A third of nonfasting plasma cholesterol is in remnant lipoproteins: Lipoprotein subclass profiling in 9293 individuals

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HIGHLIGHTS

- Using NMR, a third of plasma cholesterol was present in remnant lipoproteins.
- Of 14 lipoprotein subclasses, large LDL and IDL were containing most cholesterol.
- Directly measured remnant cholesterol was highest in older individuals.

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ABSTRACT

Background and aims: Increased concentrations of calculated remnant cholesterol in triglyceride-rich lipoproteins are observationally and genetically, causally associated with increased risk of ischemic heart disease; however, when measured directly, the fraction of plasma cholesterol present in remnant particles is unclear. We tested the hypothesis that a major fraction of plasma cholesterol is present in remnant lipoproteins in individuals in the general population.
Methods: We examined 9293 individuals from the Copenhagen General Population Study using nuclear magnetic resonance spectroscopy measurements of total cholesterol, free- and esterified cholesterol, triglycerides, phospholipids, and particle concentration. Fourteen subclasses of decreasing size and their lipid constituents were analysed: six subclasses were very low-density lipoprotein (VLDL), one intermediate-density lipoprotein (IDL), three low-density lipoprotein (LDL), and four subclasses were high-density lipoprotein (HDL). Remnant lipoproteins were VLDL and IDL combined.
Results: Mean nonfasting cholesterol concentration was 1.84 mmol/L (72 mg/dL) for remnants, 2.01 mmol/L (78 mg/dL) for LDL, and 1.83 mmol/L (71 mg/dL) for HDL, equivalent to remnants containing 32% of plasma total cholesterol. Of 14 lipoprotein subclasses, large LDL and IDL were the ones containing most of plasma cholesterol. The plasma concentration of remnant cholesterol was from ∼1.4 mmol/L (54 mg/dL) at age 20 to ∼1.9 mmol/L (74 mg/dL) at age 60. Corresponding values for LDL cholesterol were from ∼1.5 mmol/L (58 mg/dL) to ∼2.1 mmol/L (81 mg/dL).
Conclusions: Using direct measurements, one third of total cholesterol in plasma was present in remnant lipoproteins, that is, in the triglyceride-rich lipoproteins IDL and VLDL.

1. Introduction

The leading cause of lost healthy life years globally is cardiovascular disease [1,2]. This is mainly due to ischemic heart disease and stroke [1], and yearly, 18 million individuals die from cardiovascular disease worldwide [2]. Important metabolic changes associated with cardiovascular disease are hypertension, obesity, diabetes mellitus, and hyperlipidemia [3]. For the latter, clinical practice has focused on

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lowering low-density lipoprotein (LDL) cholesterol, based on the strong evidence that use of lipid-lowering statins lowers both LDL cholesterol and the risk of atherosclerotic cardiovascular disease [4,5]. Nevertheless, a considerable residual risk remains after achieving recommended LDL cholesterol targets [6,7]. Such residual risk could partly be explained by cholesterol in the triglyceride-rich lipoproteins [4,5].

Human and animal studies have shown that the triglyceride-rich lipoproteins chylomicron remnants, very low-density lipoproteins (VLDL), and intermediate-density lipoproteins (IDL) like LDL can enter the arterial intima and become trapped [5,8,9]; however, large chylomicrons and the largest VLDL particles cannot enter the arterial intima because of their size [10–12]. Resistance from the blood pressure gradient from the arterial lumen across the arterial wall to no pressure in the adventitia combined with attachment to proteoglycans and other components of the arterial intima leads to LDL entrapment, causing accumulation of cholesterol and subsequently atherosclerosis [5]. Triglyceride-rich lipoproteins likely are trapped in the arterial intima by similar mechanisms; however, and because of their larger size, returning to the arterial lumen could be more difficult than for LDL [4,5].

Lipoproteins contain different concentrations of cholesterol esters, free cholesterol, triglycerides, phospholipids, and various proteins [13]. The atherogenic part of triglyceride-rich lipoproteins is most likely the cholesterol content, also known as remnant cholesterol, while plasma triglycerides are a marker of the amount of remnant cholesterol because of the correlation between these two lipid components of the same particles [4].

Nuclear magnetic resonance (NMR) spectroscopy offers the opportunity of detailed measurements of how cholesterol is distributed in different lipoprotein subclasses. The classification of lipoproteins has traditionally been based on density and size determined by ultracentrifugation and gel filtration, ranging from the largest chylomicrons and VLDL to the smaller IDL, LDL, and HDL [14]. Exploring how cholesterol is distributed in a detailed continuum of the lipoprotein fractions in individuals in the general population may contribute to the understanding of how atherosclerosis develops.

