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Invited critical review

Small dense LDL: An emerging risk factor for cardiovascular disease

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ABSTRACT

Although low-density lipoprotein cholesterol (LDL-C) is a strong risk factor for coronary artery disease (CAD), LDL-C levels are not always elevated in CAD patients. LDL consists of several subclasses with distinct sizes, densities, and physicochemical compositions. Thus, LDL subclasses can be separated by various laboratory procedures. Among them, ultracentrifugation and electrophoresis have been used most frequently for determining LDL subclasses. Accumulating evidence has shown that a predominance of small dense LDL (sd-LDL) is closely associated with CAD. Moreover, sd-LDL-cholesterol (sd-LDL-C) concentrations are elevated in groups at a high risk for CAD, such as patients with type 2 diabetes and metabolic syndrome. Therefore, sd-LDL concentration is recognized as a surrogate marker for CAD. However, some studies failed to show therapeutic modulation of sd-LDL, likely because separating methods and sd-LDL particle definitions have not yet been standardized. Recently, a detergent-based homogenous assay for sd-LDL-C has been developed. This method does not require any pretreatment, and the measured values are highly reproducible with an automated analyzer. These features are suitable for large-scale clinical studies. This homogeneous assay is a useful tool for clarifying whether sd-LDL-C is a superior marker to LDL-C, and whether sd-LDL-C lipid-lowering therapies decrease the incidence of CAD.

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Contents

1. Introduction

Increases in the incidence of coronary artery disease (CAD) have been attributed to several classic risk factors, such as hypertension (HT), dyslipidemia, diabetes mellitus (DM), obesity, lower physical exercise, and smoking [1]. Among these, hypercholesterolemia (HC) is the most evident causal disorder for CAD [2]. Statin is a potent inhibitor of hydroxy-methyl-glutaryl-coenzyme A reductase, a rate-limiting enzyme of cholesterol synthesis in the liver, and is frequently used in the clinical management of cardiovascular disease (CVD) [3]. Extensive intervention studies have revealed that the risk of CVD events is consistently reduced by lowering cholesterol with statins. However, these risk reduction rates did not reach 30% in most clinical trials, such as the WOSCOPS (West of Scotland Coronary Prevention Study) trial, 4S (Scandinavian Simvastatin Survival Study), TNT (Treating to New Target), IDEAL (Incremental Decrease in Events through Aggressive Lipid Lowering), PROVE IT-TIMI -22 (Pravastatin or Atorvastatin Evaluation and Infection Therapy — Thrombolysis in Myocardial Infarction 22), and A-to-Z (Aggrastat to Zocor) trials [4–6]. To achieve further risk reduction, recent clinical interest has focused on the "beyond cholesterol" concept [7]. A number of non-lipid abnormalities have emerged as pro-atherogenic risk factors,

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Table 1
LDL subclasses, according to the separation methods.

