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LDL subfractions are associated with incident cardiovascular disease in the Malmö Prevention Project Study

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ABSTRACT

Background and aims: After assessing the risk for cardiovascular disease (CVD) based on traditional risk factors, decisions concerning lipid lowering therapy might remain uncertain. To investigate whether lipoprotein subfraction levels could aid these decisions, we assessed the association between lipoprotein subfractions and CVD, after adjustment for traditional risk factors including standard lipids.
Methods: Using a case-cohort design, participants were randomly drawn from the Malmö Prevention Project (MPP), a population-based prospective study of 18,240 participants, and supplemented with additional incident CVD events (5764 participants, 1784 CVD events).
Results: Low density lipoprotein particle number (LDL-P) and individual subfractions ranging in size from very-small to large were associated with CVD (continuous p value (p_{cont}) < 0.001) while adjusting for age, sex, hypertension, smoking, and diabetes. After further adjustment for LDL-C, HDL-C, and triglycerides, very small LDL subfraction (b) (LDL-VS (b)) remained associated with CVD (HR = 1.23, 95% CI, 1.06 to 1.43 for top *vs.* bottom quartile, p_{cont} = 0.03). Among participants with low/intermediate risk [without diabetes and with LDL-C <3.36 mmol/L (<130 mg/dL)], the fully adjusted HR for LDL-small (top *vs.* bottom quartile) was 1.48 (95% CI 1.02 to 2.17, p_{cont} = 0.03). Among those with very-high risk (>20% 10-year risk of CVD), LDL-VS(a) and LDL-VS(b) were associated with CVD in fully adjusted models (HR = 1.37, 95% CI 1.12 to 1.67 and HR = 1.28, 95% CI 1.07 to 1.53, respectively, $p_{cont} \leq 0.03$).
Conclusions: Smaller LDL particles are associated with incident CVD independently of traditional risk factors, including standard lipids, in participants with low/intermediate and very-high risk, who might benefit from improved risk assessment.

1. Introduction

Evidence from multiple randomized trials of statin therapy [1], as well as analysis of genetic variants associated with LDL-C levels [2], suggests that LDL-C lowering therapy effectively reduces CVD burden. Thus, LDL-C lowering therapy for CVD prevention is recommended by both European [3] and US guidelines [4]. These guidelines support matching the intensity of preventive therapy to the patient's absolute risk of CVD. Therefore, improving CVD risk assessment can lead to improved CVD prevention by more appropriately matching the intensity of therapy to the patient's risk.

Two groups of patients would particularly benefit from improved risk assessment. The first group comprises patients with low/intermediate risk of CVD and moderate levels of LDL-C, for whom a decision to initiate statin therapy requires careful assessment of the risks and benefits associated with therapy [4], as well as willingness to adhere to life-long daily pill taking [5]. The second group comprises very high risk patients. For these patients, high-intensity statin therapy was, until recently, the only pharmacological intervention available. However, these patients can now consider further reduction of LDL-C levels by PCSK9 inhibitors [6,7]. But since PCSK9 inhibitors are expensive [8], and the long-term side

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effects of PCSK9 inhibitors have not been thoroughly evaluated, further risk stratification of these very high risk patients could aid in treatment decisions.

The value of lipoprotein subfraction levels as an aid in the assessment of CVD risk has been investigated using different methods to assess lipoprotein subfraction levels. Although many studies have reported that lipoprotein subfractions in general, and LDL particle number (LDL-P) in particular, are associated with CVD events, most of these studies either found no association after adjustment for standard lipids (LDL-C, HDL-C, and triglycerides) or did not report the association results in fully adjusted models [9–17]. Recently, two studies reported that LDL-P determined by ion mobility was associated with incident CVD in models that fully adjusted for standard lipids [18,19].

To better understand whether lipoprotein subfractions are associated with CVD events independently of standard lipid measurements, we investigated these associations in a large, population-based study of middle-aged to elderly individuals. Our particular interest was in populations of special clinical interest, those for whom further risk assessment could aid in lipid lowering therapy decisions.

2. Patients and methods

2.1. Study participants

The Malmö Prevention Project (MPP) is a population-based prospective study of 33,346 men and women recruited and examined between 1974 and 1992. A reexamination of 18,240 MPP participants who responded to an invitation sent to all survivors of the original MPP cohort was carried out between 2002 and 2006. The reexamination included a physical examination, blood pressure measurement, and collection of blood samples after overnight fast [20,21]. The current case-cohort study of participants free of CVD at baseline was drawn from the 18,240 MPP participants who were included in the reexamination (Supplemental Fig. 1); first 5301 participants were randomly drawn; then the study cohort was supplemented with 1313 additional participants who experienced a CVD event during follow-up. After excluding participants with CVD at baseline and those with missing clinical information or LDL subfraction data, 5764 participants with 1784 incident CVD events were included in the analysis. The study has been approved by the regional ethics review board in Lund, and complies with the declaration of Helsinki. Participants gave informed consent.

