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Supplementary Methods

Materials

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless stated otherwise.

Sample Preparation

HDL for the experimental cohort was isolated from fresh plasma of non-fasting subjects by sequential ultracentrifugation as described.[1] In brief, the density of plasma was raised to ρ=1.063 kg/L by addition of potassium bromide (KBr) (Merck, Darmstadt, Germany). The samples were then centrifuged in a type 50.4 Ti rotor in an Optima L-90-K ultracentrifuge (Beckmann, Fullerton, CA, USA) at 50,000 rpm for 12 hours at 20°C. After removal of the supernatant containing the larger lipoproteins, the density of the infranate fraction was raised to ρ=1.210 kg/L by addition of KBr and samples were centrifuged for further 12 hours in the type 50.4 Ti rotor at 50,000 rpm. HDL was then collected from the top of the centrifuge tubes, aliquoted and stored at -80°C until further use.

Apolipoprotein B-depleted serum was prepared from thawed serum samples by precipitation of apolipoprotein B-containing lipoproteins with polyethyleneglycol (PEG). 20% PEG in 200mM glycine buffer pH 7.4 was added to serum samples diluted at 1:2.5. After incubation for 20 minutes at room temperature while shaking, samples were centrifuged at 16.000xg for 30 minutes at 4°C. The supernatant (apolipoprotein B-depleted serum) was collected and used immediately; the pellet containing lipoprotein fractions with apolipoprotein B was discarded.

Statistical Analysis

Statistical analysis was performed using the GraphPad Prism 6 software. Data were compared by using Mann-Whitney-U test or unpaired, two-tailed student's t-test. Linear regression analysis was performed when testing for the relationship of two variables. Results are expressed as means +SEM.

Protocol: enzyme-linked immunosorbent assay (ELISA)

Working volume was 50µl/well, washing was performed with 200µl/well. All working steps were carried out at room temperature unless indicated otherwise.

96-well ELISA plates (Thermo Fisher Scientific, Waltham, MA, USA) were coated with chicken α-human HDL antibody at 1 µg/ml) overnight at 4°C. The plates were washed 3 times with PBS and incubated with apolipoprotein B-depleted serum (diluted 1:50) in triplicates for 1.5 hours while shaking. 10 µg/ml HDL samples with high and low amounts of serum amyloid A (SAA) and surfactant protein B (SP-B) were used as positive and negative controls. After being washed with PBS, samples were fixed with paraformaldehyde, followed by washing the plates with PBS containing 0.1% (vol/vol) Tween 20 (PBS-T). The plates were then incubated for 1 hour with primary mouse monoclonal and rabbit polyclonal antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) against HDL-bound serum amyloid A (SAA 115) and surfactant protein B (SP-B H-300), respectively. After washing with PBS-T, plates were treated with secondary biotin-conjugated goat α-mouse or goat α-rabbit IgG (H&L) (Southern Biotech, Birmingham, AL, USA) for another hour. Following incubation and additional washing steps, streptavidin-peroxidase (Roche, Basel, Switzerland) was added to the plates for 30 minutes. Finally, HDL-bound proteins were detected with tetramethylbenzidine substrate. The reaction was stopped by addition of H_2SO_4 to each well and absorbance was measured at 450nm in a microplate reader (Anthos HT3, Anthos Labtech Instruments, Wals, Austria). The levels of HDL-bound SAA and SP-B were calculated as values normalized to the ratio of positive and negative control by the following formula:

$$sample\ value = \frac{(OD\ sample - OD\ blank)}{(OD \frac{positive}{negative} control - OD\ blank)}$$

Development and Validation of the ELISA to Detect HDL-Associated Proteins

We developed our assay for the detection of candidate proteins, which we found highly enriched in HDLs of patients with end-stage renal disease (ESRD) compared to healthy controls in a previous study.[1] SAA and SP-B were selected as most promising candidates as these two proteins were most strongly enriched in our analysis.

We tested the assay conditions in a total of 15 ESRD patients undergoing hemodialysis and 12 controls enrolled at the General Hospital of Vienna. The study was approved by the ethics committee of the Medical University of Vienna according to the declaration of Helsinki (1038/2011). We established an easy-to-perform, high-throughput laboratory test that allows specific and sensitive detection of HDL-associated proteins from sample material such as serum by the use of a HDL-specific coating antibody. We found that only HDL but not LDL was bound from serum samples by the HDL-coating antibody (Supplementary Figure S1). We setup our ELISA with HDL samples (10µg/ml) to define optimal assay conditions and to perform initial control experiments. The amount of HDL-associated SAA (SAA[HDL]) and SP-B (SP-B[HDL]) could be detected from purified HDLs individually for patients and controls by our assay (Supplementary Figures S2 and S3). Western blots of the respective HDL-samples suggested that the levels of SAA(HDL) and SP-B(HDL) measured by the ELISA corresponded to the actual protein levels present in HDL (Supplementary Figures S2 and S3). After testing the complete cohort, we could show that the range of SAA(HDL) was from 0.06 – 2.10 in patients and from 0.01 - 0.33 in controls (absolute absorbance values). The range of SP-B(HDL) were from 0.13 – 1.18 in patients and from 0.10 – 0.54 in controls (absolute absorbance values). When comparing the mean levels between patients and controls, we found a significant enrichment of both SAA and SP-B in ESRD-HDL (Supplementary Figure S4).

