Serum amyloid A: high-density lipoproteins interaction and cardiovascular risk

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Introduction

Cardiovascular disease (CVD) represents the major cause of death in Western populations. In Europe, more than four million deaths were caused by clinical complications of atherosclerosis such as myocardial infarction or stroke in 2000. A variety of risk factors including arterial hypertension, smoking, dyslipidaemia, obesity,
diabetes mellitus, and chronic kidney disease (CKD) contribute to the development of atherosclerotic vascular lesions.  

In general, high-density lipoproteins (HDLs) are considered anti-atherogenic.  

HDLs from healthy individuals have been shown to stimulate endothelial nitric oxide (NO) production, to reduce endothelial reactive oxygen species (ROS) production, and to prevent pro-inflammatory responses of the endothelium. In vivo administration of reconstituted HDL to hypercholesterolaemic men has been demonstrated to improve endothelial function.  

Accordingly, observational trials have linked high concentrations of HDL-cholesterol (HDL-C) to improve the cardiovascular outcome.  

We and others have previously shown that HDLs may lose their vasoprotective properties in disease conditions such as diabetes mellitus, coronary artery disease (CAD), and CKD.  

We assessed the association of HDL-C with all-cause and cardiovascular mortality in the LURIC study. We and others have previously shown that HDLs may lose their vasoprotective properties in disease conditions such as diabetes mellitus, coronary artery disease (CAD), and CKD.  

We assessed the impact of increasing concentrations of SAA on distinct endothelial functions (NO and ROS production, vascular cell adhesion molecule-1 (VCAM-1) expression, and endothelial mononuclear cell adhesion) was determined by incubating human aortic endothelial cells (HAEC) with HDL-SAA preparations. For a detailed description, see Supplementary material.

### Methods

#### Study populations

The Ludwigshafen Risk and Cardiovascular Health (LURIC) study enrolled 3316 patients undergoing coronary angiography between 1997 and 2000.  

3310 patients with all variables complete for current analyses were included. The study design and the examinations at baseline have been described previously.  

The median follow-up time was 9.9 (8.3–10.7) years. All information on death during follow-up was obtained from local Health Departments. Cardiovascular mortality was defined as sudden cardiac death, fatal myocardial infarction, death due to congestive heart failure, or death immediately after intervention to treat CAD and fatal stroke.

The German Diabetes and Dialysis (4D) study is a prospective, randomized, multicentre trial enrolling 1255 patients with type 2 diabetes on haemodialysis for $<2$ years between 1998 and 2002. The study design has been described in detail previously.  

The primary endpoint of the 4D study was defined as the composite of cardiac death, non-fatal myocardial infarction, and stroke, whichever occurred first (combined cardiovascular events). Here, we analysed the primary endpoint of the 4D study as well as all-cause mortality.

The Cooperative Health Research in the Region of Augsburg (KORA) S4 study is a population-based survey of inhabitants of Augsburg, Germany including 4261 participants between 1999 and 2001. We excluded subjects with previous myocardial infarction ($n = 77$), stroke ($n = 48$) or with missing values for HDL-C or SAA ($n = 102$), or those who were lost to follow-up ($n = 7$). Thus, 4027 participants were included in the present analyses. Details of the study protocol have been described.

LURIC, 4D, and KORA S4 studies were approved by the local Ethics Committees and performed in accordance with the Declaration of Helsinki. Informed consent was obtained from all participants.

#### Statistical analysis

The association among SAA, HDL-C, and their interaction (SAA $\times$ HDL-C) with all-cause and cardiovascular mortality in the LURIC study has been assessed using Cox regression analyses adjusted for age, sex, acute coronary syndrome (ACS) at presentation, Friesinger coronary score, body mass index, glycated haemoglobin, smoking, lipid-lowering therapy, cystatin C, mean systolic blood pressure, and high-sensitivity (hs) C-reactive protein. General linear models were used to estimate the marginal means (adjusted) of SAA for several cardiovascular risk factors and disease states. A formula was built to calculate biologically effective HDL-C’ on the basis of measured HDL-C and SAA. Survival analyses to examine the associations between calculated HDL-C’ and distinct outcomes as indicated were performed in the 4D and KORA S4 studies. Detailed information on the statistical methods is given in Supplementary material.