Here, we used detailed lipoprotein subclass profiles, measured by NMR spectroscopy, from 9293 individuals from the Copenhagen General Population Study to test the hypothesis that a major fraction of total cholesterol in plasma drawn nonfasting is present in lipoproteins larger than LDL, that is, in IDL, VLDL, and chylomicron remnants. As all chylomicrons secreted from the intestine and all VLDLs secreted by the liver have some triglycerides hydrolysed immediately after entrance into the bloodstream, all triglyceride-rich lipoproteins can be considered as remnants [4,5,14]. To simplify the nomenclature, we therefore use the term remnant cholesterol corresponding to cholesterol in all triglyceride-rich lipoproteins combined (IDL + VLDL + chylomicron remnants). The reference method for LDL cholesterol measurement combines ultracentrifugation to remove VLDL and chylomicrons including their remnants and heparin-Mn^{2+} precipitation to separate LDL particles from HDL [15]. With this method, the lipoprotein fraction in the density range of 1.006–1.063 g/ml is defined as LDL, and the fraction in the density range of 1.063–1.21 g/ml is defined as HDL. However, it is not widely recognized that the LDL cholesterol fraction on the reference method using ultracentrifugation also contains the cholesterol from IDL with density 1.006–1.019 g/ml [15] (see Table 1 in Nordestgaard BG [13]).

2. Materials and methods

2.1. The Copenhagen General Population Study

The Copenhagen General Population Study is an ongoing cohort study initiated in 2003. In the present study, we included individuals recruited in 2003–2004 (response rate 49%). Individuals of Danish descent (Danish citizens born in Denmark with parents also being Danish citizens born in Denmark) aged 20 or above were randomly invited, using the Danish Central Person Register, to reflect the adult white Danish population. Individuals filled in a questionnaire, underwent a physical examination, and had blood samples drawn.

Present study was conducted according to the Declaration of Helsinki. Written informed consent was obtained from all individuals and the study was approved by the Ethics Committee of the Capital Region of Denmark (H-KF-01-144/01) and by the Danish Data Protection Agency.

2.2. Nuclear magnetic resonance (NMR) spectroscopy

NMR spectroscopy was used to quantify detailed nonfasting lipoprotein measurements by the Metabolomic Core Facility at the University of Bristol operating on Nightingale Health's platform, a widely used platform in epidemiological studies that has been described previously [16–18]. Multiple plasma biomarkers are characterised by the protons in the biomarkers hydrogen atoms using a strong magnetic field exposed to electromagnetic radiation, are analysed by their resonance frequencies giving rise to a NMR spectrum, and thereby concentrations of the biomarkers in the plasma are measured [16,17]. Over 200 metabolic biomarkers were quantified in the categories cholesterol, fatty acids, glycerides, phospholipids, apolipoproteins, amino acids, glycolysis related metabolites, ketone bodies, fluid balance, inflammation, lipoprotein particle size, and lipoprotein subclasses [16–18]. The 14 lipoprotein subclasses measured included XXL VLDL, XL VLDL, L VLDL, M VLDL, S VLDL, XS VLDL, IDL, L LDL, M LDL, S LDL, XL HDL, L HDL, M HDL, and S HDL [16–18] (Supplementary Fig. 1). For each lipoprotein subclass, total cholesterol, free cholesterol, esterified cholesterol, triglycerides, phospholipids, particle concentration, and ratios of lipid contents in particles are measured [16–18]. Furthermore, the mean particle sizes for VLDL, LDL (including IDL), and HDL are calculated [16–18]. It is not possible to differentiate between chylomicrons and VLDL and their remnants by NMR spectroscopy, and chylomicrons and their remnants are therefore part of the VLDL fractions; intact chylomicrons are in the XXL VLDL fraction, while chylomicrons remnant can be in any VLDL subfraction or even in IDL, depending on their size.

Data on the analytical performance and technical variance of Nightingale's nuclear magnetic resonance spectroscopy platform on other cohorts has been published [19,20]. In short, coefficients of variations (CVs) for the NMR-based metabolic measures have previously been found to be 2.1% for total cholesterol, 2.3% for LDL cholesterol, 2.3% for HDL cholesterol, and 1.2% for total triglycerides [19]. CVs for other metabolic measures are typically below 5% [19].

2.3. Biochemical analyses

Standard hospital assays were used to measure nonfasting [21] total cholesterol, HDL cholesterol and triglycerides on fresh samples (Konelab, Helsinki, Finland). LDL cholesterol was calculated using the Friedewald equation when triglycerides were < 4 mmol/L (354 mg/dL), and otherwise measured directly (Konelab). In the original Friedewald equation, the triglyceride cutpoint of 400 mg/dL (4.5 mmol/L) was used [22]; however, for simplicity we adapted the 4 mmol/L (354 mg/dL) cutpoint as mmol/L is the unit used in Denmark.

2.4. Baseline values

Alcohol intake was self-reported drinks per week (1 drink ≈ 12 g alcohol). Physical activity in leisure time was dichotomized as being physically inactive (doing no or light physical activity in leisure time < 4 h/week) and being physically active (> 4 h/week of light physical activity or ≥ 2 h/week of vigorous physical activity in leisure time). Height and weight were measured at baseline, and body mass index calculated as weight (kg) divided by height squared (m²). Systolic

Table 1
Cholesterol and triglyceride content by lipoprotein size in individuals in the Copenhagen General Population Study.