2.2. Laboratory measurements

Standard lipid levels were measured in fasting plasma samples that had been frozen at −80 °C since the time of the MPP re-examination (2002–2006). Lipoprotein subfraction levels were assessed in these same samples by ion mobility, as previously described [19], at Quest Diagnostics Nichols Institute (San Juan Capistrano, CA).

2.3. Study end points

The primary end point of the study was time to first occurrence of CVD, a composite endpoint of fatal or non-fatal myocardial infarction, fatal or non-fatal stroke, coronary revascularization, or death from coronary heart disease. The endpoints were identified by a record linkage of the 10-digit personal identification number of each Swedish citizen with four registers: the Swedish Hospital Discharge and Cause of Death Registers, Swedish Coronary Angiography and Angioplasty Registry (SCAAR) and the Stroke Register of Malmö (STROMA). The registers have been previously described and validated for classification of outcomes [22–24]. Myocardial infarction was defined on the basis of ICD-9 code 410 and ICD-10 code I21. Death due to ischemic heart disease was defined on the basis of codes 412 or 414 (ICD-9) and I22–I23 or I25 (ICD-10). Coronary artery bypass surgery was identified from national Swedish classification systems of surgical procedures and defined as procedure codes 3065, 3066, 3068, 3080, 3092, 3105, 3127, or 3158 (the Op6 system) or procedure code FN (the KKÅ97 system). Percutaneous intervention was identified from the national Swedish Coronary Angiography and Angioplasty Registry (SCAAR). Stroke was defined on the basis of codes 430-432, or 434 (ICD-9) and code I60-I63 (ICD-10). Transient ischemic attack was not included as a stroke event. The last assessment of incident stroke events for MPP participants was performed December 31, 2010. All other events were last assessed in December 31, 2013.

2.4. Statistical methods

The estimated 10-year risk for each individual was calculated using the Pooled Cohort Equation parameters published in the 2013 ACC/AHA Guidelines on the assessment of cardiovascular risk [25]. Event rates per 100 person-years and 95% confidence intervals were estimated for the cohort portion of this case-cohort study. The cumulative incidence of CVD events was estimated as a function of the participants' age while considering non-CVD death as competing risk and the difference between the cumulative incidence in top and bottom quartile was assessed by Gray's test [26]. Cox proportional hazards regression models were used to estimate the association between incident CVD and lipoprotein subfractions and for standard lipids. p values are reported for 1 standard deviation increase (p_{cont}) as well as for top *vs.* bottom quartile. The regression models adjusted for baseline values of age, sex, hypertension, diabetes, and smoking status with or without standard lipids (LDL-C, HDL-C, and triglycerides). Hypertension was defined as systolic blood pressure $\geq$140 mmHg, diastolic blood pressure $\geq$90 mmHg, or use of anti-hypertensive medication. All analyses were performed in R [27].

3. Results

3.1. Study population

The median follow-up time in this study was 8.05 years (5.60 years for stroke events and 8.13 years for all other events). The baseline characteristics of this case-cohort study are summarized in Table 1. We also provided the baseline characteristics of two groups of participants for whom improved CVD risk assessment is of particular interest: a low/intermediate risk group and a very high risk group of participants. The low/intermediate risk group comprised participants without diabetes and with LDL-C levels <3.36 mmol/L (130 mg/dL). For this low/intermediate risk group, the CVD event rate was 1.22 per 100 person years (*versus* 1.75 per 100 person years rate observed in the entire study cohort). The very high risk group comprised participants with a 10-year risk >20% as estimated by the pooled cohort equations [25]. The event rate in the high risk group was 2.41 per 100-person years.

3.2. Lipoprotein subfractions and CVD in the entire study

In this case-cohort study of 5764 participants (1784 CVD events), the association between lipoprotein subfractions and incident CVD was investigated in a model that adjusted for age, sex, smoking, baseline hypertension, and diabetes (Table 2). Several LDL subfractions were associated with incident CVD, these included very small LDL subfraction (c) (LDL-VS(c)) to large LDL subfraction (b), as

Table 1
Baseline characteristics.

	Current study (case-cohort)	Cohort	Low/intermediate risk[a]	Very high risk[b]
Number (events)	5764 (1784)	4597 (617)	1186 (283)	3381 (1292)
Event rate (95% CI)[c]	1.75 (1.61–1.90)	1.75 (1.61–1.90)	1.22 (0.98–1.50)	2.41 (2.19–2.64)
Age, years (SD)	69.2 (6.1)	69.0 (6.2)	69.1 (6.2)	71.9 (5.3)
Men, n (%)	3939 (68.3)	3113 (67.7)	860 (72.5)	2686 (79.4)
Smoking, n (%)	1145 (19.9)	922 (20.1)	262 (22.1)	789 (23.3)
Hypertension, n (%)	4232 (73.4)	3306 (71.9)	807 (68.0)	2949 (87.2)
Prevalent diabetes, n (%)	867 (15.0)	649 (14.1)	0 (0)	808 (23.9)
HDL-C, mmol/L (SD)	1.38 (0.4)	1.39 (0.4)	1.44 (0.45)	1.31 (0.38)
LDL-C, mmol/L (SD)	3.73 (0.96)	3.71 (0.96)	2.83 (0.41)	3.73 (0.98)
Triglycerides, mmol/L (SD)	1.25 (0.63)	1.24 (0.63)	1.07 (0.59)	1.34 (0.67)