Next, we wanted to further optimize the specificity of our assay for serum samples, which was anyway given by application of the HDL-specific catching antibody. Therefore, we generated apolipoprotein B-depleted serum obtained by precipitation of LDL and VLDL with 20% polyethyleneglycol (PEG) as described in the sample preparation section. Apolipoprotein B-depleted serum has been used by Khera et al. for the determination of the cholesterol efflux capacity of HDLs[2]. Supplementary Figure S5 shows that apolipoprotein B-depleted serum is devoid of detectable LDL and VLDL (apo-B100). We then measured SAA(HDL) and SP-B(HDL) in apolipoprotein B-depleted serum from our cohort and compared it to the results from corresponding isolated HDL samples. We found that SAA(HDL) and SP-B(HDL) from apolipoprotein B-depleted serum strongly correlated to SAA and SP-B values from isolated HDL (Supplementary Figure S6). The coefficient of variance (CV) for the duplicate samples were 10.9% for SAA(HDL) and 21.6% for SP-B(HDL). In addition, our assay yielded highly reproducible results. When we measured the same apolipoprotein B-depleted serum

samples in two independent experiments on different days we obtained an inter-assay precision of R^2 = 0.9805 (CV of 15.9%) and R^2= 0.9299 (CV of 29.8%), for SAA(HDL) and SP-B(HDL), respectively (Supplementary Figure S7).

Effect modification of statin intervention by SAA(HDL) or SP-B(HDL)

We investigated whether SAA(HDL) or SP-B(HDL) modified the outcome of the atorvastatin treatment. To examine effect modification by marker quartile we fitted efficacy models stratified by marker quartile aimed to contrast the incidence rates between the atorvastatin and placebo group. If stratified efficacy analysis indicated effect modification, we fitted a model including treatment-marker quartile interaction terms.

Stratified analysis by quartiles did not indicate any significant effect modification by SP-B(HDL). However, we found that atorvastatin reduced the risk for all cardiac events combined in subjects of the second (HR 0.70; 95% CI, 0.49-0.99; P=0.045) and third (HR 0.56; 95% CI, 0.39-0.80; P=0.001) quartiles of SAA(HDL) (Supplemental Figure S11). By contrast, in the highest SAA(HDL) quartile we did not find any effect of atorvastatin treatment. The overall interaction term modeling this effect modification was not significant (P=0.27). For all other endpoints investigated we did not observe any significant interaction.

Generally, beneficial effects of statins on improving the cardiovascular risk of hemodialysis patients are controversial.[3-6] However, in a previous *post-hoc* analysis of the 4D study, atorvastatin treatment was shown to significantly reduce cardiac events and death from any cause in participants with pre-treatment LDL cholesterol levels above 145 mg/dL indicating that a selected subgroup of dialysis patients could benefit from statin therapy.[7] Our analysis suggests that atorvastatin treatment results in a reduction of cardiac events in the two middle SAA(HDL) quartiles. Therefore, it may be arguable that patients with highest SAA(HDL) levels are in an excessively morbid and inflammatory condition (Table 1), which may not allow beneficial treatment effects anymore. Direct anti-inflammatory actions of statins are widely recognized by now[8,9] and statin treatment may exert the highest effects in patients with moderate inflammation according to CRP and SAA(HDL) levels in our analysis. Supportive to this hypothesis are the results from a previous *post-hoc* analysis of the 4D cohort, in which atorvastatin was found to be more effective in preventing cardiovascular death in

patients with intermediate serum CRP levels.[10] Our data may suggest a possible stratification strategy for statin treatment in ESRD patients. Nonetheless, our current secondary analysis of the 4D study was not designed to identify effect modifications and thus it is critical to extend and validate our results in independent patient collectives.

