Low-density lipoprotein particle diameter and mortality: the Ludwigshafen Risk and Cardiovascular Health Study

Tanja B. Grammer¹, Marcus E. Kleber¹, Winfried März¹,²,³, Günther Silbernagel⁴, Rüdiger Siekmeier⁵, Heinrich Wieland⁶, Stefan Pilz⁷, Andreas Tomaschitz⁸,⁹, Wolfgang Koenig¹⁰, and Hubert Scharnagl¹²*

¹Mannheim Institute of Public Health, Social and Preventive Medicine, Mannheim Medical Faculty, University of Heidelberg, Mannheim, Germany; ²Clinical Institute of Medical and Chemical Laboratory Diagnostics, Medical University of Graz, Auenbruggerplatz 15, Graz A-8036, Austria; ³Synlab Academy, Mannheim, Germany; ⁴Department of Angiology, Swiss Cardiovascular Center, Inselspital, University of Bern, Bern, Switzerland; ⁵Federal Institute for Drugs and Medical Services, Bonn, Germany; ⁶Division of Clinical Chemistry, University Medical Center Freiburg, Freiburg, Germany; ⁷Department of Internal Medicine, Division of Endocrinology and Metabolism, Medical University of Graz, Graz, Austria; ⁸Department of Cardioiology, Medical University of Graz, Graz, Austria; ⁹Specialist Clinic for Rehabilitation PVA Bad Aussee, Bad Aussee, Austria; and ¹⁰Department of Internal Medicine II – Cardiology, University of Ulm Medical Centre, Ulm, Germany

Received 9 August 2013; revised 14 November 2013; accepted 14 January 2014; online publish-ahead-of-print 25 February 2014

Aims
The aim of the study was to examine whether differences in average diameter of low-density lipoprotein (LDL) particles were associated with total and cardiovascular mortality.

Methods and results
We studied 1643 subjects referred to coronary angiography, who did not receive lipid-lowering drugs. During a median follow-up of 9.9 years, 398 patients died, of these 246 from cardiovascular causes. We calculated average particle diameters of LDL from the composition of LDL obtained by β-quantification. When LDL with intermediate average diameters (16.5–16.8 nm) were used as reference category, the hazard ratios (HRs) adjusted for cardiovascular risk factors for death from any cause were 1.71 (95% CI: 1.31–2.25) and 1.24 (95% CI: 0.95–1.63) in patients with large (>16.8 nm) or small LDL (<16.5 nm), respectively. Adjusted HRs for death from cardiovascular causes were 1.89 (95% CI: 1.32–2.70) and 1.54 (95% CI: 1.06–2.12) in patients with large or small LDL, respectively. Patients with large LDL had higher concentrations of the inflammatory markers interleukin (IL)-6 and C-reactive protein than patients with small or intermediate LDL. Equilibrium density gradient ultracentrifugation revealed characteristic and distinct profiles of LDL particles in persons with large (approximately even distribution of intermediate-density lipoproteins and LDL-1 through LDL-6) intermediate (peak concentration at LDL-4) or small (peak concentration at LDL-6) average LDL particle diameters.

Conclusions
Calculated LDL particle diameters identify patients with different profiles of LDL subfractions. Both large and small LDL diameters are independently associated with increased risk mortality of all causes and, more so, due to cardiovascular causes compared with LDL of intermediate size.

Keywords
Cholesterol • Triglycerides • Low-density lipoproteins • Subfractions • Mortality

Introduction
Low-density lipoproteins (LDL) include a heterogeneous mixture of particles which can be separated by ultracentrifugation,¹–³ chromatography,⁴ nuclear magnetic resonance spectroscopy,⁵ or gradient gel electrophoresis.⁶ Experimental and turnover studies have raised the possibility that dense LDL may be more atherogenic than buoyant LDL. Potential mechanisms include easier transfer of dense LDL from the vessel lumen into the subintimal space,⁷ decreased binding to LDL receptors and increased plasma residence time,⁸–¹¹ and higher affinity to proteoglycans compared with buoyant LDL.¹²,¹³ Dense LDL have been linked to surrogate vascular endpoints including endothelial dysfunction¹⁴ or carotid intima media...
Methods