Lipoprotein	Subclassification	Size, nm	Mean free + esterified cholesterol			Mean triglycerides		
			mmol/L	mg/dL	%	mmol/L	mg/dL	%
Remnant lipoproteins	VLDL + IDL	≥27.2	1.84	72	32%	1.39	124	77%
Largest VLDL	XXL + XL + L	≥49.2	0.14	6	3%	0.36	32	20%
Smallest VLDL	M + S + XS	30.1–49.1	0.82	32	14%	0.89	79	49%
IDL		27.2–30	0.88	34	15%	0.14	13	8%
LDL	L + M + S	16.6–27.1	2.01	78	35%	0.23	20	13%
HDL	XL + L + M + S	≤16.5	1.83	71	32%	0.19	17	11%
Total			5.67	221	100%	1.81	161	100%

Cholesterol and triglycerides in the different lipoprotein fractions were measured by nuclear magnetic resonance spectroscopy followed by correction for recovery relative to values from standard hospital assays using fresh samples, as described in the Methods. Because of rounding of numbers, the ratio between mmol/L and mg/dL varies slightly.
HDL: high-density lipoprotein. IDL: intermediate-density lipoprotein. L: large. LDL: low-density lipoprotein. M: medium. S: small. VLDL: very low-density lipoprotein. XL: extra large. XS: extra small. XXL: extra extra large.

blood pressure was measured at baseline (mmHg). Diabetes mellitus was nonfasting glucose > 11 mmol/L (198 mg/dL), self-reported diabetes mellitus, use of antidiabetic medication, and/or a diabetes mellitus diagnosis from the national Danish Patient Registry (ICD-8: 250; ICD-10: E10, E11, E13, E14).

2.5. Statistical analyses

Stata/SE 13 was used. As is standard with respect to ultracentrifugation, cholesterol and triglycerides in the different lipoprotein fractions measured by NMR spectroscopy were corrected, separately for each individual person, for recovery relative to total plasma cholesterol and triglycerides measured on fresh samples at the day of attendance (Supplementary Fig. 2). Since we had no measurement of total phospholipids on fresh samples, the recovery for phospholipids was defined as the average of the recovery for total cholesterol and triglycerides in each individual separately. The largest VLDL was calculated by adding XXL VLDL, XL VLDL, and L VLDL while the smallest VLDL was calculated by adding M VLDL, S VLDL and XS VLDL. Remnant cholesterol was defined as cholesterol in IDL + VLDL including chylomicron remnants.

The particle size ranges were approximated as the median value between the average size for each particle subclass (see Fig. 2 in Pasi Soininen et al. [17]). For XXL VLDL and S HDL the particle sizes were given as more than the median between XXL VLDL and XL VLDL and for S HDL the particle sizes were given as less than the median between M HDL and S HDL [17].

The particle concentrations given in mol/L were converted to particles/L by multiplying with Avogadro's constant ($6.02214086 \times 10^{23}$ particles/mol). Kernel density estimation was used to estimate distribution of mean particle diameters and particle concentrations, whereas kernel-weighted local polynomial smoothing curves, with 95% confidence intervals, were used to study LDL and remnant cholesterol concentrations by age.

For VLDL, LDL + IDL (IDL was included in the measurement of mean particle diameter of LDL) and HDL the mean sizes were calculated by weighting the subclass diameters with their particle concentrations (See Supplementary Table 1 Krista Fischer et al. [23]). For IDL there is only one subclass with an average particle diameter defined as 28.6 nm [17,23] and therefore the mean particle diameter of IDL alone is drawn as an assumption with a mean of 28.6 nm.

R^2 was taken from linear regression models between LDL- and IDL-, VLDL-, and remnant cholesterol.

3. Results

Supplementary Table 1 shows baseline characteristics of 9293 individuals from the Copenhagen General Population Study recruited in

2003-2004 using standard hospital assays. Mean plasma concentrations were 5.7 mmol/L (220 mg/dL) for total cholesterol, 3.3 mmol/L (127 mg/dL) for "LDL" cholesterol including LDL + IDL, and 1.8 mmol/L (161 mg/dL) for total triglycerides.

3.1. Lipoprotein sizes and particle numbers

The mean particle diameter of the lipoproteins in each individual in the Copenhagen General Population Study was 37.1 nm (range: 33.1 nm–41.9 nm) for VLDL, 23.6 nm (23.2 nm–24.5 nm) for LDL (including IDL), and 10.0 nm (9.3 nm–11.1 nm) for HDL (Fig. 1). For comparison with the other lipoproteins, an approximation of the mean particle diameter for IDL at 28.6 nm (28.4 nm-28.8 nm) was added to Fig. 1. The mean particle diameters of the lipoproteins were approximately normally distributed.