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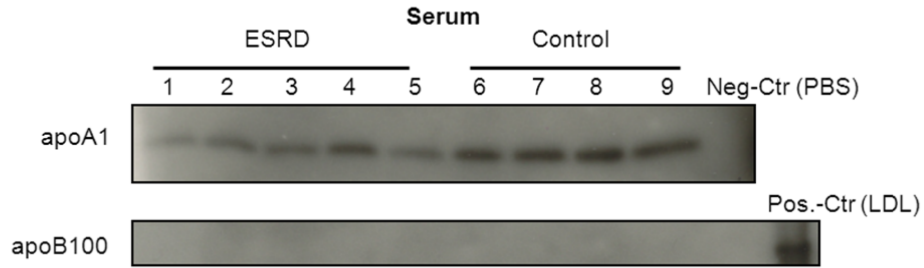
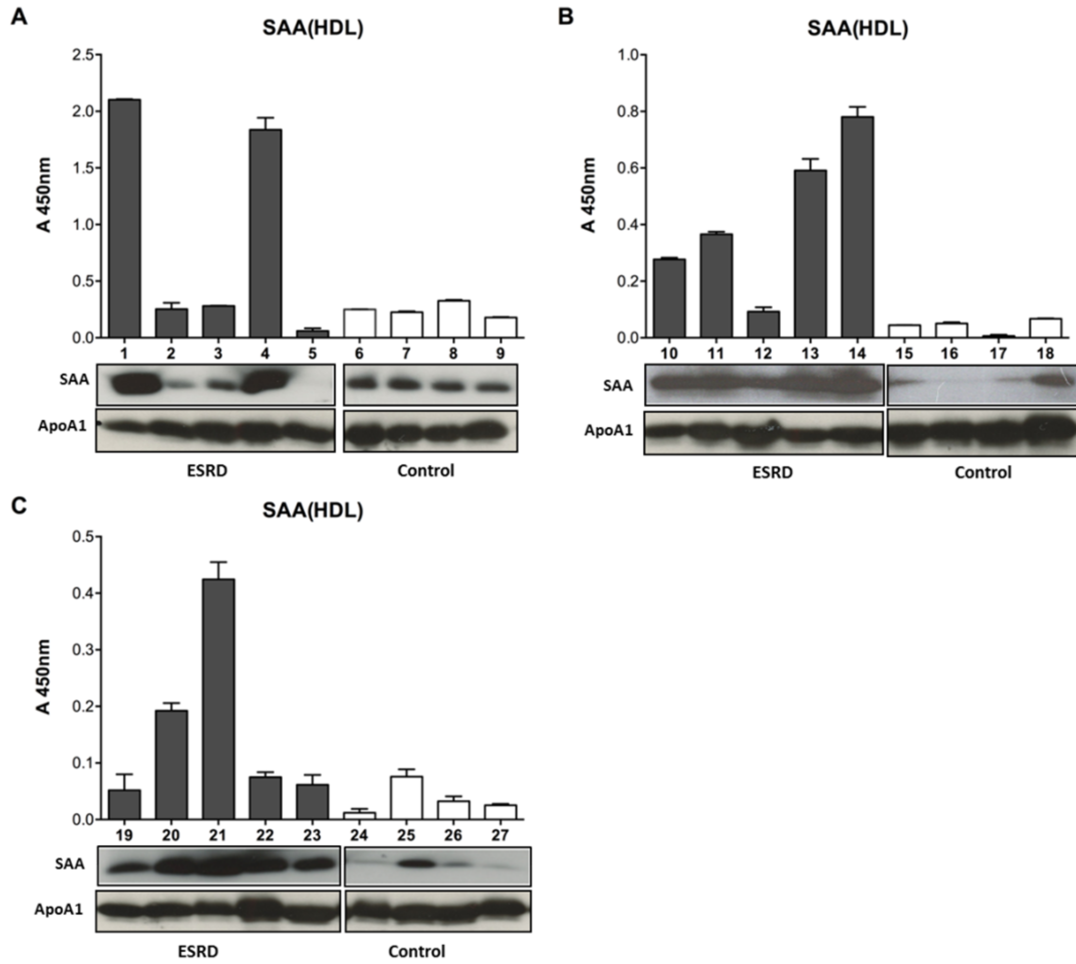
Supplementary Figure S1. Specificity of the ELISA

To check binding specificity of the HDL-catching antibody, an ELISA plate was coated with 1µg/ml α-human HDL overnight and incubated with serum samples from ESRD patients and controls. After incubation, samples were obtained directly from the plate, subjected to immunoblot and probed with the indicated antibodies against HDL (apoA1) and LDL (apoB100).



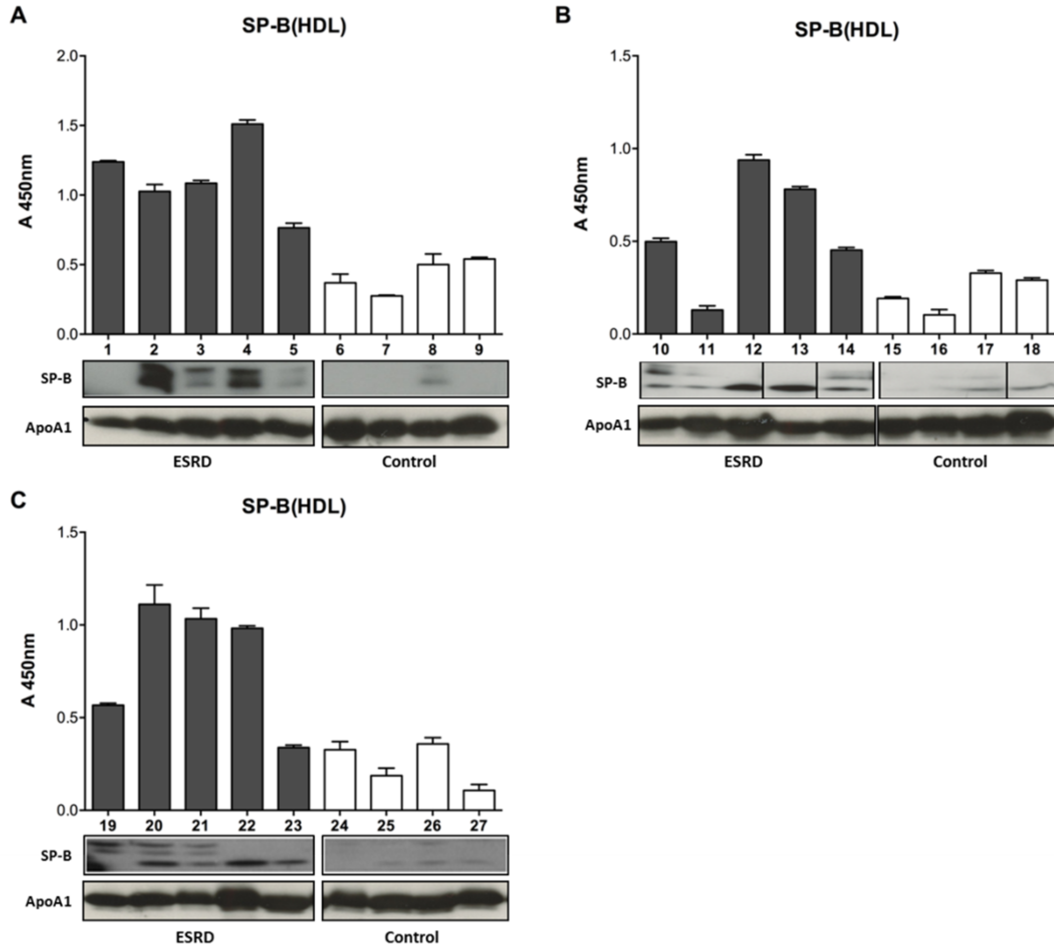
Supplementary Figure S2. Detection of SAA(HDL)

A-C: The amount of HDL-bound SAA was measured by ELISA from 10µg/ml samples in our study cohort. Corresponding samples (10µg HDL/lane) were subjected to immunoblot to confirm the assay results.