#### Results

Baseline characteristics of the LURIC study are presented in Supplementary material online, Table S1. During a median follow-up time of 9.9 years, 995 participants (30.0%) died. About 66.5% ($n = 622$) of all deaths were caused by cardiovascular events.

We assessed the impact of increasing SAA on all-cause and cardiovascular mortality. Supplementary material online, Figure S1 shows survival curves for all-cause and cardiovascular mortality, according to quintiles of SAA. Higher SAA levels were significantly associated with all-cause and cardiovascular mortality (both $P < 0.001$). By Cox regression analyses (Supplementary material online, Table S2), patients in the highest quintile of SAA had a significantly increased risk for all-cause [hazard ratio (HR) 1.58; 95% confidence interval (CI): 1.26–1.97] and cardiovascular mortality (HR 1.72; 95% CI: 1.30–2.28) after adjustment for potential confounders.

Recent experimental evidence suggests that SAA may be incorporated into HDL particles rendering HDL dysfunctional. To examine the clinical relevance of these experimental findings, we assessed the association of HDL-C with all-cause and cardiovascular mortality at SAA concentrations above or below the 80th percentile (16.9 mg/L) (Figure 1). HDL-C was inversely, dose-dependently, and significantly associated with all-cause and cardiovascular mortality below the 80th percentile of SAA after adjustment for potential confounders. In marked contrast, at SAA above the 80th percentile, HDL-C not only lost its ‘protective’ association with mortality, but was also significantly and positively associated with all-cause and
cardiovascular mortality. To analyse the interaction between HDL-C and SAA, we performed Cox regression analyses including the interaction term between HDL-C divided in quartiles and SAA divided into two groups at the 80th (16.9 mg/L) or the 90th percentile (42.9 mg/L) (Supplementary material online, Table S3). These analyses revealed a significant interaction especially between HDL-C in the highest quartile and SAA, suggesting that SAA may modulate the biological effects of HDL.

Using the procedure described in Supplementary material, an equation was derived, which allows for calculation of the ‘biologically effective’ HDL-C concentration based on concomitantly measured HDL-C and SAA concentrations:

$$\text{HDL} - C = 20 \cdot 14(0 \cdot 213 \cdot \ln(\text{SAA}) + 1) + (0 \cdot 073 \cdot \ln(\text{SAA}) + 1 - 0 \cdot 283)^2 + 0 \cdot 176$$

Figure 1 HRs of all-cause and cardiovascular mortality according to HDL-C levels at high and low SAA concentrations in the LURIC study. Multivariable-adjusted hazard functions for (A) all-cause and (B) cardiovascular mortality according to HDL-C concentrations at SAA below and above 16.9 mg/L (80th percentile). Solid lines represent the hazard functions and dashed lines the respective 95% CIs. The median of HDL-C (37 mg/dL) was chosen as reference (HR = 1.0). Adjusted for age, sex, ACS, Friesinger score, body mass index, glycated haemoglobin, smoking, lipid-lowering therapy, cystatin C, hs C-reactive protein, and mean systolic blood pressure. Green ranges represent the range of HDL-C with a HR < 1 and red ranges with a HR > 1.
The graph of the underlying function indicates that already slightly elevated SAA is sufficient to substantially reduce the proportion of biologically effective HDL-C (Figure 2 and Supplementary material online, Table S4). The acute-phase protein SAA can be associated with different cardiovascular risk factors. Therefore, we next examined clinical conditions associated with SAA (Table 1). Indeed, many cardiovascular risk factors (e.g. diabetes, smoking, age, and renal impairment) and manifest CVD (e.g. CAD and ACS) are related to significantly elevated SAA after adjustment for heterogeneity. Patients with angiographically proved CAD had more than two-fold higher SAA levels than those without CAD, and in ACS patients SAA was even increased more.

As renal impairment represents a potent cardiovascular risk factor and a clinical condition characterized by increased levels of SAA, we validated the aforementioned algorithm in participants of the 4D study. Baseline characteristics of the 4D study are shown in Supplementary material online, Table S5. During a mean follow-up of 3.9 years, 37.3% (n = 294) of the study participants died, including 2.6% (n = 105) deaths due to cardiovascular events. In survival analyses, measured HDL-C was not significantly associated with all-cause and cardiovascular mortality. However, in marked contrast, calculated biologically effective HDL-C significantly predicted all-cause and cardiovascular mortality (Figure 4).

