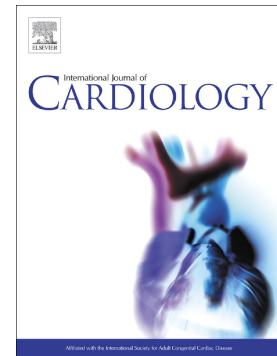


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LDL particle size and composition and incident cardiovascular disease in a South-European population: The Hortega-Liposcale Follow-up Study

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**LDL particle size and composition and incident cardiovascular disease in a  
South-European population: The Hortega-LIPOSCALE Follow-up Study**

**Short title: LDL particle composition and cardiovascular disease**

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## Disclosure

NA is employed by, is a stockholder of, and serves on the board of directors of Biosfer Teslab, a diagnostic laboratory company that performed the lipoprotein subclass analyses described herein. MAPC is employed (Chief Operations Officer) by Biosfer Teslab. The remaining authors report no relationships that could be construed as a conflict of interest.

## Author Contributions

NA, MTP and JR developed the study concept and design. MTP, GP and ADL performed statistical analyses. GP, MTP and JR drafted the manuscript. All the authors interpreted data, and critically revised and completed the manuscript. JR is the guarantor of this work and, as such, had full access to all the data in the study and take responsibility for the

integrity of the data and the accuracy of the data analysis.

### **Permissions information**

The authors do hereby declare that all illustrations and figures in the manuscript are entirely original and do not require reprint permission.

**Keywords:** LDL particles, NMR metabolomics, cardiovascular disease, prospective study

ACCEPTED MANUSCRIPT

**Abstract**

**Background:** The association of low-density lipoprotein (LDL) particle composition with cardiovascular risk has not been explored before. The aim was to evaluate the relationship between baseline LDL particle size and composition (proportions of large, medium and small LDL particles over their sum expressed as small-LDL %, medium-LDL % and large-LDL %) and incident cardiovascular disease in a population-based study.

**Methods:** Direct measurement of LDL particles was performed using a two-dimensional NMR-technique (Liposcale®). LDL cholesterol was assessed using both standard photometrical methods and the Liposcale® technique in a representative sample of 1,162 adult men and women from Spain.

**Results:** The geometric mean of total LDL particle concentration in the study sample was 827.2 mg/dL (95% CI 814.7, 839.8). During a mean follow-up of  $12.4 \pm 3.3$  years, a total of 159 events occurred. Medium LDL particles were positively associated with all cardiovascular disease, coronary heart disease (CHD) and stroke after adjustment for traditional risk factors and treatment. Regarding LDL particle composition, the multivariable adjusted hazard ratios for CHD for a 5% increase in medium and small LDL % by a corresponding decrease of large LDL % were 1.93 (1.55, 2.39) and 1.41 (1.14, 1.74), respectively.

**Conclusions:** Medium LDL particles were associated with incident cardiovascular disease. LDL particles showed the strongest association with cardiovascular events when the particle composition, rather than the total concentration, was investigated. A change in baseline composition of LDL particles from large to medium and small LDL particles was associated with and increased cardiovascular risk, especially for CHD.

## Introduction

Photometrically assessed low-density lipoprotein (LDL) cholesterol is a standard measure for cardiovascular risk stratification and is directly related to the total amount of cholesterol, which is transported by LDL particles (1). The assessment of LDL particles carrying cholesterol is feasible nowadays using sophisticated techniques of lipidomics including nuclear magnetic resonance (NMR) spectroscopy (2). The size, lipid content and number of LDL particles can be measured using NMR-based Advanced Lipoprotein Testing (ALT) and subfractionation, and the resulting LDL particle subclasses are commonly divided into large, medium and small particles according to their respective size (3). Although the determination of lipoprotein by NMR has been performed for some years, both at the level of research and commercial tests, most approaches are limited to lipid peaks and analysis of lipoproteins in one dimension. The 2D-diffusion ordered spectroscopy (DOSY)-NMR Liposcale®, a two dimensional NMR test, is a unique technique able to determine the number of LDL lipoprotein particles from direct size measurements, providing more accurate results than the commercial alternatives (4).

Over the past years, interest has grown in the predictive value of LDL particle number and size, and the determination of LDL particles has been included into the guidelines of the American Association of Clinical Endocrinologists for prevention of atherosclerosis (5). However, current evidence is mainly based on studies conducted in patients with elevated cardiovascular risk or sub-studies of clinical trials addressing the effect of lipid-lowering treatment, thus, available data on the relationship between NMR-measured LDL particles and cardiovascular events in the general population is scarce (6–10). Moreover, inconsistent findings have been reported regarding the relationship of LDL subclasses with cardiovascular disease. Small, medium and large LDL particles have been associated with a higher, lower and null risk for cardiovascular endpoints, nonetheless, the comparison of

available studies is challenging due to differences in terms of study population, outcome variables of interest, particle assessment method and statistical analysis (11–14).