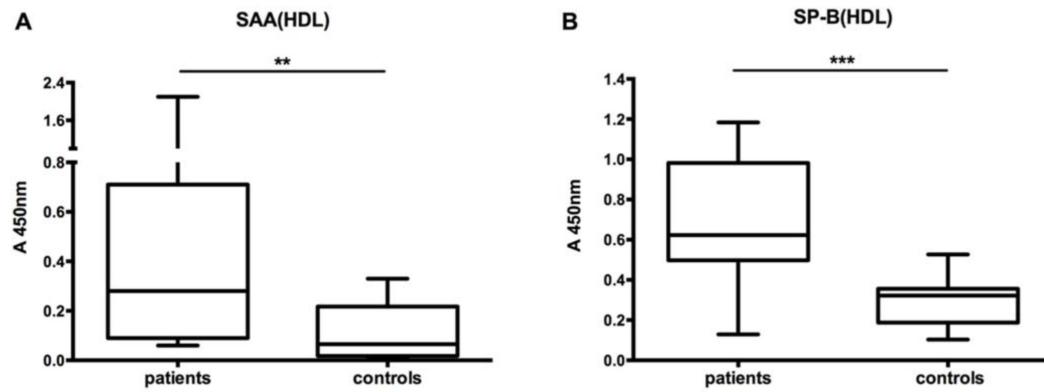
Supplementary Figure S3. Detection of SP-B(HDL)

A-C: The amount of HDL-bound SP-B was measured by ELISA from 10μg/ml samples in our study cohort. Corresponding samples (10μg HDL/lane) were subjected to immunoblot to confirm the assay results.

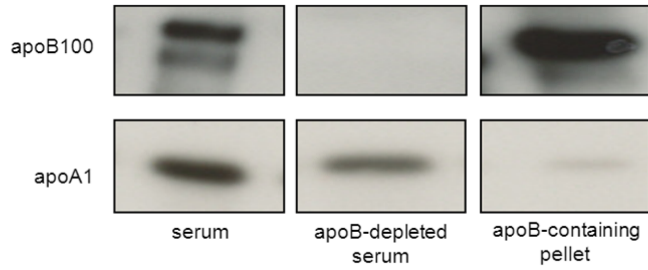


Supplementary Figure S4. SAA(HDL) and SP-B(HDL) are enriched in ESRD patients

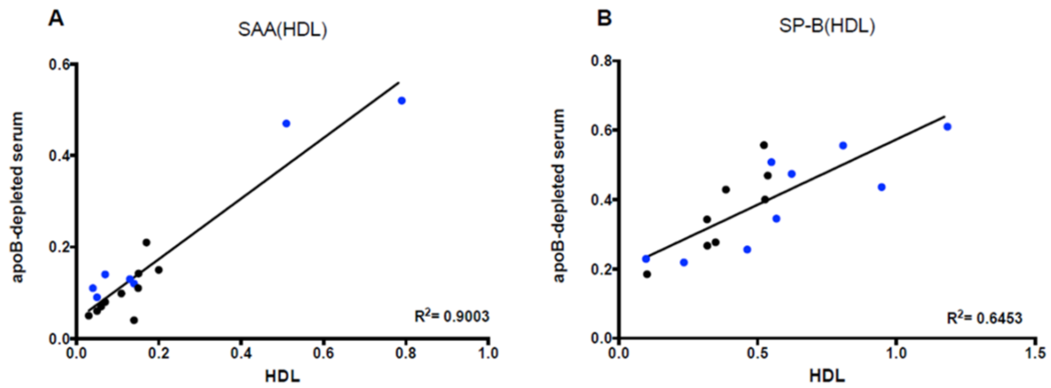
Box-Whisker-Plots with means of (A) SAA and (B) SP-B levels in HDLs of ESRD patients (n=15) and controls (n=12). $^{**}P < 0.01$, $^{***}P < 0.001$.



Supplementary Figure S5. Apolipoprotein B-depleted serum generated by PEG precipitation Apolipoprotein B-depleted serum was prepared from one representative sample, subjected to immunoblot and probed with specific antibodies to analyze the presence of apolipoprotein B-containing fractions and apolipoprotein A1-containing HDL in the supernatant and pellet.