Ludwigshafen Risk and Cardiovascular Health Study

The Ludwigshafen Risk and Cardiovascular Health (LURIC) study is an ongoing prospective cohort study of patients referred for coronary angiography. In total, 3316 subjects were recruited between June 1997 and May 2001.29 Recruitment was done consecutively on regular working days and the number of subjects included per day was only limited by the capacity of the staff involved. There was no deliberate exclusion of subjects for reasons other than the exclusion criteria specified in the study protocol. Low-density lipoprotein composition was available in 3266 individuals with coronary angiograms; of these, 1623 with lipid-lowering drugs were excluded from the analysis. Among the remaining 1643 subjects, 1070 had angiographically proven CAD (≥20% stenoses), and 573 had no CAD. Of the 1070 subjects with CAD, 743 presented with stable CAD, while the remaining patients were admitted with unstable angina pectoris (n = 243), non-ST-elevation myocardial infarction (NSTEMI) (TroponinT > 0.1 μg/L, n = 47), or ST-elevation myocardial infarction (STEMI) (TroponinT > 0.1 μg/L, n = 37). During a median follow-up of 9.9 years, 398 patients died, of these 246 from cardiovascular causes according to death certificates obtained from local health authorities. No patient was lost to follow-up, death certificates were obtained for age, sex and additional adjustment for other cardiovascular risk factors in- side the LURIC study) (VLDL, intermediate-density lipoproteins (IDL), LDL, six LDL subfractions, HDL).1 The average radius of LDL was calculated from apolipoproteins and major lipids as described.1

Results

Characteristics of study participants according to mean low-density lipoprotein diameters

The proportion of men significantly increased in parallel to decreasing LDL size (Table 1). Patients with small LDL were on average younger than those with large or intermediate LDL. Current or past smoking, diabetes mellitus and hypertension were more prevalent among patients with small LDL. There was no obvious association of LDL size with angiographic CAD. In parallel to decreasing LDL size, body mass index, diabetes mellitus, hypertension, smoking, estimated glomerular filtration rate, LDL-C, HDL-C, and TG (Figure 1, Table 2).

The assumption that residuals are normally distributed was examined using plots of observed vs. predicted values. In none of the analyses did we obtain an indication that this assumption was violated. We are reporting estimated marginal means of the dependent variables along with their 95% CI. The least significant difference was used for post hoc comparisons. The statistical tests were two-sided; P < 0.05 was considered significant. The SPSS 19.0 statistical package (SPSS Inc.) was used.

Low-density lipoprotein size, total mortality, and mortality from cardiovascular causes

Mortality from all causes was lowest in patients with intermediate LDL (16.5–16.8 nm), while it was significantly higher in patients with larger and smaller LDL. This finding was robust against adjustment for age and sex and additional adjustment for other cardiovascular risk factors including LDL-C (Figure 1, Table 2, Model 3). The hazard ratios (HRs) of the fully adjusted model were 1.71 (95% CI: 1.31–2.25) and 1.24 (95%...
Table 1  Clinical characteristics of study participants according to low-density lipoprotein size