Values are means when the SD is provided.
Study population: the case-cohort population which comprises the randomly drawn cohort and those with CVD events who were not included in the random cohort study included in this manuscript. Cohort: the random cohort that is part of the study population.
[a] Excluding participants with LDL-C> 3.36 mmol/L (130 mg/dl), lipid lowering therapy use, or diabetes.
[b] >20% estimated 10-year risk of CVD.
[c] Per 100 person-years, calculated for the cohort portion of this case-cohort study.

Table 2
Lipoprotein subfractions, lipids, and incident CVD events.

	HR (95% CI)[a]	*p* value[a]	p_{cont} (per SD)
HDL-Small	1.02 (0.89–1.17)	0.77	0.82
HDL-Large	0.92 (0.80–1.06)	0.25	0.10
LDL-Very Small (d)	1.02 (0.89–1.16)	0.81	0.21
LDL-Very Small (c)	1.14 (1.00–1.31)	0.056	6.2×10^{-4}
LDL-Very Small (b)	1.32 (1.15–1.51)	8.0×10^{-5}	2.8×10^{-4}
LDL-Very Small (a)	1.34 (1.17–1.54)	3.5×10^{-5}	5.8×10^{-4}
LDL-Very Small	1.24 (1.08–1.42)	0.002	4.1×10^{-4}
LDL-Small	1.34 (1.17–1.54)	4.0×10^{-5}	2.7×10^{-4}
LDL-Medium	1.31 (1.15–1.50)	6.5×10^{-5}	1.2×10^{-5}
LDL-Large (b)	1.17 (1.03–1.34)	0.02	2.0×10^{-4}
LDL-Large (a)	1.06 (0.92–1.21)	0.42	0.23
LDL-Large	1.13 (0.99–1.28)	0.08	0.02
LDL-P	1.39 (1.22–1.59)	9.7×10^{-7}	8.9×10^{-7}
IDL-Small	0.94 (0.82–1.08)	0.39	0.57
IDL-Large	1.24 (1.09–1.41)	0.001	0.002
VLDL-Small	1.10 (0.96–1.26)	0.16	0.48
VLDL-Medium	1.09 (0.95–1.25)	0.20	0.32
VLDL-Large	1.09 (0.95–1.25)	0.22	0.55
HDL-C	0.77 (0.67–0.89)	3.0×10^{-4}	0.002
LDL-C	1.33 (1.17–1.51)	1.8×10^{-5}	1.5×10^{-7}
Triglycerides	1.20 (1.05–1.39)	0.009	0.008

Adjusted for baseline age, sex, smoking, hypertension, and diabetes.
[a] Top *vs*. bottom quartile.

Table 3
Lipoprotein subfractions, lipids, and CVD events: adjusted for standard lipids.

	HR (95% CI)[a]	*p* value[a]	p_{cont} (per SD)
LDL-Very Small (c)	1.06 (0.92–1.23)	0.43	0.03
LDL-Very Small (b)	1.23 (1.06–1.43)	0.007	0.03
LDL-Very Small (a)	1.22 (1.04–1.43)	0.02	0.13
LDL-Very Small	1.14 (0.98–1.32)	0.10	0.05
LDL-Small	1.16 (0.98–1.38)	0.08	0.42
LDL-Medium	1.08 (0.92–1.26)	0.36	0.36
LDL-Large (b)	0.98 (0.83–1.15)	0.81	0.37
LDL-Large	0.99 (0.83–1.18)	0.90	0.87
LDL-P	1.15 (0.97–1.36)	0.10	0.16
IDL-Large	1.01 (0.86–1.18)	0.91	0.69
HDL-C	0.79 (0.68–0.92)	0.003	0.02
LDL-C	1.31 (1.15–1.49)	4.5×10^{-5}	5.6×10^{-7}
Triglycerides	1.07 (0.92–1.25)	0.39	0.37

The risk estimates and *p* values for lipoprotein subfractions were assessed in Cox regression models that included age, sex, smoking, hypertension, diabetes, LDL-C, HDL-C, triglycerides, and the lipid subfraction of interest. The risk estimates and *p* values for LDL-C, HDL-C and triglycerides were assessed in a Cox regression model that included age, sex, smoking, hypertension, diabetes, LDL-C, HDL-C, and triglycerides.
[a] Top *vs*. bottom quartile.

well as LDL-Large and LDL-P. Among non-LDL subfractions, only the large subfraction of intermediate density lipoproteins (IDL-large) was associated with incident CVD events ($p_{cont} = 0.002$). We also assessed the effect of additional adjustment for standard lipids (LDL-C, HDL-C, and triglycerides) and found that LDL-VS(c) and LDL-VS(b) (both $p_{cont} = 0.03$) remained associated with incident CVD in the fully adjusted model (Table 3). We also investigated the association of these subfractions after adjustment for non-HDL-C (rather than LDL-C), as well as HDL-C and triglycerides, and found that LDL-VS(c) and LDL-VS(b) (both $p_{cont} = 0.03$) remained associated with incident CVD in this model (Supplemental Table 1).