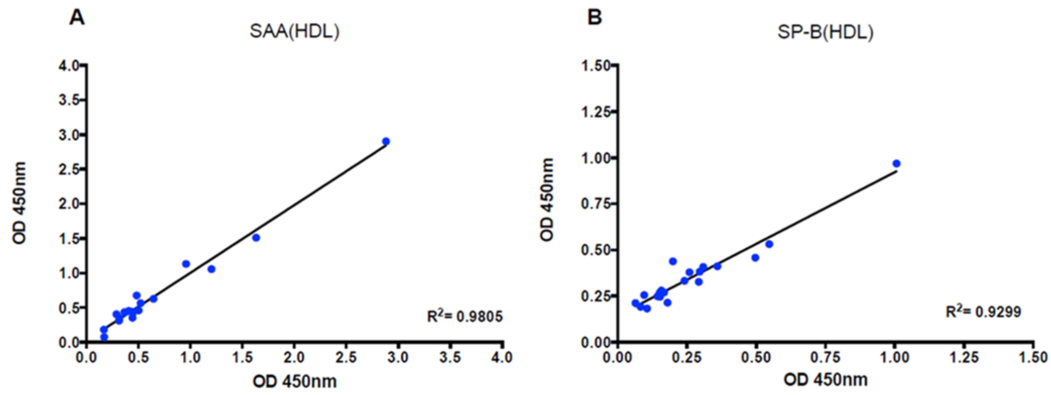


Supplementary Figure S6. SAA(HDL) and SP-B(HDL) levels in apolipoprotein B-depleted serum and HDL Scatterplot with linear regression analysis of (A) SAA(HDL) and (B) SP-B(HDL) levels from apolipoprotein B-depleted serum and corresponding HDL samples of ESRD patients (n= 9) and controls (n= 8). Values in blue represent patients.

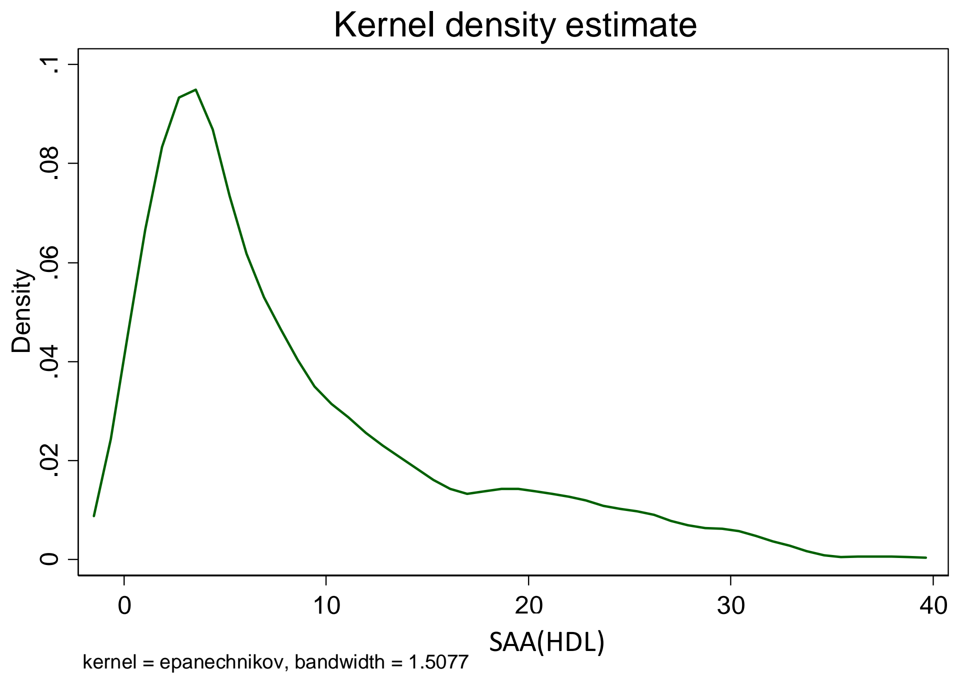


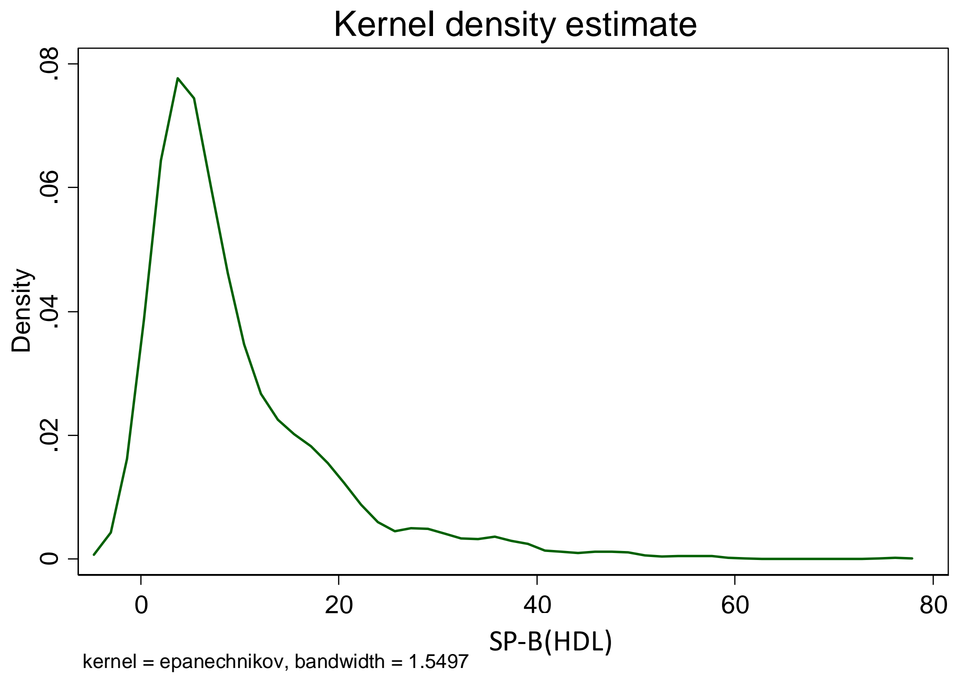
Supplementary Figure S7. Inter-assay variation of the SAA(HDL) and SP-B(HDL) ELISA

Scatterplot with linear regression analysis of (A) SAA(HDL) and (B) SP-B(HDL) levels in apolipoprotein B-depleted serum. Samples from ESRD patients (n=20) were measured in two independent experiments to check for reproducibility of the assay settings.