Furthermore, the potential role of LDL particle subclasses' composition (proportions of large, medium and small LDL particles over their sum expressed as small-LDL %, medium-LDL % and large-LDL %), rather than absolute concentrations, on cardiovascular disease has not been explored before.

The objective of the present study was to evaluate the association of total LDL cholesterol, LDL particle size, concentration and LDL particle composition with incident cardiovascular disease in adult men and women participating in the Hortega Follow-up Study, a cohort study representative of a general population from Spain.

## **Methods**

### *Study Population*

The Hortega Study is a population-based survey among adults 15-85 years old residing in the conscription area of the Rio Hortega University Hospital in Valladolid (central Spain). The Hortega Study started in 1997 as a mail survey on a random sample of 20% of public health system beneficiaries (Hortega Phase I), followed by a pilot examination of randomly selected individuals who responded to the questionnaire (Hortega Phase II). In 2001-2002 the Hortega study (phase III) examined and interviewed a representative sample of 1502 phase I participants (including re-examination of the phase II- pilot study- participants). In 2001-2002, urine and blood samples were collected from all Hortega Study participants and immediately stored at -80 °C at the Hospital Rio Hortega, Valladolid (Spain), with no sample losses and following standardized protocols and standard quality control procedures. Incident health endpoints and mortality during follow-up were assessed in 2015 by reviews of electronic health registers including primary care, hospitalization and death



records (phase IV). The study sample consisted of 1,502 total participants with stored plasma samples for Liposcale® determinations. 144 participants were excluded due to prevalent CVD, 10 participants due to missing Liposcale® measures and 186 participants due to missing data in other relevant covariates, leaving 1,162 participants for the present study. The research protocol was approved by the Ethics Committee of the Rio Hortega University Hospital and all participants provided written informed consent.

### *Lipid profiling*

For the measurement of “standard” lipid levels, plasma total cholesterol, high-density lipoprotein (HDL)-cholesterol, and total triglycerides (TG) concentrations in non-fasting samples were determined using a Hitachi 917 analyzer. LDL cholesterol was calculated using the Friedewald formula.

Elevated total cholesterol, LDL cholesterol and TG were defined as  $> 200$  mg/dl,  $> 130$  mg/dl, and  $> 150$  mg/dl, respectively. Low HDL cholesterol was defined as  $< 40$  mg/dl in men or  $< 50$  mg/dl in women.

In 2015, 500  $\mu$ l of blood plasma samples were shipped on dry ice to the Biosfer Teslab facilities (Reus, Spain) for Liposcale® lipoprotein analysis by Nuclear Magnetic Resonance (NMR), contracting a reliable shipping service to maintain the cold chain and guarantee that they arrive frozen. Before the NMR analysis, the samples were completely thawed, homogenized and transferred to the NMR tubes in a controlled temperature of 4°C. The size, lipid content and the number of the LDL particles were determined as previously reported (4). Briefly, particle concentration and the diffusion coefficients were obtained from the measured amplitudes and attenuation of their spectroscopically distinct lipid methyl group NMR signals, using the 2D diffusion-ordered  $^1\text{H}$  NMR spectroscopy (DSTE) pulse. LDL subtypes were identified according to their diffusion coefficients. The area of each lorentzian function was related to the lipid concentration of each lipoprotein subtype, and the size of each subtype was calculated from their diffusion coefficient. The particle numbers of

each lipoprotein subtype (p) were calculated by dividing the lipid volume by the particle volume of a given class. The lipid volumes were determined by using common conversion factors to convert concentration units into volume units. The lower and upper detection limits for total LDL-p, large LDL-p, medium LDL-p and small LDL-p were, respectively, 100-3000 nmol/L, 0.5-500 nmol/L, 0.5-1000 nmol/L, and 100-2000 nmol/L. No sample had levels below or above these limits. The inter-assay coefficient of variation for total LDL-p, large LDL-p, medium LDL-p, and small LDL-p were 3, 4, 5, and 5 %, respectively.

#### *Cardiovascular endpoints definitions*

The primary outcome of this study was all cardiovascular disease incidence, including both fatal and non-fatal events, which was defined as mortality or the first episode of hospitalization by any cardiovascular cause (International Classification of Diseases, 10th Revision (ICD-10) codes I00-I78). Secondly, findings for incident coronary heart disease (CHD) and stroke were reported. Detailed definitions of fatal and nonfatal events follow the guidelines published by the 2014 American College of Cardiology and American Heart Association Task Force on Clinical Data Standards Committee (15). Incident CHD was defined as the first occurrence of definite fatal myocardial infarction, sudden death due to CHD, nonfatal myocardial infarction, or definite non-fatal CHD. Incident cases of stroke were defined as the first occurrence of definite or possible fatal or definite non-fatal stroke. Time to event was calculated as the difference between the date of the baseline examination and the date of the cardiovascular event, the date of death, or 30 November 2015 (administrative censoring), whichever occurred first.