3.3. Lipoprotein subfractions and CVD in low/intermediate and very high risk groups

Because a previous study had found that LDL subfractions were associated with incident CVD in individuals with LDL-C <3.36 mmol/L (<130 mg/dL) and without diabetes [19], we investigated the association between lipoprotein subfractions and CVD in participants without diabetes, with baseline LDL-C <3.36 mmol/L (<130 mg/dL), after excluding those who received lipid lowering therapy at baseline. In this low/intermediate risk population LDL-Small, LDL-VS as well as subfractions of LDL-VS, (c), (b), and (a) were associated with incident CVD after adjustment for traditional risk factors including standard lipid (Table 4). In a top *vs*. bottom quartile analysis, those in the top quartile of LDL-Small had 48% greater risk than those in the bottom quartile (HR = 1.48, 95% CI 1.02 to 2.17) in a fully adjusted model.

We also investigated the association of lipoprotein subfraction with CVD in the very high risk group, those with >20% 10-year risk of CVD. In this group, we found that LDL-VS, as well as subfractions of LDL-VS, (c), (b), and (a), were associated with CVD. In a top *vs*. bottom quartile analysis, we found that those in the top quartile of LDL-VS(a) had 37% greater risk of CVD than those in the bottom quartile (HR = 1.37, 95% CI 1.12 to 1.67) after adjustment for traditional risk factors including standard lipids.

3.4. Cumulative events by lipoprotein subfraction quartile

LDL-VS(b) was associated with incident CVD events for the entire study population, as well as for those in the low/intermediate risk group and those in the very high risk group, in models that adjusted for standard lipids (p_{cont}<0.05 for all, Tables 3 and 4). We observed higher cumulative incidence of CVD events (while considering non-CVD death as competing risk) as a function of the

Table 4
Lipoprotein subfractions and CVD events in risk groups.

Subfraction	Low/intermediate risk[a]			Very high risk[b]		
	HR (95% CI)[c]	*p* value[c]	p_{cont} (per SD)	HR (95% CI)[c]	*p* value[c]	p_{cont} (per SD)
LDL-Very Small (c)	1.44 (1.00–2.06)	0.05	0.004	1.09 (0.92–1.29)	0.33	0.009
LDL-Very Small (b)	1.33 (0.93–1.89)	0.12	5.4×10^{-4}	1.28 (1.07–1.53)	0.007	0.004
LDL-Very Small (a)	1.26 (0.88–1.78)	0.20	0.003	1.37 (1.12–1.67)	0.002	0.03
LDL-Very Small	1.32 (0.93–1.90)	0.12	7.4×10^{-4}	1.19 (0.99–1.43)	0.06	0.01
LDL-Small	1.48 (1.02–2.17)	0.04	0.03	1.28 (1.05–1.57)	0.02	0.10
LDL-Medium	0.89 (0.62–1.29)	0.55	0.82	1.12 (0.93–1.35)	0.24	0.09
LDL-Large (b)	0.79 (0.55–1.13)	0.19	0.27	1.00 (0.83–1.22)	0.97	0.39
LDL-Large	0.75 (0.52–1.10)	0.14	0.33	0.89 (0.72–1.09)	0.26	0.49
LDL-P	1.48 (1.02–2.16)	0.04	0.12	1.26 (1.04–1.52)	0.02	0.10
IDL-Large	1.29 (0.90–1.85)	0.17	0.55	0.96 (0.79–1.15)	0.65	0.80
HDL-C	0.95 (0.66–1.37)	0.78	0.72	0.90 (0.75–1.07)	0.24	0.27
LDL-C	1.06 (0.77–1.48)	0.71	0.74	1.24 (1.06–1.46)	0.007	5.1×10^{-5}
Triglycerides	1.20 (0.83–1.73)	0.33	0.11	1.04 (0.87–1.24)	0.68	0.79

The risk estimates and *p* values for lipoprotein subfractions were assessed in Cox regression models that included age, sex, smoking, hypertension, diabetes, LDL-C, HDL-C, triglycerides, and the lipid subfraction of interest. The risk estimates and *p* values for LDL-C, HDL-C and triglycerides were assessed in a Cox regression model that included age, sex, smoking, hypertension, diabetes, LDL-C, HDL-C, and triglycerides.

[a] Excluding participants with LDL-C> 3.36 mmol/L (130 mg/dl), lipid lowering therapy use, or diabetes.

[b] >20% estimated 10-year risk of CVD.

[c] Top *vs.* bottom quartile.

participants' age, for those in the top quartile of LDL-VS(b) compared to those in the bottom quartile (Fig. 1) for the entire study, ($p < 0.0001$), as well as for the very-high risk group ($p < 0.0001$) and the low/intermediate risk group ($p = 0.003$).