Finally, we addressed the mechanisms underlying these findings on the cellular level by incubating cultured HAECs with HDL alone and with HDL supplemented with different concentrations of SAA. Although HDL alone significantly increased endothelial production of NO, an important regulator of the vascular tone, HDL supplemented with SAA not only lost its stimulatory effect, but also significantly inhibited endothelial NO production in a concentration-dependent fashion (Figure 5A). Vice versa, HDL supplemented with SAA significantly increased endothelial production of ROS, whereas HDL without SAA supplementation did not affect basal endothelial ROS production (Figure 5B). HDL also significantly reduced the expression of VCAM-1 on the surface of endothelial cells in contrast to SAA-supplemented HDL (Figure 5C). Accordingly, HDL significantly decreased the adhesion of mononuclear cells to tumour necrosis factor alpha (TNF-α)-treated endothelial cells (Figure 5D), whereas HDL supplemented with SAA lost this inhibitory activity.

Discussion
We found a statistically significant association between the SAA concentration and all-cause and cardiovascular mortality in a large
cohort of patients with high cardiovascular risk. Even more important, our data provide strong evidence both in vivo and in vitro that SAA transforms HDL from a protective into a noxious lipoprotein. In subjects with elevated SAA, higher HDL-C was associated with increased mortality, revealing that SAA significantly modifies vascular properties of HDL.

Atherosclerotic CVD is characterized by inflammatory reactions of the vessel wall including accumulation of leucocytes. Consistently, several acute-phase proteins such as C-reactive protein, interleukin-6, or TNF-α have emerged as predictors for adverse outcomes in patients with clinically manifest or subclinical CVD. Previous studies have also linked elevated SAA to poorer outcomes in patients with prevalent CVD. Interestingly, in this study, higher SAA was not only associated with manifest CVD, but also with several clinical conditions associated with increased cardiovascular risk including CAD, diabetes mellitus, smoking, ageing, and renal impairment.

Exactly in these conditions, HDL has been suggested to be dysfunctional, whereas HDL from healthy subjects preserves vascular integrity and endothelial function. The molecular mechanisms leading to dysfunctional HDL are only incompletely understood. Alterations of the protein and lipid composition of HDL have documented to be crucially related to abnormal vascular function of HDL. Indeed, we recently showed that HDL from patients with CKD inhibits endothelial NO production and, thereby, increases arterial blood pressure. It is in line with the current findings that SAA accumulates in dysfunctional HDL particles of patients with CAD and/or CKD, although the clinical relevance of these findings has been elusive so far.

Therefore, we examined the association between HDL-C and all-cause and cardiovascular mortality in patients with low and high SAA. In patients with low concentrations of SAA, HDL-C was related to reduced all-cause and cardiovascular mortality. In marked contrast, HDL-C was significantly and positively associated with all-cause and cardiovascular mortality at high SAA, even after adjustment for other inflammatory markers such as C-reactive protein. These results suggest that even moderately elevated SAA (>16.9 mg/L) is sufficient to substantially interfere with the beneficial vascular properties of HDL. These findings may not only be relevant to patients with CAD, as recent studies suggest, but to all conditions that are associated with subclinical inflammation and, concomitantly, elevated SAA such as diabetes, CKD, or smoking.

Table 1  Association of cardiovascular risk factors and markers of disease severity with SAA concentrations in the LURIC study