#### *Other Variables*

Information on age, sex, education, smoking status, and lipid-lowering medication was based on self-report. Blood samples were obtained after an average of 3 h since the last intake of food (range 0–17). Glucose was measured by the glucose oxidase method with an

auto-analyzer (Hitachi 704, Boehringer Mannheim, Germany), and a second fasting glucose determination was obtained only in subjects with non-fasting glucose levels equal to or higher than 140 mg/dl. Glycosylated hemoglobin (HbA1c) levels were measured from capillary blood samples with a DCA 2000 HbA1c analyzer (Bayer Diagnostics, Tarrytown, NY). Type 2 diabetes (T2D) was defined as a fasting glucose levels of 126 mg/dL or greater, HbA1c > 6.5%, by physician diagnosis or as a use of glucose-lowering medications in the health records. Blood pressure was measured up to three times in sitting position and 'hypertension' was defined as a mean SBP/DBP of at least 140/90 mmHg, a recorded physician diagnosis, or medication use. Urine albumin was measured by automated nephelometric immunochemistry (Behring Institute). Chronic kidney disease was defined according to the Kidney disease improving global outcomes (KDIGO) criteria as an estimated glomerular filtration rate lower than 60 ml/min/1.73m<sup>2</sup> (16) or as albuminuria levels greater than 20 mg/g creatinine in men and 30 mg/g creatinine in women.

### *Statistical Methods*

The survey package in R software (version 3.0.3; R Development Core Team 2014) was used to account for the sampling design and survey weights. Baseline data are presented as means and standard deviation for continuous variables and N (%) for categorical variables. LDL particles were not normally distributed and have been, thus, log-transformed for all statistical analyses. Subgroup analyses were performed to evaluate the characteristics of individuals with predominantly large, medium or small LDL particles. Spearman's correlation among lipid biomarkers was calculated. The prospective association of lipid biomarkers with mortality and cardiovascular incidence endpoints was evaluated using Cox-proportional hazard models. Lipid biomarkers were introduced in the models using previously defined categories for altered lipid levels and as continuous concentrations to compare the 75<sup>th</sup> and 25<sup>th</sup> percentiles (equivalent to an interquartile range increase).

The potential role of lipoprotein particle composition on mortality and cardiovascular incidence endpoints was evaluated examining the relationship between a 5% increase in the relative amount of lipoprotein particle subtypes over the sum of lipoproteins (expressed as small LDL-p %, medium LDL-p % and large LDL-p %) and the study endpoints. In the conventional approach, each lipoprotein particles biomarker proportion alone was entered into the regression model, however, findings from the conventional approach are challenging to interpret. For instance, the increase in small LDL-p% could be related to a decrease in either medium LDL-p% or large LDL-p%, as the sum of small LDL-p%, medium LDL-p and large LDL-p equal 1. To address this issue, a “leave-one-out” approach was used (17,18). In this method, two LDL-p particles concentration variables are entered at a time. For example, small LDL-p% and medium LDL-p % are entered together leaving out large LDL-p %. The regression coefficients thus estimate the hazard ratio associated with an increase in small LDL-p % by a corresponding decrease in large LDL-p%, and with an increase in medium LDL-p% by a corresponding decrease in large LDL-p%, respectively.

## Results

### *Study population and lipid profile*

A total of 1,162 subjects (49% male, mean age 49.7 years) was included into the study. Main demographic characteristics are shown in table 1. In terms of cardiovascular risk factors, the characteristics of the study population followed a distribution as expected in the Spanish general population. Subjects with incident stroke – as compared to subjects with incident CHD - were older, had a higher rate of obesity and dyslipidemia but a lower rate of former or current smoking status, diabetes and hypertension.

Distributions of standard lipids and NMR lipoproteins are shown in table 1. The geometric mean levels of NMR-measured total cholesterol and LDL cholesterol were similar to the

geometric mean levels of standard lipid equivalents, whereas a higher mean HDL cholesterol concentration and a lower mean triglyceride concentration was found when lipids were assessed with NMR. The correlation between standard and NMR-measured lipids showed Spearman's correlation coefficients of around 0.90 (supplemental figure 1).