3.5. Sensitivity analysis

The inclusion and exclusion criteria of this study resulted in the inclusion of 36 participants, who had also been enrolled in the portion of the population-based Malmö Diet and Cancer Study (MDC) that was the subject of a previously published analysis of lipoprotein subfractions [18]. The MDC blood sample collection and physical examination occurred approximately 10 years prior to those of the MPP Study. We found that excluding these 36 participants did not change the results (Supplementary Tables 2–4).

4. Discussion

We investigated the association between lipoprotein subfractions and incident CVD events in a population-based study of individuals without CVD at baseline and found that very small low density lipoprotein subfractions (c) and (b) were associated with

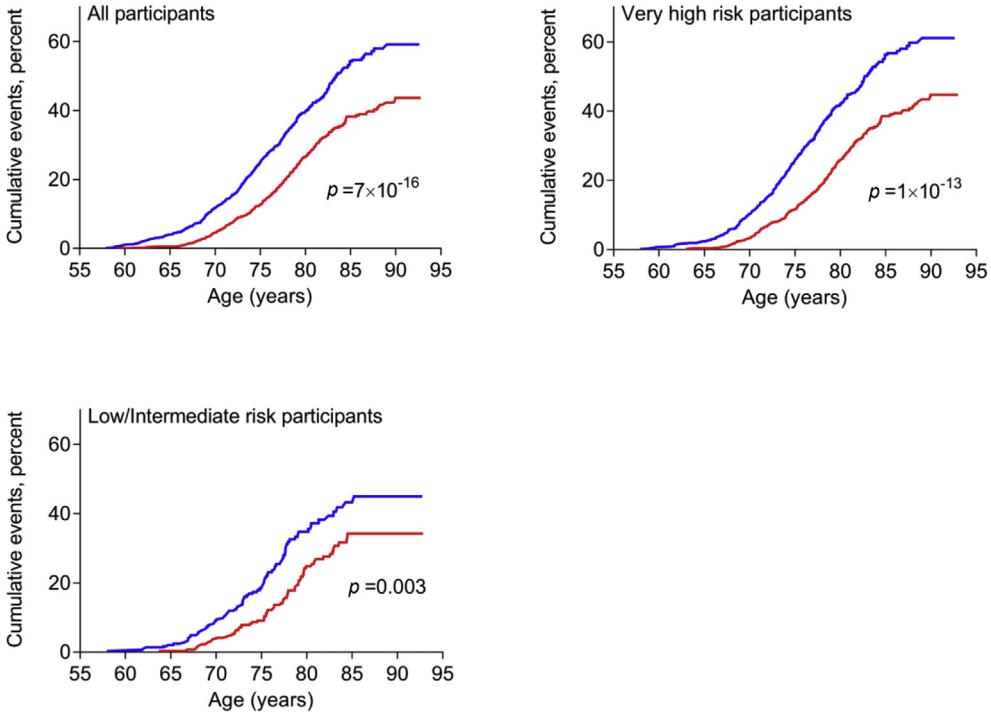

Fig. 1. Cumulative incidence of CVD events for those in the top and bottom quartiles of LDL-VS(b) lipoprotein subfraction.
The cumulative incidence of CVD events was estimated at the participants' age while considering non-CVD death as competing risk, and the difference between the cumulative incidence in top and bottom quartile was assessed by Gray's test. Blue, top quartile; red, bottom quartile.

CVD independently of traditional risk factors, including standard lipids (LDL-C, HDL-C, and triglycerides). For participants with LDL-C levels below 3.36 mmol/L (130 mg/dL) and without diabetes, we found that the LDL-Small and LDL-VS, as well as subfractions of LDL-VS, (a), (b), and (c) were associated with CVD events independently of standard lipids. Similar results (except for LDL-Small) were observed for very high risk participants: those with >20% 10-year risk of CVD.

While characterizing the association between lipoprotein subfractions and CVD could further our understanding of the biology of atherosclerosis, the clinical utility of measuring lipoprotein subfractions levels depends on whether, after traditional risk factors have been considered, the risk associated with lipoprotein subfractions remains actionable. If a definite decision on whether treatment with lipid lowering medication has been made after considering traditional risk factors, lipoprotein subfraction levels may add no value. However, if the treatment decision remains uncertain after full consideration of traditional risk factors, then the risk estimate for the patient's lipoprotein subfraction levels could aid in the treatment decision. But only if that risk is independent of the already considered traditional risk factors.

To assess whether the association between lipoprotein subfractions and CVD is independent of traditional risk factors, the association should be evaluated in models that fully adjust for standard lipids, even when the study population is stratified by LDL-C levels. For example, we assessed the association of lipoprotein subfractions with CVD events in intermediate/low risk patients who had LDL-C levels <3.36 mmol/L (130 mg/dL). And since physicians would consider LDL-C levels when assessing CVD risk in these patients, the question of whether lipoprotein subfractions provide additional information about CVD risk can only be answered in a model that adjusts for standard lipids. Such additional information about CVD risk could aid the treatment decision discussions between clinicians and patients, discussions that consider the estimated risk of CVD risk, the risks and benefits of statin therapy, and the patient's willingness to adhere to a daily pill-taking routine.