<table>
<thead>
<tr>
<th>Risk Factor</th>
<th>SAA (mg/L)</th>
<th>Mean difference, %</th>
<th>P-value</th>
<th>P-value</th>
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<td>Acute coronary syndrome</td>
<td></td>
<td></td>
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<tr>
<td>No</td>
<td>17.1 (12.2–22.0)</td>
<td>+234.1</td>
<td>&lt;0.001</td>
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<tr>
<td>Yes</td>
<td>57.1 (50.0–64.3)</td>
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<td>Coronary artery disease</td>
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<tr>
<td>No</td>
<td>14.8 (6.1–23.5)</td>
<td>+132.3</td>
<td>&lt;0.001</td>
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<tr>
<td>Yes</td>
<td>34.3 (29.4–39.2)</td>
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<td>Chronic kidney disease</td>
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<tr>
<td>eGFR &gt; 90 mL/min</td>
<td>24.3 (17.0–31.7)</td>
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<tr>
<td>eGFR 60–90 mL/min</td>
<td>28.8 (23.0–34.5)</td>
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<tr>
<td>eGFR ≤ 60 mL/min</td>
<td>43.0 (32.1–53.9)</td>
<td>+43.4</td>
<td>0.008</td>
<td>0.019</td>
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<td>Smoking</td>
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<tr>
<td>No</td>
<td>21.7 (15.3–28.2)</td>
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<tr>
<td>Yes</td>
<td>35.1 (29.5–40.7)</td>
<td>+61.5</td>
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<td>Diabetes</td>
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<tr>
<td>No</td>
<td>24.9 (19.3–30.6)</td>
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<tr>
<td>Yes</td>
<td>36.1 (28.7–43.4)</td>
<td>+44.5</td>
<td>0.028</td>
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<tr>
<td>Sex</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Female</td>
<td>26.4 (19.4–33.4)</td>
<td></td>
<td>0.182</td>
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<tr>
<td>Male</td>
<td>32.2 (27.6–36.8)</td>
<td>+21.7</td>
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<td>Age (years)</td>
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<tr>
<td>≤56.3</td>
<td>15.7 (7.3–24.0)</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>56.3–63.5</td>
<td>24.5 (16.6–32.4)</td>
<td>+56.3</td>
<td>0.117</td>
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<td></td>
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<tr>
<td>63.6–70.5</td>
<td>31.6 (23.7–39.4)</td>
<td>+101.5</td>
<td>0.006</td>
<td>0.204</td>
<td></td>
</tr>
<tr>
<td>≥70.6</td>
<td>45.6 (37.7–53.5)</td>
<td>+190.9</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>0.012</td>
</tr>
</tbody>
</table>

*Estimated marginal means and 95% CIs as calculated in a general linear model, adjusted for age, sex, lipid-lowering therapy, ACS, body mass index, mean systolic blood pressure, cystatin C, glycated haemoglobin, Friesinger score, smoking, and hs C-reactive protein (where appropriate).

bComparison with the first category of each variable.

cComparison with the second category of each variable.

dComparison with the third category of each variable.
Notably, our results led to the development of an algorithm that can be used to calculate biologically effective HDL-C. By using the well-described 4D cohort of dialysis patients as well as the KORA S4 cohort, the clinical relevance of this formula was proved. Although measured HDL-C had no significant impact on cardiovascular outcomes as reported earlier, higher levels of the calculated biologically effective HDL-C were associated with reduced risk all-cause mortality as well as cardiovascular endpoints. Although the LURIC study includes patients at intermediate-to-high risk of CVD, KORA represents the general population and 4D is a cohort with a very high risk. Thus, our formula has been validated over a very broad range of CVD risks from standard to extraordinary high.

Earlier studies have suggested that SAA might replace the main structural protein of HDL apolipoprotein A–I, which may mediate some of the vasoprotective properties of HDL. Additionally, the accumulation of SAA in HDL was linked to reduced cholesterol efflux capacity and anti-inflammatory activity of HDL. Moreover, SAA-enriched HDL may be associated with the development of atherosclerotic lesions in mice. In experimental studies, we observed that SAA-supplemented HDL reduced endothelial NO production and concomitantly increased endothelial production of ROS. Thus, SAA turned HDL into a pro-inflammatory particle. These findings underscore the important role of SAA in inverting the beneficial vascular properties of HDL.

Our findings have several important clinical implications. Currently, all assays to measure the biological properties of HDL require time-consuming isolation of HDL by ultracentrifugation or immunological methods. Our data suggest that concomitantly measuring SAA and HDL may reflect the vascular function of HDL. This may be of importance as a plethora of clinical conditions are associated with inflammation and increased SAA. SAA, therefore, may aid in the identification of patients with increased risk for cardiovascular events, despite high HDL-C concentrations. Our algorithm to calculate the effective HDL-C can be easily implemented in routine diagnostics and can be used as a convenient tool to estimate the functionality of HDL without time-consuming isolation of HDL and demanding functional assays.