The distribution of LDL cholesterol particle (LDL-p) composition was as follows: small LDL-p was predominant (median proportion 49.6%, (interquartile range 47.4% - 52.5%)), followed by medium LDL-p (median proportion 35%, (interquartile range 32.5% - 36.8%)) and large LDL-p (median proportion 15.7%, (interquartile range 14.5% - 16.9%)). Individuals below median levels of large LDL-p % showed a less favourable cardiovascular risk profile compared to subjects above median levels of large LDL-p % (supplemental Table 1).

#### *Association of lipoproteins with CVD*

During a mean-follow up of  $12.4 \pm 3.3$  years, the total number of events was 159 for all CVD, 42 for CHD and 60 for stroke. The Hazard Ratios of lipid parameters for all CVD, CHD and stroke are shown in supplemental figure 2. In fully adjusted models (see description in supplemental figure 2), LDL concentrations and the total cholesterol/HDL ratios were positively associated with the risk for all events, whereas the association with HDL concentrations was inverse. Observed risk ratios were similar for both standard and NMR-measured lipids except for the association of total cholesterol and CHD, which showed a statistically significant association only when the lipid was measured with the standard method. Overall, the magnitude of the association of lipid concentration and events was attenuated but remained significant in NMR-measured lipids compared to standard lipids.

#### *Association of LDL particles with CVD*

The Hazard Ratios for LDL particle concentration and size are shown in table 2. Total LDL particle concentration was related to all events when traditional cardiovascular risk

factors were controlled for. Medium LDL-p, but not small LDL-p, were positively and linearly associated with the risk of all CVD, CHD and stroke in fully adjusted statistical models.

The relevance of LDL particles was further evaluated in a compositional analysis using a leave-one-out approach (table 3). The strongest associations were observed for LDL-p and CHD when the proportion of medium and small LDL-p were entered simultaneously into the model. A 5% increase in the proportion of medium LDL-p by a corresponding decrease in the proportion of large LDL-p was associated with an increase in risk for all events (HRs 1.19 (1.07, 1.32), 1.93 (1.55, 2.39) and 1.20 (1.00, 1.44) for all-CVD, CHD and stroke, respectively). A 5% increase in the proportion of small LDL-p by a corresponding decrease in the proportion of large LDL-p was associated with an increase in risk for CHD (HR 1.41 (1.14, 1.74)) (table 3). In other words, a change in baseline LDL composition from large to medium and small was associated with a 2-fold and 40% increase in risk for CHD, respectively (figure 1). In addition, a 5% increase in the proportion of medium LDL-p by a corresponding decrease in the proportion of small LDL-p was associated with a statistically significant increase in risk for all events (table 3).

## Discussion

In a representative sample of a general population from Spain, NMR-measured LDL particle concentration and composition were associated with cardiovascular disease incidence. For total lipoprotein concentrations, the magnitude of the observed associations was similar comparing the standard and NMR measurements, after adjustment for cardiovascular and lipid risk factors. Among the LDL particle subclasses, medium LDL particles showed the strongest association with cardiovascular events, particularly for CHD. The observed risk ratios of LDL particles and cardiovascular endpoints were more pronounced when particle

composition rather than the absolute particle number was assessed. Specifically, a decrease in large LDL particles by a corresponding increase in medium or small LDL particles was associated with a 2-fold and 40% increase in risk for CHD, respectively.

The present study was performed in a representative sample of the general population. Study participants were recruited from the catchment area of the Rio Hortega University Hospital in Valladolid, Spain, a hospital with a low rate of external patient admission. The levels of measured LDL-cholesterol in this population was in agreement with other population-based studies (19). Cross-sectional studies conducted in the US, including data from the Cardiovascular Health Study, the Women's Health Study and the Framingham Offspring Study, support the predictive role of LDL particle number for cardiac events, especially in women (11,20–22). In Europe, the Finnish FINRISK study included more than 7000 individuals without cardiovascular disease at baseline and showed that NMR-measured LDL particle concentration was associated with cardiovascular events (12). A nested case-control study in the EPIC-Norfolk, conducted in more than 2800 individuals from United Kingdom, related LDL particle concentration to CHD in healthy subjects (23). The Hazard Ratios for cardiovascular events in these studies were generally higher than the risk ratio of total LDL particle concentration observed in the present study, and it has been hypothesized that the predictive power of LDL particles might be superior to risk management based on standard cholesterol values (24). Nonetheless, the adjustments in statistical models vary considerably from one study to another. The increased risk attributed to particles is largely attenuated when lipids and variables related with metabolic syndrome, including obesity, hyperglycemia, hypertriglyceridemia, low HDL cholesterol and hypertension are entered into the regression models (25,26). Furthermore, discrepancies regarding the relationship between LDL particle subclasses and cardiovascular risk have been. In fully adjusted models, large LDL particles have been related with higher (6,12,13), lower (9,11) and null (7,14,21,27) risk for cardiovascular disease. Likewise, the association of medium LDL particles with cardiovascular outcomes has been