For very-high risk patients, novel PCSK9 inhibitors could be considered as a treatment option. However, given the high cost of PCSK9 inhibitors and the relatively limited clinical experience in long term use of these drugs, considering only the highest risk patients as candidates for PCSK9 inhibitor therapy would be prudent. This study supports the use of lipoprotein subfraction to identify those with elevated risk of CVD among those with >20% 10-year risk of CVD. Patients treated with the PCSK9 inhibitor alirocumab have been reported to have reduction in both small and large LDL particles [28].

The association we observed between small LDL particles (LDL-Small, and subclasses of LDL-Very Small) after adjustment for LDL-C is consistent with the hypothesis that small LDL particles are more atherogenic than large LDL particles [29]. Small LDL particles could be more atherogenic than larger LDL particles because they are more susceptible to oxidation and have greater affinity for proteoglycans in the arterial wall [30]. Among individuals with similar levels of LDL-C, those with smaller LDL particles would have more particles, increasing the probability of subendothelial retention [31]. Thus, for individuals who have similar LDL-C levels, those with fewer, larger LDL particles, have lower risk than those with a greater number of smaller LDL particles.

A recent analysis of the placebo arm of the JUPITER trial, a study of patients with baseline LDL-C levels below 3.36 mmol/L (130 mg/dL), hsCRP level above 2 mg/L and without diabetes, found that LDL-Large, -Medium, -Small, and LDL-Very Small (d) were associated with CVD after adjustment for traditional risk factors including standard lipids [19]. Moreover, an analysis of the Malmö Diet and Cancer study participants, who were not in a statin benefit group (according to the ACC/AHA guideline), found that LDL-Small, and -Medium were associated with CVD in a fully adjusted model [18]. However, contrary to these previous studies, the current study did not find that total number of LDL particles (LDL-P) was associated with CVD after adjustment for standard lipids.

Other studies have also reported the association of LDL particles with CVD events using other assessment methods. Apolipoprotein B (apoB) has been widely used to assess LDL particle number, regardless of their size or density. Recently Pencina et al. [32] reported that in the Framingham Offspring Cohort, apoB improved risk assessment of incident coronary heart disease in models that adjusted for traditional risk factors, including LDL-C and HDL-C. However, they did not report results from models that also adjusted for triglycerides. Others have interpreted the nuclear magnetic resonance (NMR) signal of the terminal methyl groups of triglycerides and cholesterol esters to infer lipoprotein subfraction concentration without a physical separation of lipoproteins [33]. Several studies have reported that NMR-assessed LDL-P is a better predictor of coronary events in those with high LDL-P but low LDL-C or *vice versa* [34,35].

In this study, high levels of HDL-C were associated with fewer CVD events, but high levels of HDL subfractions were not. Similarly, HDL-C was associated with fewer incident CVD events in an analysis of the placebo group of the JUPITER study [19]. Since ion mobility separates particles by size, we speculate that other, uncharacterized, HDL-sized particles were counted as HDL particles and might have diluted the true HDL subfraction effect.

Non-fasting remnant cholesterol has been shown to be a causal risk factor for ischemic heart disease using a Mendelian randomization strategy [36], and it was also found to be associated with all-cause mortality, whereas LDL-C was not [37]. Since individuals with high levels of fasting triglycerides are more likely to have small-dense LDL particles [38,39], it would be interesting to investigate whether non-fasting, triglyceride-rich remnants are correlated with lipoprotein subfractions measured in fasting samples.

This study has several strengths and limitations. The main strength is that this is a large prospective study with a large number of CVD events, and is therefore less likely to produce false positive results or be subject to biases that are more common in small case-control studies. The large age-span in the current study reflects a population in whom risk stratification is frequently needed, and improved stratification and subsequent improved treatment of this population could help prevent CVD events. Other lipoprotein-related measures, including apoB, apolipoprotein A1, lipoprotein(a), and pre-beta-HDL have been shown to be associated with CVD. These lipoprotein-related measures were not assessed in this current study. Thus, we were unable to assess what would be the effect of including them in our analysis. The size categorization of the lipoprotein subfractions in this study uses a standard industrial technique for directly measuring particle sizes, and the analyses included full adjustment for standard lipids. However, the method we used to assess lipoprotein subfraction sizes and levels is one of several methods in use. Although different methods are likely to provide similar results, we have no direct evidence that specific subfractions assessed by different methods may not differ. The samples we used to assess both lipids and lipoprotein subfractions levels have undergone 5 or fewer freeze-thaw cycles prior to assessment. However, it has been reported that lipid levels a little changed after 10 freeze-thaw cycles [40] and lipoprotein subfraction were reported to be stable after 5 freeze thaw cycles [41] Finally, the study population is middle-age to elderly individuals from Southern Sweden. Therefore, our results may not be generalizable to populations with different ethnic and socioeconomic characteristics.