<table>
<thead>
<tr>
<th>LDL size</th>
<th>Large</th>
<th>Intermediate</th>
<th>Small</th>
<th>P*</th>
<th>P#</th>
<th>P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Particle diameter (nm)x</td>
<td>&gt;16.8</td>
<td>16.5–16.8</td>
<td>&lt;16.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N</td>
<td>469</td>
<td>470</td>
<td>704</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Men</td>
<td>246 (53%)</td>
<td>289 (62%)</td>
<td>563 (80%)</td>
<td>&lt;0.001*</td>
<td>0.005*</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Women</td>
<td>223 (47%)</td>
<td>181 (38%)</td>
<td>168 (20%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>64 ± 11</td>
<td>63 ± 10</td>
<td>61 ± 12</td>
<td>&lt;0.001f</td>
<td>0.752f</td>
<td>0.001f</td>
</tr>
<tr>
<td>Smoking</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Never</td>
<td>209 (45%)</td>
<td>210 (45%)</td>
<td>228 (32%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Previous</td>
<td>161 (34%)</td>
<td>170 (36%)</td>
<td>311 (44%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Current</td>
<td>99 (21%)</td>
<td>90 (19%)</td>
<td>165 (23%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Coronary artery disease</td>
<td></td>
<td></td>
<td></td>
<td>0.270g</td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>185 (39%)</td>
<td>161 (34%)</td>
<td>227 (32%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stable CAD</td>
<td>196 (42%)</td>
<td>210 (45%)</td>
<td>337 (48%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unstable (TnT−)</td>
<td>63 (13%)</td>
<td>76 (16%)</td>
<td>104 (15%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NSTEMI (TnT+)</td>
<td>15 (3%)</td>
<td>15 (3%)</td>
<td>17 (2%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>STEMI (TnT+)</td>
<td>61 (2%)</td>
<td>8 (2%)</td>
<td>19 (3%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>26.5 ± 4.0</td>
<td>27.3 ± 4.2</td>
<td>28.0 ± 4.2</td>
<td>&lt;0.001h</td>
<td>0.005h</td>
<td>&lt;0.001h</td>
</tr>
<tr>
<td>Diabetes mellitus (%)</td>
<td>124 (26%)</td>
<td>129 (27%)</td>
<td>253 (36%)</td>
<td>&lt;0.001i</td>
<td>0.632i</td>
<td>&lt;0.001i</td>
</tr>
<tr>
<td>LDL-C (g/L)</td>
<td>1.24 ± 0.33</td>
<td>1.31 ± 0.32</td>
<td>1.19 ± 0.30</td>
<td>&lt;0.001h</td>
<td>0.001h</td>
<td>0.001h</td>
</tr>
<tr>
<td>HDL-C (g/L)</td>
<td>0.44 ± 0.13</td>
<td>0.42 ± 0.11</td>
<td>0.37 ± 0.09</td>
<td>&lt;0.001h</td>
<td>0.030h</td>
<td>0.001h</td>
</tr>
<tr>
<td>Triglycerides (g/L)x</td>
<td>1.23 (1.18–1.29)</td>
<td>1.31 (1.26–1.37)</td>
<td>1.73 (1.67–1.79)</td>
<td>&lt;0.001h</td>
<td>0.042h</td>
<td>&lt;0.001h</td>
</tr>
<tr>
<td>Hypertension</td>
<td>325 (69%)</td>
<td>341 (73%)</td>
<td>531 (75%)</td>
<td>0.068i</td>
<td>0.190i</td>
<td>0.016i</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>140 ± 23</td>
<td>142 ± 23</td>
<td>144 ± 23</td>
<td>0.001k</td>
<td>0.228k</td>
<td>0.001k</td>
</tr>
<tr>
<td>Diastolic blood pressure (mmHg)</td>
<td>81 ± 12</td>
<td>82 ± 11</td>
<td>82 ± 12</td>
<td>0.101k</td>
<td>0.133k</td>
<td>0.608k</td>
</tr>
</tbody>
</table>

*P for trend: large, intermediate, and small LDL.
#Post hoc P (least square difference): large vs. intermediate LDL.
$Post hoc P (least square difference): small vs. intermediate LDL.
\(^{x}\)Calculated from apoB and major lipids determined by β-quantification.
\(^{f}\)Logistic regression adjusted for age.
\(^{g}\)ANOVA adjusted for sex.
\(^{h}\)ANOVA adjusted for age and sex.
\(^{i}\)ANOVA adjusted for age and sex.
\(^{j}\)ANOVA adjusted for age, sex, and for use of antihypertensive drugs.

CI: 0.95–1.63) for large and small LDL, respectively. Comparable results were obtained for the association with cardiovascular mortality (Figure 1, Table 2). Adjustment for apoB instead of LDL-C did not substantially change the association of LDL size with total and cardiovascular mortality (Supplementary material online, Table S1). There was no relationship between LDL-C and LDL-apoB with mortality, neither in the entire study population nor in any of the LDL size groups.

The association of small LDL with total mortality was stronger in women than in men (P for interaction = 0.023), while there was no significant interaction between sex and LDL diameter for the association with cardiovascular mortality (P for interaction = 0.759). The HRs for total and cardiovascular mortality were similar in patients with and without type 2 diabetes mellitus (P for interaction: 0.182 and 0.745, respectively).

With the β-quantification method, lipoprotein(a) is in the LDL fraction. Thus, we calculated HRs in a subgroup with lipoprotein(a) concentrations <0.30 g/L (n = 1183). The resulting HRs were only slightly different compared with the entire study population indicating that lipoprotein(a) did not impact on our findings (Supplementary material online, Table S2).

The size and composition of LDL are related to the metabolism of TG-rich lipoproteins.33 However, VLDL-apoB and VLDL-C were not significantly associated with total or cardiovascular mortality in the current study (Supplementary material online, Table S3).