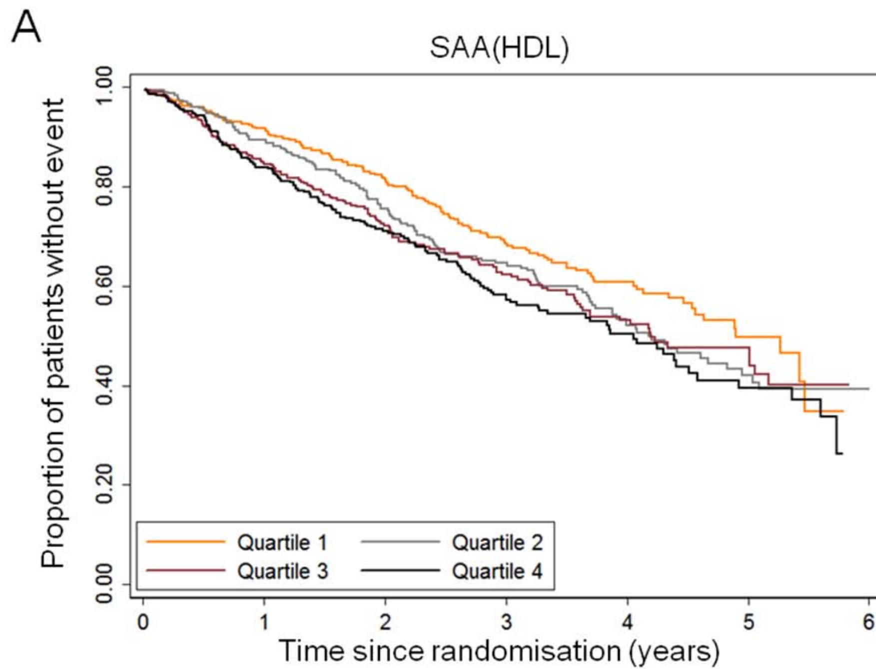
Supplementary Figure S8. Distribution of SAA(HDL) and SP-B(HDL) by Kernel density estimation

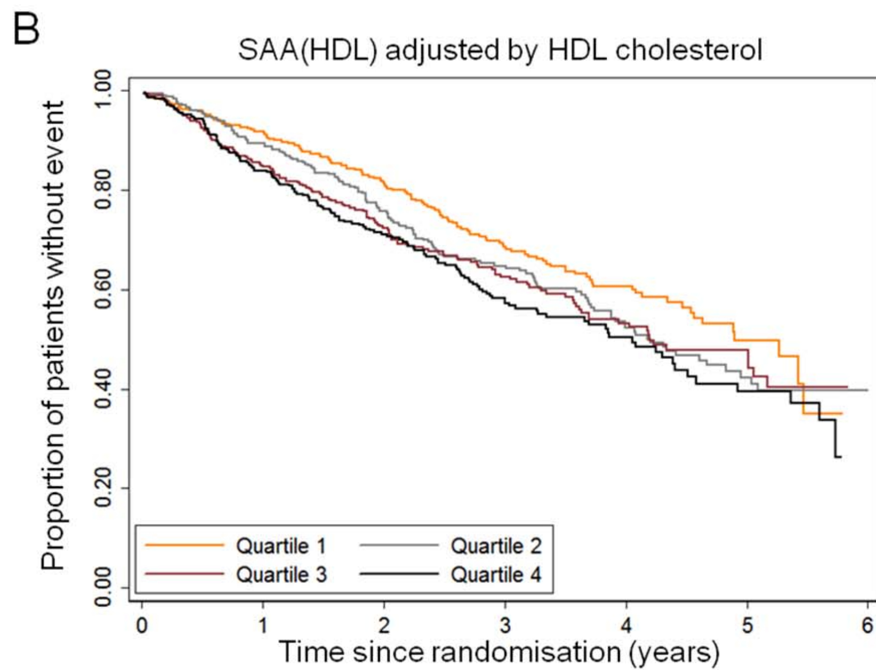




Supplementary Figure S9. Kaplan-Meier estimates for cardiac events in subgroups of patients according to quartiles of baseline SAA(HDL)