In conclusion, small LDL subfractions were associated with CVD after adjustment for LDL-C, HDL-C, and triglycerides in a population of middle-age to elderly individuals and also in subpopulations that could benefit from improved risk assessment.

Conflict of interest

DS, JZL, MPC, and JJD are employees of Quest Diagnostics. The other authors have nothing to disclose.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.atherosclerosis.2017.07.003.

References

[1] Cholesterol Treatment Trialists, C J. Fulcher, R. O'Connell, et al., Efficacy and safety of LDL-lowering therapy among men and women: meta-analysis of individual data from 174,000 participants in 27 randomised trials, Lancet 385 (2015) 1397–1405.
[2] B.F. Voight, G.M. Peloso, M. Orho-Melander, et al., Plasma HDL cholesterol and risk of myocardial infarction: a mendelian randomisation study, Lancet 380 (2012) 572–580.
[3] M.F. Piepoli, A.W. Hoes, S. Agewall, et al., 2016 European guidelines on cardiovascular disease prevention in clinical practice: the sixth joint task force of the European society of cardiology and other societies on cardiovascular disease prevention in clinical practice (constituted by representatives of 10 societies and by invited experts)Developed with the special contribution of the European association for cardiovascular prevention & rehabilitation (EACPR), Eur. Heart J. 37 (2016) 2315–2381.
[4] N.J. Stone, J.G. Robinson, A.H. Lichtenstein, et al., 2013 ACC/AHA guideline on the treatment of blood cholesterol to reduce atherosclerotic cardiovascular risk in adults: a report of the American College of Cardiology/American Heart Association task force on practice guidelines, Circulation 129 (2014) S1–S45.
[5] R. Hutchins, A.J. Viera, S.L. Sheridan, et al., Quantifying the utility of taking pills for cardiovascular prevention, Circ. Cardiovasc Qual. Outcomes 8 (2015) 155–163.
[6] R.T. Dadu, C.M. Ballantyne, Lipid lowering with PCSK9 inhibitors, Nat. Rev. Cardiol. 11 (2014) 563–575.
[7] E.P. Navarese, M. Kolodziejczak, V. Schulze, et al., Effects of proprotein convertase subtilisin/kexin type 9 antibodies in adults with hypercholesterolemia: a systematic review and meta-analysis, Ann. Intern Med. 163 (2015) 40–51.
[8] D.S. Kazi, A.E. Moran, P.G. Coxson, et al., Cost-effectiveness of PCSK9 inhibitor therapy in patients with heterozygous familial hypercholesterolemia or atherosclerotic cardiovascular disease, JAMA 316 (2016) 743–753.
[9] G.J. Blake, J.D. Otvos, N. Rifai, et al., Low-density lipoprotein particle concentration and size as determined by nuclear magnetic resonance spectroscopy as predictors of cardiovascular disease in women, Circulation 106 (2002) 1930–1937.
[10] L. Kuller, A. Arnold, R. Tracy, et al., Nuclear magnetic resonance spectroscopy of lipoproteins and risk of coronary heart disease in the cardiovascular health study, Arterioscler. Thromb. Vasc. Biol. 22 (2002) 1175–1180.
[11] J.D. Otvos, D. Collins, D.S. Freedman, et al., 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 113 (2006) 1556–1563.
[12] W.C. Cromwell, J.D. Otvos, M.J. Keyes, et al., LDL particle number and risk of future cardiovascular disease in the Framingham offspring study - implications for LDL management, J. Clin. Lipidol. 1 (2007) 583–592.
[13] K. El Harchaoui, W.A. van der Steeg, E.S. Stroes, et al., Value of low-density lipoprotein particle number and size as predictors of coronary artery disease in apparently healthy men and women: the EPIC-Norfolk prospective population study, J. Am. Coll. Cardiol. 49 (2007) 547–553.
[14] J. Hsia, J.D. Otvos, J.E. Rossouw, et al., Lipoprotein particle concentrations may explain the absence of coronary protection in the women's health initiative hormone trials, Arterioscler. Thromb. Vasc. Biol. 28 (2008) 1666–1671.
[15] S. Mora, J.D. Otvos, N. Rifai, et al., Lipoprotein particle profiles by nuclear magnetic resonance compared with standard lipids and apolipoproteins in predicting incident cardiovascular disease in women, Circulation 119 (2009) 931–939.
[16] R.H. Mackey, P. Greenland, D.C. Goff Jr., et al., High-density lipoprotein cholesterol and particle concentrations, carotid atherosclerosis, and coronary events: MESA (multi-ethnic study of atherosclerosis), J. Am. Coll. Cardiol. 60 (2012) 508–516.
[17] B.T. Steffen, W. Guan, A.T. Remaley, et al., Use of lipoprotein particle measures for assessing coronary heart disease risk post-American Heart Association/American College of Cardiology guidelines: the multi-ethnic study of atherosclerosis, Arterioscler. Thromb. Vasc. Biol. 35 (2015) 448–454.