reported to be positive (11–13) although in some cases non- statistically significant (9,27). Small LDL particles are generally considered to be strongly related to cardiovascular disease due to augmented oxidation and delivery of cholesterol to atheromatous plaques, but the statistical significance is frequently lost after adjustment for lipoproteins (24). Moreover, LDL particles were not associated with future cardiovascular events in patients with metabolic syndrome, a condition characterized by an increase in small, dense LDL particles with typically normal LDL cholesterol values (14,28). It has been postulated that an elevated small LDL particle concentration – at normal total LDL cholesterol levels - might be representative of the so-called residual cardiovascular risk. Nevertheless, the exact role of LDL particles and their subclasses on cardiovascular risk is not fully understood (29).

In the present study, conducted in a representative sample of a general South European population, medium LDL particles showed the highest association with cardiovascular events in conventional Cox-models, even after adjustment for total LDL particle subclass concentration. Small LDL particles as a continuous variable were positively related to stroke in models adjusting for CV risk factor but surprisingly showed an inverse association with all-CVD and CHD. Gender (30), age (31), ethnicity (32–34) and body fat composition (35,36) are main determinants of lipoprotein subclasses distribution in the population. Currently available studies were mainly conducted in high risk patients or healthy subjects, including predominantly women. Therefore, differences regarding the concentration and predictive value of particles can be expected when a general population is investigated. The discrepancies regarding the predictive value of individual LDL particle subclasses across studies could be also partly due to methodological issues. For instance, comparison between the different studies performed on lipoprotein profiles is limited by the lack of standardization between the different analytical methods used for the determination of lipoprotein profiles.

Lipoprotein particles can be ascertained using NMR, gradient gel electrophoresis, vertical auto profile ultracentrifugation or ion mobility (2). In this study, we used the Liposcale® test for quantifying lipoprotein subclasses based on two-dimensional (2D) diffusion-ordered  $^1\text{H}$



NMR spectroscopy (DOSY), which directly measures the size of lipoprotein particles. This methodology has been previously validated and compared to the established one-dimensional (1D) NMR method developed by Jeyarajah *et al.* (37). The LDL particle measurements obtained by using 1D and 2D-NMR techniques were highly correlated, although the LDL particle number obtained by 2D-NMR method (the Liposcale® test) were more in agreement with biochemical values than those obtained using the 1D-NMR test (4). A direct determination of the size and number of particles cannot be performed using 1D NMR techniques, since the molecular profiles base their results on empirical models, developed by correlations between the crude NMR spectrum and biochemical measurements (2). Alternatively, the approximation using 2D NMR experiments, whose signal is modulated by the diffusion of the particles in the mixture (2D-DOSY-NMR), allows the hydrodynamic characteristics of the molecules, such as the size, to be incorporated in the results (38).

In addition, the NMR-measured LDL particle distribution of any given subject is determined by the relative content of cholesterol ester and triglycerides and can vary up to 2-fold across individuals (39). In contrast, photometrically assessed standard LDL cholesterol reflects the total amount of cholesterol which is transported by LDL particles (1). Total LDL particles concentration and standard LDL cholesterol concentrations are highly correlated, but an increase or decrease in one LDL particle subtype does not necessarily follow a parallel change in the LDL concentrations. For instance, cross-sectional studies have shown that individuals with high LDL particle number and low LDL cholesterol concentrations (reflecting that the size of the particles is small) or vice versa have a higher risk for cardiovascular disease compared to subjects with “concordant” lipoprotein values (26–28, 40–42). The risk for cardiovascular disease in patients with an altered relationship of LDL lipoproteins was more than 2-fold higher than the risk observed for LDL particle concentration at baseline, indicating that an altered composition among LDL lipoproteins rather than the total concentration at baseline confers an increase in cardiovascular risk. In our data, the compositional analysis revealed that both medium and small LDL particles are associated

with cardiovascular risk, especially for CHD, when the baseline composition, rather than individual LDL particles subclasses, is jointly investigated. The strongest association for CHD was observed for an increase in the relative amount of medium LDL particles with a corresponding decrease in the relative amount of large LDL particles (2-fold risk increase), followed by an increase in the relative amount of small LDL particles with a corresponding decrease of the relative amount of large LDL particles (41% risk increase), independent of total LDL and HDL particle concentration.