Distributions of concentrations in particles/L in all 14 lipoprotein subclasses are shown in Fig. 2. Overall, the particle number per liter increased with increasing density of the lipoprotein and thus with decreasing size. Mean particles/L were 1.1×10^{14} for XXL VLDL, 5.7×10^{14} for XL VLDL, 3.5×10^{15} for L VLDL, 1.2×10^{16} for M VLDL, 1.8×10^{16} for S VLDL, 2.3×10^{16} for XS VLDL, 6.4×10^{16} for IDL, 1.0×10^{17} for L LDL, 8.2×10^{16} for M LDL, 9.5×10^{16} for S LDL, 2.7×10^{17} for XL HDL, 7.3×10^{17} for L HDL, 1.2×10^{18} for M HDL, and 2.8×10^{18} for S HDL.
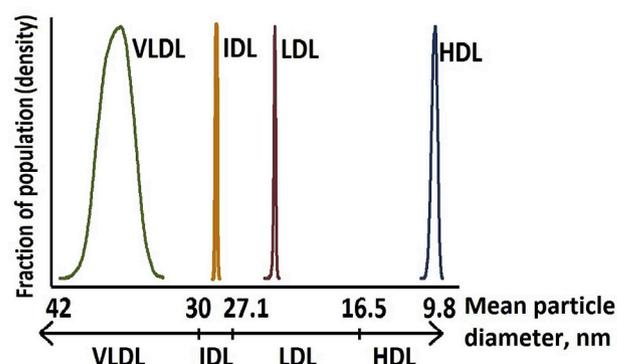

Fig. 1. Distribution of mean particle diameter in 9293 individuals in the Copenhagen General Population Study.
For VLDL, LDL + IDL (IDL was included in the measurement of mean particle diameter of LDL) and HDL, the mean sizes were calculated by weighting the subclass diameters with their particle concentrations. For IDL, there is only one subclass with an average particle diameter defined as 28.6 nm and therefore the mean particle diameter of IDL alone is drawn as an assumption with a mean on 28.6 nm. HDL: high-density lipoprotein. IDL: intermediate-density lipoprotein. LDL: low-density lipoprotein. VLDL: very low-density lipoprotein.
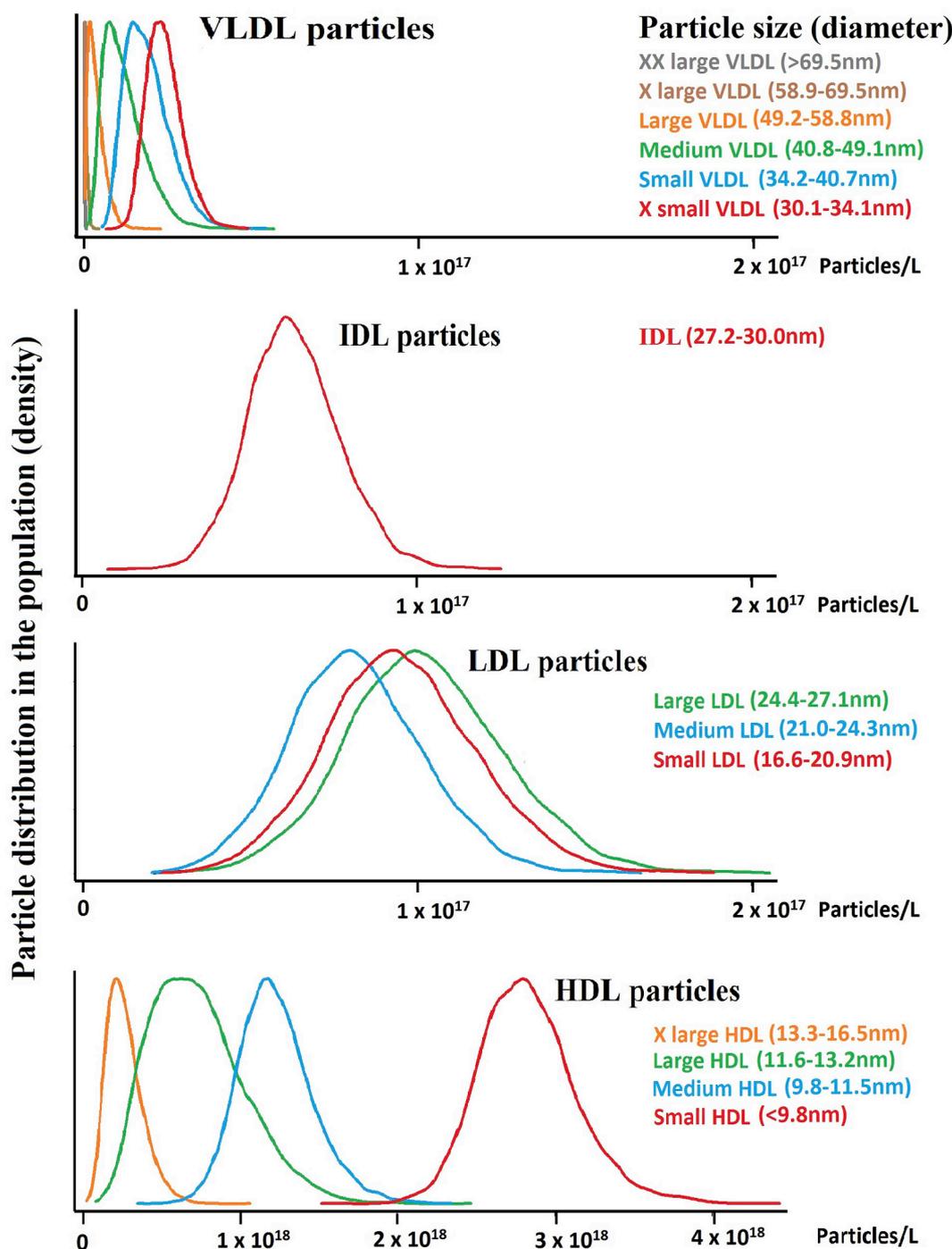
Fig. 2. Distribution of particle numbers in 9293 individuals in the Copenhagen General Population Study.
Number and size of lipoprotein particles were measured by nuclear magnetic resonance spectroscopy. Note that the scale for the x-axis for HDL particles is approximately 20-fold that for the x-axes used for LDL, IDL, and VLDL particles. HDL: high-density lipoprotein. IDL: intermediate-density lipoprotein. LDL: low-density lipoprotein. VLDL: very low-density lipoprotein. X: extra.

