with high NGAL and high proBNP; in fact NGAL at discharge was an even stronger predictor of readmission than proBNP.11

It could be a significant finding that the 2 biomarkers do not correlate, reflect different pathophysiological mechanisms, and are both elevated in CVD.

In conclusion, plasma NGAL is associated to biomarkers of inflammation and independently predicts mortality and MACE adding incremental value to traditional cardiovascular risk factors and hsCRP.

Study Limitations and Strengths

The present findings are based on an epidemiological approach including the use of nationwide registries for ascertainment of MACE. Although highly validated, these findings cannot show causality. Body mass index in this Danish cohort from the General population was relatively low (25.9±3.4) compared with similar cohorts from US or other European countries. Accordingly, results may not be generalizable. Blood samples were collected during a 2-year period from 2001 to 2003. It is possible that the absolute concentration of NGAL has increased a little over time because blood samples are >10 years old; however, it is unlikely that this has affected our results because all samples have been treated in the same way in terms of freezing/thawing and storage.

Study strengths include the longitudinal community-based study design with 10 years follow-up, which to date is the largest study with measurements of plasma NGAL. In addition, no participants were lost to follow-up because of the high quality Danish registry data.

Sources of Funding

The study was supported by the Danish Heart Foundation.

Disclosures

None.

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**Significance**

Neutrophil gelatinase-associated lipocalin (NGAL) is a protein stored in neutrophil leukocytes associated with acute kidney injury. The present study shows that in the general population, plasma NGAL and neutrophil leukocyte count are strongly associated, indicating neutrophil leukocytes as the main contributor of plasma NGAL. By contrast, estimated glomerular filtration rate contributed little to plasma NGAL, thus emphasizing NGAL as a marker of inflammation in the general population. During 10-year follow-up in 5599 participants from the general population, increasing NGAL associated with increased risk of all-cause mortality and cardiovascular events. Compared with hsCRP, high NGAL conferred more risk than hsCRP, whereas participants with the combination of high hsCRP and high NGAL had the worst prognosis.
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Materials and Methods

Study population: The present study included 5,599 men and women (20-94 years of age) with measurements of NGAL from the 4th Copenhagen City Heart Study, a longitudinal cohort study of CVD and risk factors which has been described previously. Patients were followed for 10 years, and follow-up was 100% complete. Endpoints were all-cause mortality and major adverse cardiovascular events (MACE) consisting of cardiovascular mortality or hospitalization due to AMI, ischemic stroke or heart failure. Data on mortality were collected from the National Person Identification Registry at the Danish National Board of Health’s which holds information on vital status (alive, death or emigration). Data on a new MI, ischemic stroke or admission with symptomatic heart failure were collected using the highly validated Danish National Board of Health’s National Patient Registry, using ICD-10 codes.

Health examination at baseline: CVD was defined as a history of hospital admission due to AMI, percutaneous coronary intervention, coronary artery bypass grafting, ischemic stroke or heart failure. Hypertension was defined as systolic blood pressure ≥ 140mmHg, diastolic blood pressure ≥ 90mmHg or use of antihypertensive medication. Diabetes was defined as plasma glucose concentration ≥11.1mmol/L, use of anti-diabetic medicine, self-reported disease or HbA1c level ≥ 7.0%. Hypercholesterolemia was defined as use of cholesterol lowering medicine or total-cholesterol ≥ 7.0mmol/L. Alcohol consumption was calculated as grams of ethanol pr. week using standard ethanol concentrations in beer, wine, liquor and spirits.

All subjects gave informed consent to participate, and the study was performed in accordance with the second Helsinki Declaration and approved by the regional ethics committee.

Blood samples were immediately centrifuged at 3000 rpm for 10 min and EDTA-plasma was stored at -80°C until subsequent analysis. Plasma NGAL was determined using an in-house time resolved immunofluorometric (TRIFMA) assay based on NGAL antibodies and recombinant NGAL from R&D Systems (Abingdon, UK). Samples and controls were diluted 1:50 and analyzed in duplicate. Intra assay coefficient of variation of standards, controls, unknown samples and non-specific background controls averaged less than 5%. Inter assay coefficient of variation averaged < 9 % based on standards, non-specific background controls, internal controls and repeated samples. Plasma NGAL concentrations were determined using a five-parameter standard curve fit implemented in the WorkOut 2.5 Data Analysis software (PerkinElmer Inc.). Mean values were calculated and used for statistical analyses. Plasma NGAL has been shown to be stable using repetitive freezing (-80°C) and thawing (RT) cycles and to remain stable for at least 11 months at -80°C.

Plasma proBNP concentration was quantified using a processing-independent assay fully comparable to the commercially available Modular N-terminal-proBNP assay by Roche (Karlsruhe, Germany), with the analytical validation already reported elsewhere. Other blood tests including high sensitivity C-reactive protein (hsCRP), HbA1c, blood glucose, lipids and creatinine were assayed with routine laboratory methods in non-fasting blood samples. Estimated glomerular filtration rate (eGFR) was calculated on the basis of serum creatinine, age and gender using the CKD-EPI formula. The Framingham risk score was calculated from published equations using age, gender, systolic blood pressure, antihypertensive treatment, smoking, total cholesterol and high-density lipoprotein cholesterol. The Framingham risk score was used to divide patients into 5 risk groups (<5%, 5-10%, 10-20%, 20-30% and ≥ 30%). Due to the fact that the Framingham risk score only should be calculated in patients < 80 years, is was only possible to calculate the risk score in 89% (n=4978).

Statistics: Plasma NGAL (and other biomarkers including hsCRP and plasma proBNP) concentrations were positively skewed and therefore logarithmically transformed using the base logarithm of 2 before analysis. Accordingly hazard ratios (HR) for a linear increase in NGAL on the log-scale are reported per doubling. Associations between log-transformed NGAL and baseline variables were examined using regression analysis. The association of log-transformed NGAL with outcome was examined by Kaplan-Meier curves and Cox-proportional hazards regression analyses. Deviation from linearity was assessed by simultaneous assessment of linear and quadratic effects. Evaluation of first order interactions was made in the final model. We specifically
investigated a possible interaction between NGAL and eGFR. All other possible interactions were adjusted for multiple testing by the Bonferroni method. Misspecification of the functional form of the covariates and the assumption of proportional hazards were evaluated by plots of the cumulative martingale residuals. Discrimination was evaluated using c-statistic. In order to address the clinical issues of reclassification and risk stratification, we divided all patients into risk groups of <5%, 5-10%, 10-20%, 20-30% and >30% using the Framingham-risk score. We then calculated the proportion of patients reclassified into either higher or lower risk-categories by adding NGAL to the Framingham risk-score. In the statistical tests p-values ≤0.05 were considered of statistical significance. C-statistic and reclassification calculations were performed in Stata/IC 13.0 for Windows using the “incrisk” package, whereas all other statistical calculations were performed using the SAS statistical software (SAS for Windows, release 9.2, SAS Institute Inc., Cary, NC, USA).