While chance cannot be discarded as a potential explanation for these findings, it is well known that the concentrations of LDL particle subclasses are prone to short- and long term changes. For example, aerobic exercise is followed by a shift from smaller to larger LDL particles, whereas the levels of total cholesterol and LDL cholesterol remain unchanged (43,44). Likewise, weight change is associated with a corresponding change in LDL particles independent of baseline lipoprotein values (45,46). The beneficial metabolic consequences of exercise and weight reduction may therefore be partially explained by a reduction in medium and small LDL particles, or an increase in large LDL particles. In the present study, individuals above median levels of large LDL particles percent showed a more favourable cardiometabolic profile compared to subjects below median levels of large LDL particles percent. While findings from our compositional analysis of baseline particles data with respect to cardiovascular incidence events collected after more than 12 years of follow-up suggest that differences in the baseline LDL particles composition are associated to cardiovascular risk, longitudinal studies with repeated measures of LDL particle concentrations are desirable in order to obtain an additional insight into the relationship of LDL particle subclasses changes over time with cardiovascular outcomes.

The present study needs to be viewed within its strengths and limitations. For instance, one important limitation is the unavailability of all lipid variables that are known to be related to cardiovascular disease, such as apo B and apo A-I. In addition, although lipid-lowering

treatment was controlled for in regression models, statin type, dosage, and changes in statin use during follow-up with its possible effects on cardiovascular events were not available. On the other hand, the availability of incident cardiovascular endpoints after more than 12 years of follow-up, and the lipoprotein particles determination using state-of-the-art two-dimensional NMR methods, are major strengths of our study. Other strengths of this study include the representative sampling design, which allows the findings to be extrapolated to a general population framework.

In conclusion, NMR-measured LDL particles and photometrically assessed LDL cholesterol were prospectively associated with cardiovascular risk in a representative sample of a general population from Spain. LDL particle subclasses showed a stronger association with cardiovascular events when the baseline composition of particle subtypes, rather than the absolute concentration of individual subtypes, was investigated. Particularly, the strongest positive association with CHD was observed for a decrease in large LDL particles, with a corresponding increase in medium or small LDL particles. While the reported associations provide some insight into the relation of LDL particles and cardiovascular disease, further mechanistic research addressing LDL particle composition changes is needed to understand the causal pathways underlying our findings.

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**Table 1.** Participant characteristics and overall description of lipoproteins by cardiovascular incidence status.

	Overall (N=1162)	Incident CVD (N=159)	No incident CVD (N=1003)
Age, years	49.7 ± 18.6	56.9 ± 12	49.4 ± 17.7*
Male, %	49	60.5	49
Obesity, %	16.5	25.5	15.9°
BMI, kg/m <sup>2</sup>	26.1 ± 4.2	27.4 ± 4.4	26.1 ± 4.1°
Former smoking, %	27.2	20.2	27.3
Current smoking, %	27.7	33.3	27.4
Diabetes, %	5.9	16.2	4.7*
Glucose lowering medication, %	2.8	9.5	2.1*
Hypertension, %	35.7	65.3	33.9*
Antihypertensive medication, %	13	22.8	11.7*
Systolic blood pressure, mmHg	127.6 ± 21.1	139 ± 22.2	126.9 ± 19.7*
Diastolic blood pressure, mmHg	79.1 ± 10.6	82.2 ± 11.5	79 ± 10.4*
Chronic kidney disease, %	8.2	12.5	7.6*
Dyslipidemia, %	51	69.7	50.6°
Lipid lowering medication, %	4.8	13.5	4.4°
<b>Standard lipid biomarkers</b>			
Total cholesterol, mg/dL	201.4 ± 37.4	211.2 ± 37.5	201.5 ± 37.3°
LDL-cholesterol, mg/dL	114.5 ± 33.6	127.2 ± 34.3	114.4 ± 33.4°
HDL-cholesterol, mg/dL	52.4 ± 14.2	49 ± 12.9	52.5 ± 14.4 <sup>†</sup>
<b>NMR lipid biomarkers</b>			
Total cholesterol, mg/dL	202.4 ± 39.6	213 ± 14.8	202.6 ± 39.3°
LDL-cholesterol, mg/dL	116.3 ± 32.3	127.8 ± 33.3	116.3 ± 32°
HDL-cholesterol, mg/dL	61 ± 17.9	57.7 ± 16.3	61.2 ± 18.1 <sup>†</sup>
<b>Particle concentration (p)</b>			
Total LDL-p, nmol/L <sup>§</sup>	827.2 (814.7, 839.8)	915.8 (859.8, 975.3)	827.8 (814.9, 841)°
Large LDL-p, nmol/L <sup>§</sup>	128.5 (126.4, 130.7)	138.3 (129.7, 147.4)	128.6 (126.4, 130.9) <sup>†</sup>
Medium LDL-p, nmol/L <sup>§</sup>	275.2 (269.3, 281.3)	314.3 (293.1, 337.1)	275.1 (268.7, 281.7)°
Small LDL-p, nmol/L <sup>§</sup>	415.2 (408.5, 422)	455.8 (426.6, 486.9)	415.7 (408.7, 422.8)°

Data are arithmetic mean ± SD.