Methods	Principle	Detection	No[a] of subclass	Classification [subclass or pattern type]		Definition			Reagents	Sample volume[b]	Separation time	Cost
I. Ultracentrifugation												
A. Analytical UC Refs [9,15–19]	Flotation rate (S_f)	Cholesterol	3	IDL	–	S_f 12–20 (d 1.008–1.022) [9,17]			High-salt solution	>10 ml	−78 h	Low
				LDL I	(Large, buoyant)	S_f 7–12 (d 1.019–1.023) [9,17]						
				LDL II–IV	(Small, dense)	S_f 0–7 (d 1.023–1.060) [9,17]						
B. DGUC Refs [9,10,12,13,18–24]	Density	Cholesterol	3–4 (7)	LDL I	(Large, buoyant)	1.019–1.023 [9,22]	1.025–1.034 [21,23]	g/ml	High-salt density gradient solution	3–5 ml	24–40 h	Low
				LDL II	(Intermediate)	1.023–1.034 [9,22]	1.034–1.044 [21,23]					
				LDL III	(Small, dense)	1.034–1.044 [9,22]	1.044–1.060 [21,23]					
				LDL IV	(Very small)	1.044–1.060 [9,22]						
						(Predominant subclass)						
			2 (3)	Pattern A	(Large)	LDL I and II [22,24]						
				Pattern I	(Intermediate)	LDL II and III (40–50%) [22,24]						
				Pattern B	(Small)	LDL III (>50%) [22,24]						
C. IDGU Refs [24,25]	Density	Cholesterol or protein	3–4 (6,7)	LDL I and II		1.022–1.028 [24]	1.016–1.028 [25]	g/ml	Iodixanol density gradient solution	3–6 ml	3–4 h	Low
				LDL III		1.028–1.041 [24]	1.028–1.043 [25]					
II. Electrophoresis												
A. GGE Refs [11,14,19,21,24,26–28]	Size (electro phoretic mobility)	Protein or lipid	4 (7)	LDL I	(Large)	26.35–28.5 [14]	26.0–28.5 [19,28]	nm	2–16% gradient PAG	3–10 μl	24–30 h	Low
				LDL II	(Intermediate)	25.75–26.34 [14]	25.5–26.4 [19,28]					
				LDL IIIA	(Small)	22.0–25.74 [14]	24.7–25.5 [19,28]					
				IIIB		–	24.2–24.6 [19,28]					
				LDL IVA	(Very small)	–	23.3–24.1 [19,28]					
				IVB		–	22.0–23.2 [19,28]					
						(Peak diameter)						
			2 (3)	Pattern type A	(Large, buoyant)	≥25.8 [11]	>25.5 [26,27]	nm				

				Pattern type AB	–	–	–					
				Pattern type B	(Small dense)	<25.8 [11]	≤25.5 [26,27]					
B. TGE	Size	Protein or	4 (7)	–		–			3% non-gradient PAG	25 μl	3–4 h	Mode
Refs [29,30]		lipid				(LDL subfraction score)						rate
			2 (3)	Phenotype A	–	<5.5 [29,30]						
				Phenotype intermediate	–	5.5–8.5 [29,30]						
				Phenotype B	–	>8.5 [29,30]						
III. NMR												
Refs [29,31,32]	Size	Cholesterol	3–4 (3–5)	L3	(Large, buoyant)	21.3–22.7 [31,32]		nm	–	0.5–1 ml	<5 min	High
				L2	(Intermediate)	19.8–21.2 [31,32]						
				L1	(Small)	18.3–19.7 [31,32]						
						(Peak size)	(Average size)					
			2 (3)	Phenotype A	–	20.6–22.0 [29]	>20.5 [32]	nm	–			
				Phenotype AB	–	20.4–20.5 [29]						
				Phenotype B	–	19.0–20.3 [29]	≤20.5 [32]					
IV. HPLC												
Ref [33]	Size	Cholesterol	4	–		–			Tris buffer containing salt	5 μl	<5–20 min	High
V. Homogenous assay												
A. Precipitation (+)	Reactivity with surfactants	Cholesterol	1	–		–			Polyanion + divalent cation, surfactants	0.1–0.2 ml	1–2 h	Mode rate
B. Precipitation (−) (direct assay)	and enzyme								Surfactants with sphingomyelinase	50 μl	0.5–1 h	Mode rate
Refs [34–38]												
VI. Ion mobility analysis												
Refs [39,40]	Size (charge)	Ion mobility	4	–		–			Volatile solution	5 μl	2–3 h	High
VII. Dynamic light scattering (DLS)												
Refs [41,42]	Brownian motion (size)	Scattering light	2	–		–			Latex particle solution	1 ml	Short	Mode rate

UC, ultracentrifugation; S_f, Svedberg flotation rate; DGUC, density gradient UC; IDGU, iodixanol density gradient UC; GGE, gradient gel electrophoresis; PAG, polyacrylamide gel; TGE, tube gel electrophoresis; NMR, nuclear magnetic resonance; HPLC, high-performance liquid chromatograph; IDL, intermediate-density lipoprotein; LDL, low-density lipoprotein.

[a] Main and (total) numbers of classified subclasses or pattern types.

[b] Required sample volume as serum or plasma.

including chronic inflammation, insulin resistance, endothelial dysfunction, and adipocytokine abnormalities [8]. However, accumulating evidence strongly suggests that in addition to the amount of lipoproteins, their specific nature is associated with the development and progression of coronary atherosclerosis [9–12].