3.2. Lipoprotein particle composition

Mean fractions of cholesterol, including both cholesterol esters and free cholesterol, were 18% for XXL VLDL, 22% for XL VLDL, 24% for L VLDL, 28% for M VLDL, 35% for S VLDL, 46% for XS VLDL, 62% for IDL, 66% for L LDL, 64% for M LDL, 60% for S LDL, 48% for XL HDL, 47% for L HDL, 47% for M HDL, and 39% for S HDL (Fig. 3, bottom section). Corresponding triglyceride mean fractions were 70%, 61%, 58%, 51%, 41%, 23%, 11%, 9%, 7%, 7%, 5%, 4%, 6%, and 5%, respectively, while corresponding phospholipid mean fractions were

11%, 16%, 18%, 20%, 24%, 30%, 27%, 25%, 28%, 32%, 47%, 49%, 47%, and 56%, respectively.

The lipoprotein subclasses containing most of the plasma cholesterol were L LDL with a median cholesterol ester concentration of 0.75 mmol/L (29 mg/dL) and a median free cholesterol concentration of 0.30 mmol/L (12 mg/dL), followed by IDL with a median cholesterol ester concentration of 0.62 mmol/L (24 mg/dL) and a median free cholesterol concentration of 0.24 mmol/L (9 mg/dL) (Fig. 3, top section). The lipoprotein subclass containing most of the triglycerides was M VLDL with a median triglyceride concentration of 0.34 mmol/L
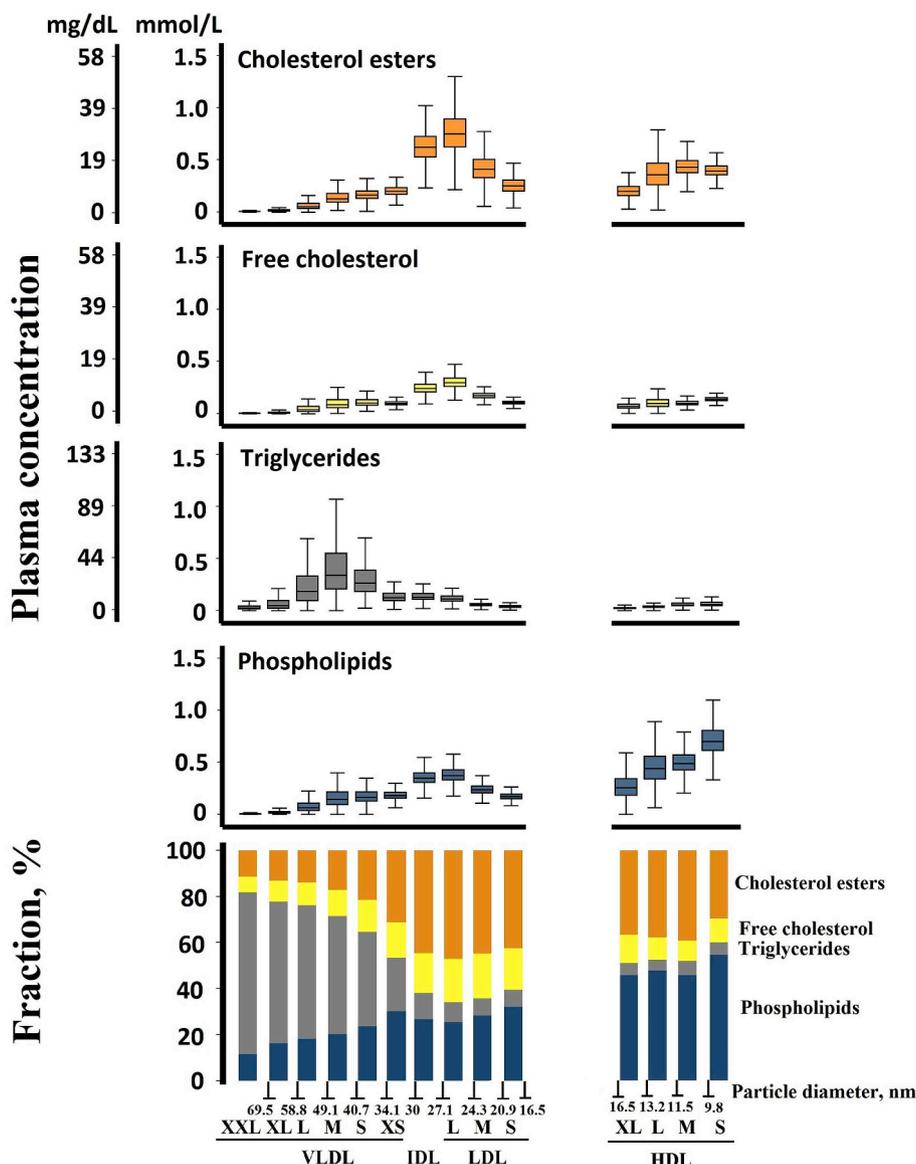
Fig. 3. Lipoproteins sizes and contents in 9293 individuals in the Copenhagen General Population Study.
Cholesterol, triglycerides, and phospholipids in different lipoprotein fractions were measured by nuclear magnetic resonance spectroscopy, and corrected for recovery as described in the Methods section. Phospholipids are only given in mmol/L only as unit conversion to mg/dL cannot be done unambiguously. HDL: high-density lipoprotein. IDL: intermediate-density lipoprotein. L: large. LDL: low-density lipoprotein. M: medium. S: small. VLDL: very low-density lipoprotein. XL: extra large. XS: extra small. XXL: extra extra large.