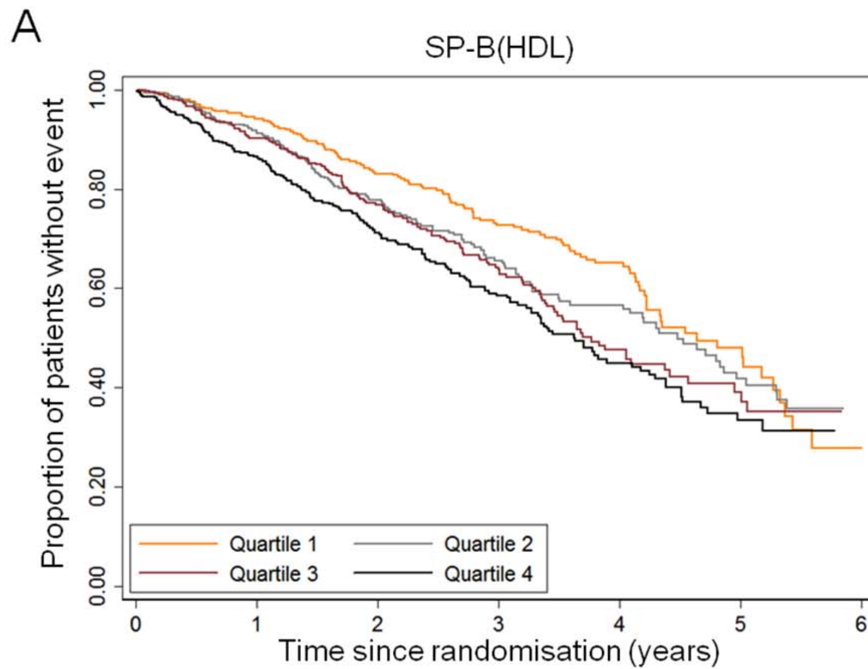
(A) adjusted for predictor variables. (B) adjusted for predictor variables and HDL cholesterol.

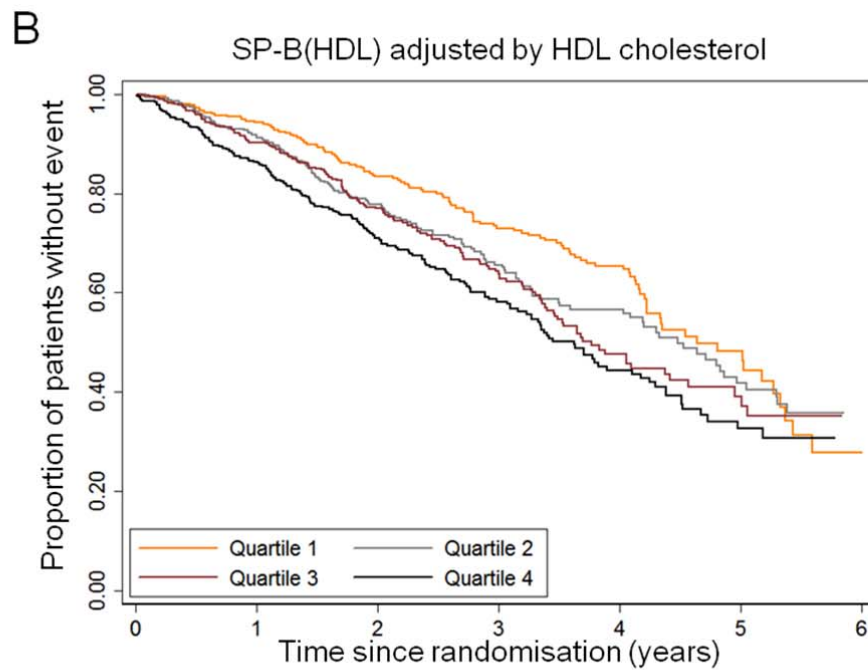




Supplementary Figure S10. Kaplan-Meier estimates for all-cause mortality in subgroups of patients according to quartiles of baseline SP-B(HDL)

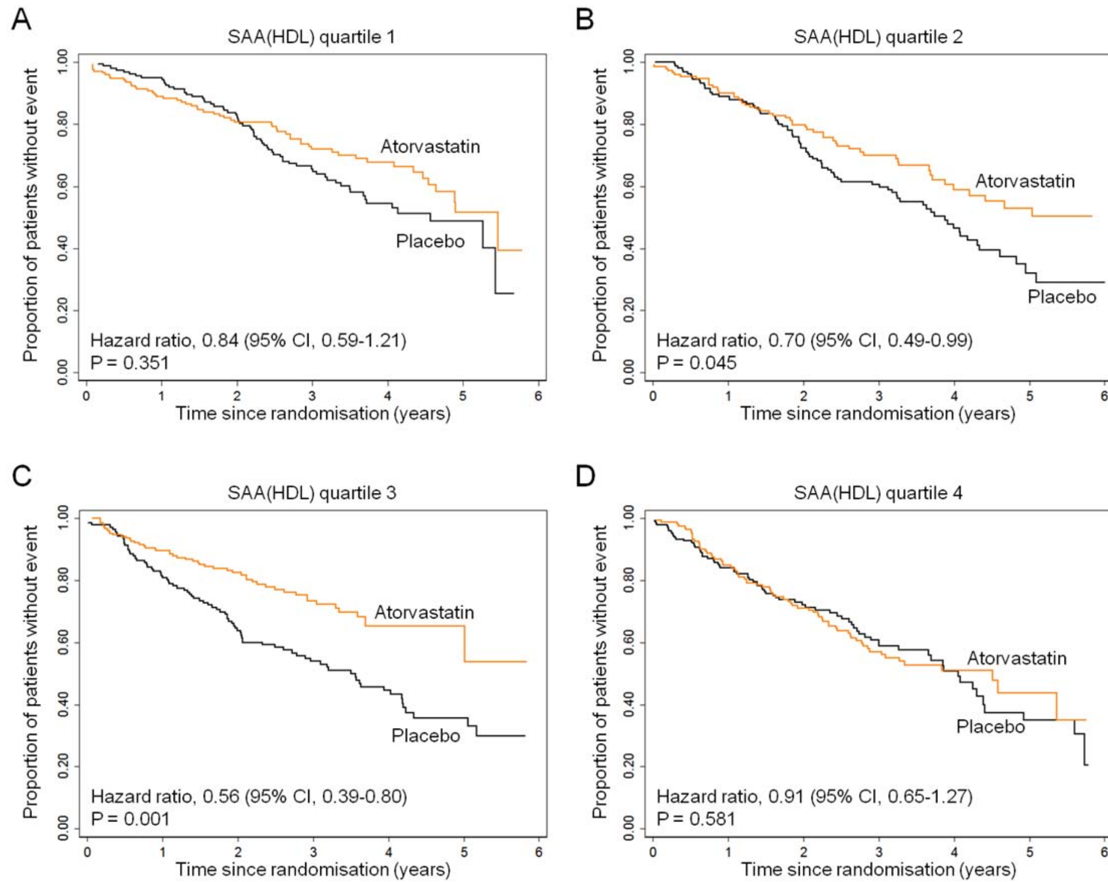
A) adjusted for predictor variables. (B) adjusted for predictor variables and HDL cholesterol.