**Low-density lipoprotein mean particle diameter and low-density lipoprotein composition**

We investigated the composition of LDL in the strata of LDL size (Supplementary material online, Table S4). In patients with small LDL, the concentration of LDL-C was lower compared with those
with intermediate-sized LDL. At the same time, the concentration of LDL-apoB was not decreased compared with intermediate LDL. Thus, at a given LDL-C the molar concentration of LDL particles was higher in individuals with small LDL. Consistently, small LDL contained less lipid molecules per particle than LDL in patients with intermediate-sized LDL (on average 1503 compared with 1675, respectively). In patients with large LDL, LDL-C was slightly lower than in patients with intermediate LDL, but LDL-apoB was much lower, indicating that these individuals have larger, lipid-rich LDL (on average 1851 molecules vs. 1675 per particle).

Low-density lipoprotein mean particle diameter and markers of inflammation and oxidative stress

Oxidized LDL and Lp-PLA2 were significantly higher in individuals with small LDL compared with the groups with large and intermediate LDL. Interestingly, the inflammatory markers C-reactive protein (CRP) and interleukin (IL)-6 were highest in the group with large LDL (Table 3). Adjustment for inflammatory markers slightly attenuated the association of large LDL with mortality, suggesting that inflammation is involved in the atherogenic effects of these particles (Table 2, Supplementary material online, Table S5).

Low-density lipoprotein mean particle diameter and low-density lipoprotein subfractions

We examined the relationship between the three categories of mean LDL particle diameters and the distribution of LDL subfractions. Because LDL subfractions have not been determined by equilibrium density gradient ultracentrifugation in the LURIC study, we exploited baseline results obtained in participants of two independent, previously completed, randomized clinical studies of lipid lowering. We calculated mean LDL diameters in these patients from the composition of the entire LDL fraction (1.0063 kg/L through 1.063 kg/L) and subsequently stratified the results according to the particle diameter thresholds used LURIC. As LDL contain one molecule apoB per particle, we considered the apoB contents of LDL subfractions to reflect the concentrations of particles in each of the density fractions LDL-1 to LDL-6. Low-density lipoprotein subfraction profiles were clearly distinct between the groups (Figure 2). In patients with large LDL, the LDL particle concentrations were almost equal across the density spectrum with LDL-1 assuming the highest concentration. In patients with LDL of intermediate mean size, LDL particle concentration reached a peak at LDL-4. Finally, in patients with the smallest calculated LDL diameters, the concentration of LDL particles was highest in LDL-6, followed by LDL-5. The compositions of LDL subfractions hardly differed across the strata with different average LDL sizes, with the exception of an enrichment of TG in the LDL subfractions in patients with large LDL (Supplementary material online, Tables S6 and S7). This overall indicates that changes in the calculated average size of LDL are mainly due to changes in the relative proportions of each of the subfractions rather than to compositional differences in individual subfractions.

Discussion

This study demonstrates that the mean particle diameter of LDL is associated with long-term total and cardiovascular mortality in patients undergoing coronary angiography, essentially independent of the main acknowledged cardiovascular risk factors. Using the composition of LDL inferred from a β-quantification procedure, we stratified patients into those with large, intermediate, and small
average calculated calculated particle size. Intriguingly, the relationship between particle size and mortality was U-shaped, placing both patients with large and small LDL particles at a higher risk of fatal events.

It has been known for long that LDL include a mixture of particles differing regarding density and size. Experimental studies have suggested that small dense LDL are particularly atherogenic. Epidemiological evidence based on clinical endpoints for a role of small dense LDL in the development or progression of vascular disease has, however, been surprisingly sparse. Some studies have relied on surrogate vascular endpoints only, such as endothelial dysfunction or carotid intima media thickness. In epidemiological studies using nuclear magnetic resonance spectroscopy to determine LDL size, either small LDL were predictive for CAD or both small and large LDL were associated with subclinical atherosclerosis or CAD, but only in one study the risk estimates were independent of other lipid parameters. Some prospective epidemiological studies using gradient gel electrophoresis have found small dense LDL associated with vascular disease after adjusting for either low HDL or high TG. The majority of studies, however, did not show such an independent association. This is evidently due to the fact that high concentrations of small dense LDL are part of the atherogenic lipid triad (in addition characterized by high TG) which in turn is a feature of the metabolic syndrome. It is, in this regard, worth to note that we were able to demonstrate that small LDL were essentially independently associated with mortality from all causes and from cardiovascular causes in the current study. Of interest, we also obtained evidence for adverse effects not only of predominantly small but also large LDL compared with LDL of intermediate size. This finding is consistent with studies claiming that buoyant rather than small LDL are associated with atherosclerosis and an increased CAD risk.