(30 mg/dL) followed by S VLDL with a median triglyceride concentration of 0.26 mmol/L (23 mg/dL). The lipoprotein subclass contributing most to the plasma phospholipid concentration was S HDL with a median concentration of 0.70 mmol/L (27 mg/dL).

3.3. Remnant cholesterol and LDL cholesterol

Higher concentrations of LDL cholesterol were associated with higher concentrations of remnant cholesterol, VLDL cholesterol, and IDL cholesterol (Supplementary Fig. 3). As LDL cholesterol increased, VLDL cholesterol increased with a correlation coefficient of 0.34 and an R^2 of 0.23, IDL cholesterol with a correlation coefficient of 0.37 and an R^2 of 0.94, and remnant cholesterol with a correlation coefficient of 0.71 and an R^2 of 0.52.

The plasma concentration of remnant cholesterol and LDL cholesterol was lowest for individuals at age 20, highest for individuals at age 60 and thereafter slightly lower (Fig. 4). The plasma concentration of remnant cholesterol was ~1.4 mmol/L (54 mg/dL) at age 20 and

~1.9 mmol/L (74 mg/dL) at age 60. Corresponding values for LDL cholesterol were ~1.5 mmol/L (58 mg/dL) and ~2.1 mmol/L (81 mg/dL).

3.4. Cholesterol and triglycerides in different lipoprotein fractions

For plasma total cholesterol (=free cholesterol + esterified cholesterol), the distribution was 32% in remnant lipoprotein particles (largest VLDL, smallest VLDL, and IDL combined), 35% in LDL particles, and 32% in HDL particles (Table 1). The mean cholesterol concentration was 1.84 mmol/L (72 mg/dL) for remnant lipoproteins, 2.01 mmol/L (78 mg/dL) for LDL, and 1.83 mmol/L (71 mg/dL) for HDL. For plasma total triglycerides the distribution was 77% in remnant lipoprotein particles, 13% in the LDL particles, and 11% in the HDL particles (Table 1). For triglycerides the mean concentration was 1.39 mmol/L (124 mg/dL) for remnant lipoproteins, 0.23 mmol/L (20 mg/dL) for LDL, and 0.19 mmol/L (17 mg/dL) for HDL.
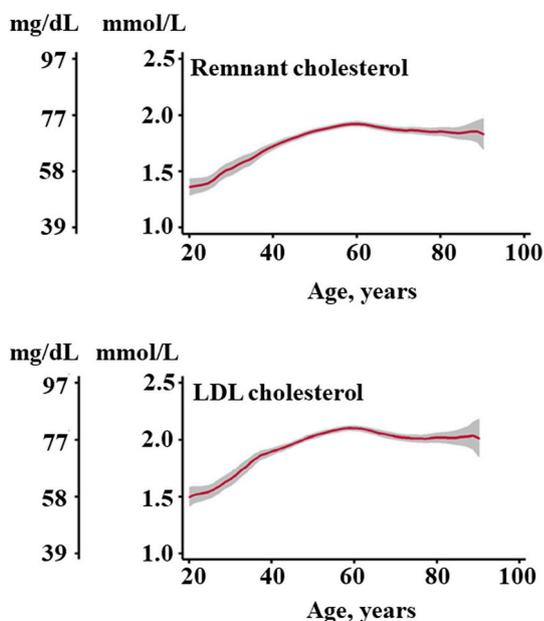
Fig. 4. Cholesterol concentrations at different age groups in 9293 individuals in the Copenhagen General Population Study.
The figure shows kernel-weighted local polynomial smoothing curves. Remnant cholesterol = intermediate-density lipoprotein cholesterol + very low-density lipoprotein cholesterol (including chylomicron remnants). LDL: low-density lipoprotein.