Supplementary Figure S11. Subgroup analysis Atorvastatin vs. Placebo stratified by SAA(HDL) quartile (endpoint: all cardiac events combined).

Cumulative proportion of patients without cardiac events for each quartile of baseline SAA(HDL) according to medication group in participants of the 4D study (A-D). The hazard ratio is shown as inset.



Supplementary Table S1: Cardiac events according to quartiles of SAA(HDL)

		Hazard ratio for all cardiac events combined (95% CI)									
	Number of events	Adjusted for predictor variables*	P value	Adjusted for predictor variables and HDL cholesterol	P value	Adjusted for predictor variables, HDL cholesterol, and SP-B(HDL)	P value	Adjusted for predictor variables and CRP	P value	Adjusted for predictor variables and apoA-I	P value
Quartile 1 (n=288)	122	1.00		1.00		1.00		1.00		1.00	
Quartile 2 (n=288)	134	1.22 (0.95-1.56)	0.12	1.22 (0.95-1.56)	0.12	1.22 (0.95-1.56)	0.12	1.22 (0.95-1.56)	0.12	1.22 (0.95-1.56)	0.12
Quartile 3 (n=288)	132	1.28 (1.00-1.64)	0.05	1.28 (1.00-1.64)	0.05	1.27 (0.99-1.64)	0.06	1.28 (1.00-1.64)	0.05	1.27 (0.99-1.63)	0.06
Quartile 4 (n=288)	146	1.43 (1.12-1.82)	0.004	1.43 (1.12-1.82)	0.004	1.39 (1.09-1.78)	0.01	1.44 (1.12-1.85)	0.01	1.41 (1.11-1.81)	0.01
P value		0.04		0.04		0.07		0.04		0.05	
Hazard ratio per 1-SD higher		1.09 (1.01-1.19)	0.04	1.09 (1.01-1.19)	0.04	1.09 (1.00-1.19)	0.05	1.09 (1.00-1.19)	0.05	1.09 (1.00-1.18)	0.05

No, Number of events; CI, Confidence interval; CRP, C-reactive protein; HR, Hazard ratio; SD, Standard deviation; P value of multivariate Cox regression model comparing the HR across the four groups.

*Risk factors included in the multivariate regression model were age, gender, phosphate, use of calcium antagonists, history of coronary artery disease, arrhythmia, congestive heart failure, peripheral vascular disease, and atorvastatin treatment.

Supplementary Table S2: All-cause mortality according to quartiles of SP-B(HDL)

	Number of events	Hazard ratio for all-cause mortality (95% CI)									
		Adjusted for predictor variables*	P value	Adjusted for predictor variables and HDL cholesterol	P value	Adjusted for predictor variables, HDL cholesterol and SAA(HDL)	P value	Adjusted for predictor variables and CRP	P value	Adjusted for predictor variables and apoA-I	P value
Quartile 1 (n=288)	117	1.00		1.00		1.00		1.00		1.00	
Quartile 2 (n=288)	128	1.14 (0.88-1.47)	0.32	1.14 (0.88-1.47)	0.32	1.13 (0.88-1.46)	0.34	1.15 (0.89-1.49)	0.28	1.14 (0.88-1.48)	0.31
Quartile 3 (n=288)	148	1.33 (1.04-1.71)	0.03	1.33 (1.04-1.71)	0.03	1.32 (1.03-1.70)	0.03	1.35 (1.05-1.73)	0.02	1.34 (1.04-1.72)	0.02
Quartile 4 (n=288)	171	1.47 (1.15-1.88)	0.002	1.48 (1.16-1.89)	0.002	1.44 (1.12-1.84)	0.01	1.49 (1.17-1.91)	0.001	1.48 (1.16-1.90)	0.002
P value		0.01		0.01		0.02		0.01		0.01	
Hazard ratio per 1-SD higher		1.10 (1.02-1.19)	0.01	1.10 (1.02-1.19)	0.01	1.09 (1.01-1.18)	0.02	1.11 (1.02-1.19)	0.01	1.10 (1.02-1.19)	0.01

No, Number of events; CI, Confidence interval; CRP, C-reactive protein; HR, Hazard ratio; SD, Standard deviation; P value of multivariate Cox regression model comparing the HR across the four groups.

*Risk factors included in the multivariate regression model were age, gender, body mass index, total cholesterol, albumin, hemoglobin, glycated hemoglobin, phosphate, leukocytes, serum creatinine, diastolic blood pressure, duration of dialysis, duration of diabetes, use of statins, use calcium antagonists, use of ACE inhibitors, history of coronary artery disease, ischemia, arrhythmia, congestive heart failure, peripheral vascular disease, and atorvastatin treatment.