Efforts have recently been made to increase HDL-C by pharmacological means, e.g. using inhibitors of the cholesteryl-ester transfer protein (CETP). Large trials using the CETP inhibitors torcetrapib and dalcetrapib did not show a reduction of cardiovascular outcomes. In these study programmes, a substantial proportion of
the patients had ACS, diabetes mellitus, and/or renal impairment, i.e. conditions with increased SAA. Based on our findings, we speculate that raising HDL concentrations in patients with high SAA may be detrimental. Measuring SAA could potentially help to identify patients in whom HDL-increasing therapies may be advantageous. Although speculative, the decrease of SAA seen during statin treatment may contribute to their atheroprotective effects and lowering may turn out a useful approach to restore HDL functionality.

The main strengths of the present study are the large study populations with long-term follow-up. Accurate and comprehensive clinical characterization of the participants made it possible to adjust for potential confounding variables. Confirmation of our results in the 4D study, including patients on dialysis treatment with a high risk for cardiovascular events, as well as in the KORA S4 study, comprising apparently healthy subjects in a population-based setting, further supports our conclusions. The clinical

Figure 4 Univariate survival analyses for all-cause and cardiovascular mortality in the KORA S4 study according to quartiles of measured and calculated biologically effective HDL-C'. Quartiles for measured HDL-C: quartile 1, ≤45.3 mg/dL; quartile 2, 45.3–55.7 mg/dL; quartile 3, 55.7–68.2 mg/dL; and quartile 4, >68.2 mg/dL. Quartiles for calculated biologically effective HDL-C': quartile 1, ≤46.3 mg/dL; quartile 2, 46.3–61.3 mg/dL; quartile 3, 61.3–82.2 mg/dL; and quartile 4, >82.2 mg/dL.)
Figure 5  Endothelial effects of HDL supplemented with increasing concentrations of SAA. (A) Endothelial NO production as determined by electron spin resonance (ESR) spectroscopy in HAECs incubated with HDL (50 μg/mL) or HDL supplemented with SAA (100, 1000, or 2000 ng/mL) for 1 h. (B) Endothelial ROS production as determined by ESR spectroscopy in HAECs incubated with HDL (50 μg/mL) or HDL supplemented with SAA (100, 1000, or 2000 ng/mL) for 1 h. (C) Endothelial VCAM-1 expression as determined by western blot in HAECs incubated with HDL (50 μg/mL) or HDL supplemented with SAA (100, 1000, or 2000 ng/mL) for 5 h and TNF-α (0.1 ng/mL) for 4 h. (D) Adhesion of Dil-labelled THP-1 cells to HAECs counterstained with DAPI and incubated with HDL (50 μg/mL) or HDL supplemented with SAA (100, 1000, or 2000 ng/mL) for 5 h and TNF-α (0.1 ng/mL) for 4 h. n = 5–7 per group. Respective means and standard deviation are shown. P-values have been adjusted for multiple comparisons.
observations are further strongly supported by the mechanistic cell culture studies. In summary, these findings indicate that the concept of calculating biologically effective HDL-C′ may be applicable to patients with manifest, prevalent, and without CVD. Limitations of our study are that the LURIC, 4D, and KORA S4 studies primarily included patients of Caucasian ancestry, which limits the extension of the present findings to other ethnicities. In addition, despite careful analysis, confounding due to differences of the baseline variables across SAA concentrations cannot be fully excluded in a post hoc analysis. Additional studies are necessary to determine the effect of different treatment strategies on the interaction between SAA and HDL-C and to assess the performance of calculated biologically effective HDL-C′ in currently available scoring algorithms to predict cardiovascular risk. Moreover, the time course and reversibility of SAA accumulation in HDL have to be determined.

In conclusion, SAA is a strong marker for cardiovascular and all-cause mortality, interacting with the functionality of HDL. SAA transforms HDL from a vasoprotective into a pro-atherosclerotic lipoprotein, which is associated with the substantially worsened cardiovascular outcome at least partially mediated by promoting endothelial dysfunction (Figure 6). Concomitantly measuring SAA concentrations may represent a convenient method to estimate HDL functionality in clinical routine.

Supplementary material

Supplementary material is available at European Heart Journal online.

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Conflict of interest: W.K. has received consulting fees from the Medicines Company and was member of the Steering Committee of Dal-Outcomes.

References