4. Discussion

In this cohort study of 9293 individuals from the Copenhagen General Population Study using direct measurements, we found that one third of total cholesterol in plasma was present in remnant lipoproteins, which is a fraction corresponding to the cholesterol content in LDL. These findings add new knowledge to the understanding of the cholesterol distribution in different lipoprotein subfractions and may help refocus attention in cardiovascular prevention toward other lipoproteins than LDL alone.

Remnant lipoproteins enter the arterial intima, though at a slightly slower speed than LDL particles; however, due to their large size, remnant lipoproteins seem to get trapped in the intima, preferentially to LDL particles, by attachment to components such as proteoglycans, and by the constant lumen to intima fluid flow due to blood pressure in the lumen [4,5,8,9,12,24]. In the intima, remnants are taken up directly by macrophages without need for modification and thereby macrophages become foam cells that develop into atherosclerotic plaques; this is unlike for LDL that needs to be modified before uptake into macrophages [4,5,25]. Furthermore, hydrolysis of triglycerides in remnant lipoproteins may generate local inflammation in the arterial wall and through this contribute to atherogenesis with vulnerable plaques [4,5,26]. Besides cholesterol and triglyceride content, particle number *per se* may also be important [27]. For individuals with similar concentrations of VLDL cholesterol, IDL cholesterol and LDL cholesterol, the lipoprotein particle number can vary, and individuals with the largest number of VLDL, IDL and/or LDL particles might be at higher risk of developing cardiovascular disease [7,28,29].

In a prospective study of 106,216 individuals from the Copenhagen General Population Study, where 1565 individuals had a myocardial infarction, stepwise higher calculated remnant cholesterol was associated with stepwise higher risk of myocardial infarction across the examined body mass index subcategories, suggesting that remnant cholesterol is a risk factor for myocardial infarction independent of overweight and obesity [30]. Furthermore, a recent study reported that individuals with high calculated remnant cholesterol concentrations

had higher risk of ischemic stroke [31]. Given such data it seems valuable simply to calculate remnant cholesterol, and that reduction of remnant cholesterol no matter how low (except for < 0.5 mmol/L (19 mg/dL)) or high may benefit to reduce cardiovascular disease; however, this needs to be tested in randomized trials.

A previous study suggests that increased concentrations of both calculated and directly measured remnant cholesterol with an assay by Denka Seiken were associated with increased all-cause mortality in patients with ischemic heart disease [32]. It could be speculated that calculated remnant cholesterol and remnant cholesterol measured with nuclear magnetic resonance spectroscopy provide similar credibility of all-cause mortality risk, and maybe also risk of cardiovascular disease. If this were the case, calculated remnant cholesterol is most easily accessible and therefore could be preferred in the clinic; however, nuclear magnetic resonance spectroscopy can be used to examine the cholesterol distribution in lipoproteins in detail.

There are different ways of determining the concentration of remnant cholesterol, including calculating it from a standard lipid profile and measuring it directly by ultracentrifugation, direct remnant cholesterol assays, and as in the present study by NMR spectroscopy [5,14,30,33,34]. A Chinese population based study, also using the Nightingale Health's platform as in the present study, found similar fractions of cholesterol in remnants in nonfasting samples as in the present study [20].

Debate over lipoprotein fractions has been ongoing for decades. Early studies used density cuts, but density gradient, and other methods, have demonstrated that lipoproteins are better characterised by a continuous function, i.e. that fractions are not discrete populations, but rather a collection of particles that overlap in biochemical and metabolic properties. However, in clinical practice density cuts for LDL cholesterol and HDL cholesterol are used throughout, making the present data relevant for evaluation of lipids and lipoproteins in clinical practice.

A study of 4756 white men and women recruited in 1971–1976 used ultracentrifugation to measure cholesterol in VLDL, LDL (including IDL) and HDL, and found mean VLDL cholesterol concentrations that were about half of the VLDL cholesterol concentrations in the present study, and LDL cholesterol concentrations that were ~1,5 times higher than in the present study [35]; however, that study excluded individuals older than 59 years and recruited in a time-period with much less obesity than today, which, together with using a different method, could explain the different results compared to our study. Furthermore, that study did not estimate IDL cholesterol separately, and IDL cholesterol was therefore included in LDL cholesterol.

In previous studies estimating remnant cholesterol by calculation from a standard lipid profile (measured by standard hospital assays), concentrations were lower than in the present study using NMR spectroscopy [30,36,37]. Also, LDL cholesterol concentrations were higher since IDL cholesterol was included in LDL cholesterol. In the present study, mean LDL cholesterol measured using a standard hospital assay was 3.3 mmol/L, while LDL cholesterol and IDL cholesterol measured with nuclear magnetic resonance spectroscopy added together were 2.0 mmol/L + 0.9 mmol/L = 2.9 mmol/L.

Furthermore, previously used direct remnant assays only measures cholesterol in a subset of chylomicron remnants and VLDLs, and in a previous study we found that one of these assays only quantified around 13% of the concentration of calculated remnant cholesterol [5,30,34]. There has recently been developed a direct assay that measures the cholesterol content of all remnants (all triglyceride-rich lipoproteins), with concentrations comparable to calculated remnant cholesterol [30].