<sup>§</sup> Data are geometric mean (95% CI)\* P<0.0001; ° p<0.01; <sup>†</sup> p<0.05.

**Table 2.** Hazard ratios for cardiovascular incidence endpoints by NMR-measured particles concentrations comparing the 75th versus 25th percentile of particles distributions (N=1,162).

	All-CVD incidence (N events=159)			CHD incidence (N events=42)			Stroke incidence (N events=60)		
	Model 1	Model 2	Model 3	Model 1	Model 2	Model 3	Model 1	Model 2	Model 3
<b>Particle size (p), nmol/L</b>									
LDL-p	0.99 (0.94, 1.05)	1.10 (1.04, 1.16)	--	1.02 (0.92, 1.13)	1.11 (1.01, 1.23)	--	1.20 (1.09, 1.31)	1.28 (1.17, 1.41)	--
Large LDL-p	0.96 (0.91, 1.01)	1.06 (1.00, 1.11)	0.94 (0.88, 1.01)	0.88 (0.80, 0.97)	0.96 (0.88, 1.06)	0.69 (0.60, 0.79)	1.06 (0.97, 1.16)	1.13 (1.03, 1.24)	0.92 (0.80, 1.04)
Medium LDL-p	1.03 (0.98, 1.09)	1.15 (1.09, 1.21)	1.07 (0.98, 1.17)	1.20 (1.09, 1.31)	1.38 (1.25, 1.51)	1.34 (1.14, 1.58)	1.28 (1.18, 1.39)	1.38 (1.27, 1.51)	1.50 (1.29, 1.76)
Small LDL-p	0.98 (0.92, 1.03)	1.03 (0.98, 1.09)	0.92 (0.86, 0.98)	0.95 (0.86, 1.04)	0.98 (0.89, 1.07)	0.76 (0.67, 0.86)	1.11 (1.01, 1.22)	1.15 (1.05, 1.26)	0.98 (0.87, 1.11)

Model 1 is adjusted for age and sex.

Model 2 is Model 1 further adjusted for smoking status, obesity, diabetes, hypertension, CKD, anti-hypertensive medication, glucose lowering medication, lipid-lowering medication.

Model 3 is Model 2 further adjusted for LDL concentration standard biochemistry.

**Table 3.** Compositional analysis (leave one-out approach) for NMR-measured LDL cholesterol particles per 5% increase.

	% LDL-P Small HR (95%CI)	% LDL-P Medium HR (95%CI)	% LDL-P Large HR (95%CI)
<b>All-CVD incidence</b>			
Standard approach	0.89 (0.85, 0.93)	1.14 (1.09, 1.19)	0.87 (0.79, 0.95)
Leave-one-out approach			
% LDL-P Small	-	1.13 (1.09, 1.18)	0.95 (0.86, 1.06)
% LDL-P Medium	0.88 (0.85, 0.92)	-	0.84 (0.76, 0.93)
% LDL-P Large	1.05 (0.94, 1.17)	1.19 (1.07, 1.32)	-
<b>CHD incidence</b>			
Standard approach	0.76 (0.71, 0.83)	1.40 (1.29, 1.53)	0.61 (0.50, 0.73)
Leave-one-out approach			
% LDL-P Small	-	1.37 (1.25, 1.49)	0.71 (0.57, 0.88)
% LDL-P Medium	0.73 (0.67, 0.8)	-	0.52 (0.42, 0.65)
% LDL-P Large	1.41 (1.14, 1.74)	1.93 (1.55, 2.39)	-
<b>Stroke incidence</b>			
Standard approach	0.85 (0.79, 0.91)	1.19 (1.11, 1.28)	0.88 (0.74, 1.04)
Leave-one-out approach			
% LDL-P Small	-	1.19 (1.10, 1.28)	0.99 (0.82, 1.19)
% LDL-P Medium	0.84 (0.78, 0.91)	-	0.83 (0.69, 1.00)
% LDL-P Large	1.01 (0.84, 1.21)	1.20 (1.00, 1.44)	-