[18] O. Melander, D. Shiffman, M.P. Caulfield, et al., Low-density lipoprotein particle number is associated with cardiovascular events among those not classified into statin benefit groups, J. Am. Coll. Cardiol. 65 (2015) 2571–2573.
[19] S. Mora, M.P. Caulfield, J. Wohlgemuth, et al., Atherogenic lipoprotein subfractions determined by ion mobility and first cardiovascular events after random allocation to high-intensity statin or placebo: the justification for the use of statins in prevention: an intervention trial evaluating rosuvastatin (JUPITER) trial, Circulation 132 (2015) 2220–2229.
[20] C. Fava, M. Sjogren, M. Montagnana, et al., Prediction of blood pressure changes over time and incidence of hypertension by a genetic risk score in Swedes, Hypertension 61 (2013) 319–326.
[21] O. Melander, M. Belting, J. Manjer, et al., Validation of plasma proneurotensin as a novel biomarker for the prediction of incident breast cancer, Cancer Epidemiol. Biomarkers Prev. 23 (2014) 1672–1676.
[22] P. Jerntorp, G. Berglund, Stroke registry in malmo, Sweden, Stroke 23 (1992) 357–361.
[23] B. Lagerqvist, J.S. Swedeheart, in: SCAAR Årsrapport, 2008.
[24] J.F. Ludvigsson, E. Andersson, A. Ekbom, et al., External review and validation of the Swedish national inpatient register, BMC Public Health 11 (2011) 450.
[25] D.C. Goff Jr., D.M. Lloyd-Jones, G. Bennett, et al., 2013 ACC/AHA guideline on the assessment of cardiovascular risk: a report of the american college of cardiology/american heart association task force on practice guidelines, Circulation 129 (2014) S49–S73.
[26] R.J. Gray, A class of K-sample tests for comparing the cumulative incidence of a competing risk, Ann. Stat. (1988) 1141–1154.
[27] R.C. Team, in: R: a Language and Environment for Statistical Computing, R Foundation for Statistical Computing, Vienna, Austria, 2012.
[28] M.J. Koren, D. Kereiakes, R. Pourfarzib, et al., Effect of PCSK9 inhibition by alirocumab on lipoprotein particle concentrations determined by nuclear magnetic resonance spectroscopy, J. Am. Heart Assoc. (2015) 4.
[29] K.K. Berneis, R.M. Krauss, Metabolic origins and clinical significance of LDL heterogeneity, J. Lipid Res. 43 (2002) 1363–1379.
[30] M.R. Diffenderfer, E.J. Schaefer, The composition and metabolism of large and small LDL, Curr. Opin. Lipidol. 25 (2014) 221–226.
[31] I. Tabas, K.J. Williams, J. Boren, Subendothelial lipoprotein retention as the initiating process in atherosclerosis: update and therapeutic implications, Circulation 116 (2007) 1832–1844.
[32] M.J. Pencina, R.B. D'Agostino, T. Zdrojewski, et al., Apolipoprotein B improves risk assessment of future coronary heart disease in the Framingham Heart Study beyond LDL-C and non-HDL-C, Eur. J. Prev. Cardiol. 22 (2015) 1321–1327.
[33] E.J. Jeyarajah, W.C. Cromwell, J.D. Otvos, Lipoprotein particle analysis by nuclear magnetic resonance spectroscopy, Clin. Lab. Med. 26 (2006) 847–870.
[34] S. Mora, J.E. Buring, P.M. Ridker, Discordance of low-density lipoprotein (LDL) cholesterol with alternative LDL-related measures and future coronary events, Circulation 129 (2014) 553–561.
[35] J.D. Otvos, S. Mora, I. Shalaurova, et al., Clinical implications of discordance between low-density lipoprotein cholesterol and particle number, J. Clin. Lipidol. 5 (2011) 105–113.
[36] A. Varbo, M. Benn, A. Tybjaerg-Hansen, et al., Remnant cholesterol as a causal risk factor for ischemic heart disease, J. Am. Coll. Cardiol. 61 (2013) 427–436.
[37] A.M. Jepsen, A. Langsted, A. Varbo, et al., Increased remnant cholesterol explains part of residual risk of all-cause mortality in 5414 patients with ischemic heart disease, Clin. Chem. 62 (2016) 593–604.
[38] M.A. Austin, J.L. Breslow, C.H. Hennekens, et al., Low-density lipoprotein subclass patterns and risk of myocardial infarction, JAMA 260 (1988) 1917–1921.
[39] H. Campos, J.J. Genest Jr., E. Blijlevens, et al., Low density lipoprotein particle size and coronary artery disease, Arterioscler. Thromb. 12 (1992) 187–195.
[40] L. Paltiel, K.S. Ronningen, H.M. Meltzer, et al., Evaluation of freeze thaw cycles on stored plasma in the biobank of the Norwegian mother and child cohort study, Cell Preserv Technol. 6 (2008) 223–230.
[41] M.P. Caulfield, S. Li, G. Lee, et al., Direct determination of lipoprotein particle sizes and concentrations by ion mobility analysis, Clin. Chem. 54 (2008) 1307–1316.