Other advanced lipoprotein profiling methods are difficult to compare with NMR spectroscopy, since the nomenclature is not uniform among the methods and the number of lipoprotein particles also differ [29]. Additionally, to our knowledge, no large-scale study has focused on the amount of cholesterol in triglyceride-rich lipoproteins using these methods, and the present study gives the most detailed

quantitative description of remnant cholesterol so far.

In the present study, directly measured remnant cholesterol was higher for individuals aged 60 compared to individuals aged 20. Previous ultracentrifugation studies recruiting participants from the 1970s to the 1990s have suggested that LDL cholesterol was higher in older individuals for both sexes, whereas VLDL cholesterol was also higher in individuals with a higher age for women and men, but only until the age of around 50 years for men [35,38]. Other NMR spectroscopy studies also show a possible association between aging and metabolomic changes, including subclasses of lipoprotein cholesterol [39–41].

Strengths of the present study include the large sample size representing the adult white Danish general population. Furthermore, the Nightingale Health platform offers a direct measurement of the cholesterol, triglyceride, and phospholipid distribution in 14 lipoprotein subfractions, allowing for a detailed and precise estimation of remnant cholesterol.

Some limitations should be mentioned. In the present study using the nuclear magnetic resonance platform, there are scaling issues compared with established biochemical laboratory measurements that need to be clarified. The measurements of plasma total cholesterol and triglycerides were slightly lower using NMR spectroscopy compared to the use of standard hospital biochemical assays; however, this is a previously known limitation not only applying to our study [20,29,42]. To account for this, cholesterol and triglycerides in the different lipoprotein fractions were corrected for recovery relative to total plasma cholesterol and triglycerides measured by standardized hospital biochemical assays on fresh samples collected at the day of attendance for each person. The standardized hospital biochemical assays are traceable to WHO standard calibrators and monthly checked for accuracy. Correction for recovery is a known method commonly used in ultracentrifugation, and the mean average recoveries of 88% for triglycerides and 76% for cholesterol in the present NMR spectroscopy are corresponding to the mean average recoveries in ultracentrifugation [43,44]; that said, differences in calibrations from measurement of triglycerides and cholesterol between standard hospital assays and NMR spectroscopy may also lead to some measurement differences between the two methods. In addition, long-term storage might give rise to subtle degradation of lipoproteins even though the samples were kept at −80 °C [29], and this could also explain some of the difference in concentrations between NMR spectroscopy and standard hospital assays in our study. Furthermore, it is a limitation that it is not possible to differentiate between chylomicrons and their remnants and VLDL and their remnants using the NMR spectroscopy method.

Another limitation is that the present study included white individuals of Danish descent only and therefore the distribution of lipoprotein subclasses, lipid content, sizes, and particle numbers may not necessarily be generalisable to other ethnicities; however, we are not aware of any data suggesting that the present results should not apply to other populations in affluent countries with similar fractions of overweight and obese individuals in the general population.

It could be argued that the term "remnant cholesterol" covering cholesterol in all triglyceride-rich lipoproteins is too simplified and that we instead should have used the term "triglyceride-rich lipoprotein cholesterol". There is no common definition of remnant cholesterol, and some experts think that remnant cholesterol intuitively should only comprise lipoprotein particles arising from the chain of delipidation of triglyceride-rich lipoproteins that in some way or another are stuck or are no longer following through the expected conversion to particles that have a defined and efficient removal mechanism. It could also be argued that the proportion of triglyceride-rich lipoproteins that are in a sequence of successful delipidation destined for a final state of receptor-mediated uptake, or some rapidly removed delipidated chylomicron remnants, should not be defined as remnant lipoproteins. However, as soon as chylomicrons from the intestine or VLDL from the liver enter the bloodstream, lipoprotein lipase will immediately start degrading some of the triglycerides in these particles. Therefore, essentially all triglyceride-rich lipoproteins in plasma, except for the rare case of complete lipoprotein lipase deficiency, can be considered some form of a remnant. We therefore prefer to use the simplified "remnant cholesterol" as this for most clinicians will be easier to understand than the term triglyceride-rich lipoprotein cholesterol.

In conclusion, in this study of 9293 individuals from the general population using direct measurement, we found that one third of total cholesterol in plasma was present in remnant lipoproteins, that is, in the triglyceride-rich lipoproteins IDL + VLDL + chylomicron remnants. Randomized controlled trials are needed to examine if lowering remnant cholesterol will reduce the risk of cardiovascular disease.

Conflicts of interest

The authors declared they do not have anything to disclose regarding conflict of interest with respect to this manuscript.

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Author contributions

Mie Balling, Anne Langsted, Shoaib Afzal, Anette Varbo, George Davey Smith, and Børge G. Nordestgaard designed the study. Mie Balling, Anne Langsted and Shoaib Afzal analysed the data. All the authors contributed to interpretation of data. Mie Balling wrote the first draft of the paper and Anne Langsted, Shoaib Afzal, Anette Varbo, George Davey Smith, and Børge G. Nordestgaard edited the paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.atherosclerosis.2019.05.011.

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