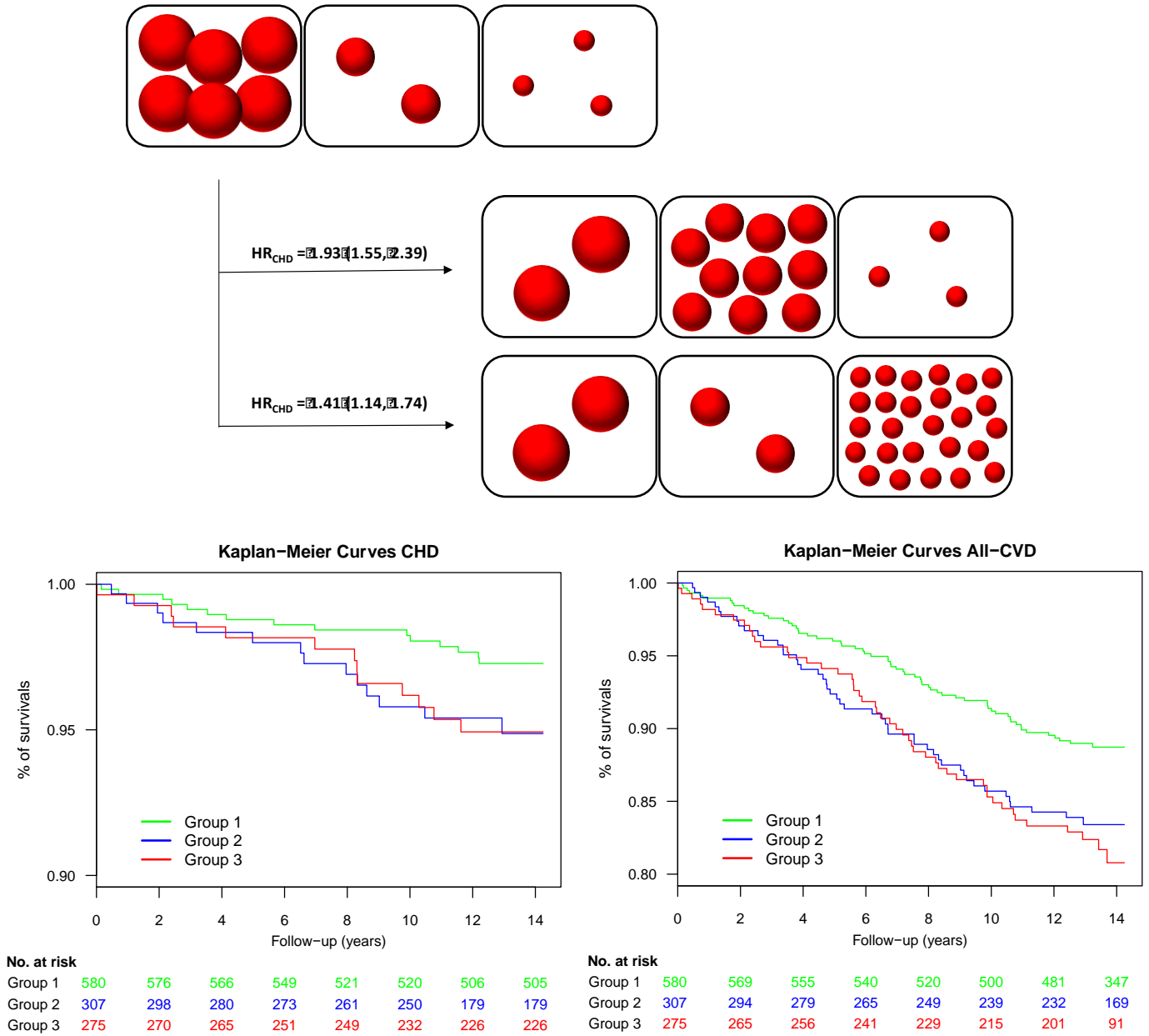
All models are adjusted for age, sex, smoking status, obesity, diabetes, hypertension, CKD, anti-hypertensive medication, glucose lowering medication, lipid-lowering medication, total LDL concentration (NMR) and total HDL concentration (NMR).

% LDL-P Small, Small LDL particles proportion

% LDL-P Medium, Medium LDL particles proportion

% LDL-P Large, Large LDL particles proportion

**Figure 1.** Risk of incident CHD for a decrease in large LDL particles by a corresponding increase in medium or small LDL particles. Kaplan-Meier plots for CHD and all-cause CVD according to predominant LDL particle size.



Group 1: Large LDL particles proportion > Median of Large LDL particles proportion.  
 Group 2: Large LDL particles proportion ≤ Median of Large LDL particles proportion and Medium LDL particles proportion > Median of Medium LDL particles proportion.  
 Group 3: Large LDL particles proportion ≤ Median of Large LDL particles proportion and Medium LDL particles proportion ≤ Median of Medium LDL particles proportion.

**Highlights**

- Absolute medium LDL particle concentrations were associated with all cardiovascular disease, coronary heart disease and stroke incidence in a representative sample of a general population from Spain
- A change in baseline composition of LDL particles from large to medium and small LDL particles was associated with and increased cardiovascular risk, especially for CHD.
- Subjects with a relative amount of large LDL particles below the median were characterized by a less favourable cardiometabolic risk profile compared to individuals with a relative amount of large LDL particles above the median.