To validate our approach of using the average calculated diameter of LDL as a surrogate for the distribution of LDL subfractions, we examined the relationship between calculated LDL particle diameters and actual LDL profiles obtained by equilibrium density gradient ultracentrifugation. As expected, patients with low-LDL diameters exhibited distinct preponderance of dense LDL. In patients with LDL of intermediate size, the LDL particle concentration had a peak at LDL-4 (1.037–1.040 kg/L). At the largest average LDL size, the distribution of LDL particles was almost even, with LDL-1 (1.019–1.031 kg/L) reaching the highest concentration. The three groups of our mortality study thus had distinct patterns of LDL subfractions.

Low-density lipoprotein particles can be synthesized via two pathways: the lipolysis of TG-rich lipoproteins and direct hepatic secretion. In our study, the composition of LDL in patients with large, intermediate, and small LDL differed characteristically. In patients with large LDL, the LDL-TG concentration and the number of lipid molecules per particle were highest among the three groups; LDL were also relatively enriched in TG. It is thus reasonable to assume that incompletely catabolized remnants of TG-rich lipoproteins accumulate in these individuals. On the other hand, patients with smaller LDL had LDL poor in lipids but relatively enriched in cholesteryl esters. Those small LDL are the product of hydrolysis of TG-rich precursors by hepatic lipase. However, some of them may directly originate from the liver, because there is a substantial contribution of hepatic synthesis of small LDL to plasma LDL in hypertriglyceridemia.
We sought to evaluate mechanisms underlying the association of buoyant and dense LDL with adverse vascular outcomes. We therefore compared markers of inflammation and oxidative stress between the strata of different average LDL sizes. We observed elevated concentrations of oxidized LDL in individuals with small LDL compared with the other groups. Further, Lp-PLA₂, which by large promotes destabilization of atherosclerotic lesions, was also higher expectedly, however, remains elusive at this time. In particular, it is hard to decide whether systemic inflammation causes the accumulation of buoyant LDL or whether these lipoproteins are themselves responsible for the inflammatory reaction. Disorders of LDL metabolism and low-grade inflammation have so far been conceived to be atherogenic by completely distinct pathways. However, the TG contents of LDL may be implicated in inflammation and atherogenesis. In humans, LDL-TG are mainly hydrolyzed by hepatic lipase, which is subject to modulation by inflammatory cytokines. Downregulation of lipases is considered to explain, at least in part, the elevation of TG during the acute phase response. Low-grade systemic inflammation could thus be the cause rather than the consequence of the accumulation of buoyant LDL. The observation, however, that LDL-TG were associated with both CAD and circulating adhesion molecules independent of CRP is in favor of the idea that TG-enrichment of LDL might itself be pro-inflammatory.

Our study has three major limitations. First, it included patients scheduled for angiography. This hospital-based population may be not representative of a random population sample. Second, we excluded subjects receiving lipid-lowering drugs and thus may have selected individuals with low-LDL-C. Third, estimating the atherogenicity of LDL with the β-quantification method used here is still technically demanding and the clinical application of our findings may therefore not be straightforward. Future investigations will therefore have to address the question whether other methods to estimate mean LDL particle size would be equally informative.

In summary, this is the first study to show a significant association of both, large and small LDL particle size on total and cardiovascular mortality. Our results underline the impact of markers of the LDL metabolism on clinical endpoints beyond elevated LDL-C concentration.

### Supplementary Material

Supplementary material is available at European Heart Journal online.

### Acknowledgements

The technical assistance of Isolde Friedrich and Brigitte Kreisel, University of Freiburg, is gratefully acknowledged. Mercodia AB
(Uppsala, Sweden) provided reagents for the determination of oxidized LDL free of charge, but did not assume any other role in this research. We thank the participants of the Ludwigshafen Risk and Cardiovascular Health Study and the study team either temporarily or permanently involved in patient recruitment, sample and data handling, and the laboratory staff at the Ludwigshafen General Hospital and the Universities of Freiburg (Germany), Ulm (Germany), and Graz (Austria).

Funding
This work was supported by the 6th Framework Program (integrated project Bloodomics, grant LSHM-CT-2004-503485) and 7th Framework Program (integrated project AtheroRemo, Grant Agreement number 201668) of the European Union.

Conflict of interests: none declared.

References
31. Ottovs JD, Collins D, Freedman DS, Shalauerova I, Schaefer J, McNamara JR, Bloomfield HE, Robins SJ. Low-density lipoprotein and high-density lipoprotein particle subclasses predict coronary events and are favorably changed by gemfibrozil therapy in the veterans affairs high-density lipoprotein intervention trial. Circulation 2006;113:1556–1563.