Low-density lipoprotein (LDL) is defined as the ultracentrifugally isolated fraction with a density between 1.006 and 1.063 g/ml, which includes intermediate-density lipoprotein (IDL). LDL is considered as the most atherogenic type of lipoprotein. Cholesterol accounts for nearly half of all LDL by weight, representing the greatest proportion of all lipoproteins. LDL can be separated into multiple distinct subclasses that differ in size, charge, density, and physicochemical composition [9–14]. Interestingly, atherogenicity differs among LDL subclasses. LDL subclasses are generated during the delipidation process from very-low-density lipoprotein (VLDL) to IDL and LDL particles [9,10,13]. In addition to the increase in large buoyant LDL, the predominance of small dense LDL (sd-LDL) has been accepted as a risk factor for cardiovascular events by the National Cholesterol Education Program Adult Treatment Panel III (NCEP III) [1]. Some studies have shown that the modulation of LDL particle size by hypolipidemic agents reduces CVD risk; however, some studies have failed to confirm their effectiveness [10,11]. These discrepancies may be explained by differences in patient characteristics, lipid and non-lipid risk factors for coronary atherosclerosis, and techniques used for separating LDL subclasses [14]. Therefore, it is necessary to pay greater attention to such potential confounding factors when evaluating the effects of lipid-lowering therapies on the size and distribution of sd-LDL particles and/or sd-LDL-cholesterol (sd-LDL-C) levels in previous studies.

In this review, we first summarize the classification and definition of LDL subclasses in earlier studies from the viewpoint of methodological techniques. We then describe the advantages and potential problems of individual methods. Taking these issues into consideration, we further discuss the effectiveness of therapeutic modulation of LDL particles by lipid-lowering agents, particularly statins.

2. LDL subclasses: classification and methods for separating LDL subclasses

2.1. Classification of LDL subclasses

LDL heterogeneity was first identified in the late 1940s and 1950s [15,16]. Since then, conventional procedures such as analytical ultracentrifugation (UC) or gradient gel electrophoresis (GGE) have been used to separate LDL subclasses. Currently, LDL particles are classified into large buoyant and smaller particles based on density, size, charge, and composition of lipid and apolipoprotein (physicochemical composition) [9–14]. However, these LDL subclass classifications are not consistent among studies, which may cause serious confusion (Table 1).

LDL particles are separated into three or four major LDL subclasses by sequential ultracentrifugation based on density. These subclasses are termed LDL I to LDL IV [9,10,12–25]. When LDL particles are classified into three subclasses (LDL I–III), LDL I (large buoyant LDL), LDL II (intermediate density LDL), and LDL III (smaller dense LDL) have densities of 1.025–1.034 g/ml, 1.034–1.044 g/ml, and 1.044–1.060 g/ml, respectively [21,23]. In some studies, the very small LDL particles were also separated, and designated as LDL IV. It should be noted, however, that definitions of the densities for individual LDL subclasses sometimes differ slightly among studies, even in those adopting the "4-classification system" (Table 1). For example, the density of small LDL particles separated in an iodixanol gradient is lower than that in a traditional salt gradient [21–25] (Table 1-I). Because the iodixanol gradient is iso-osmotic, protein molecules maintain their native hydration, in contrast to their loss of water in hyper-osmotic salt gradient [24,25]. Although respective LDL subclasses may not precisely match in earlier studies, LDL III and LDL IV are generally considered as sd-LDL.

According to the distribution of LDL subclasses, the predominance of LDL I and II was termed pattern type A, while that of LDL III (>50%) was termed pattern type B [22,24] (Table 1-I-B). The prevalence rates of pattern type B are approximately 30% in adult men, 5–10% in young men and women under 20 years, and approximately 15–25% in postmenopausal women [9–11]. Furthermore, subjects with pattern type B have an elevated risk of myocardial infarction (MI) and often exhibit atherogenic lipoprotein profiles, such as high triglycerides and low high-density lipoprotein cholesterol (HDL-C) levels [26].

Based on the electrophoretic mobilities, seven distinct LDL subclasses have been identified by GGE under non-denaturing conditions. These mobilities are determined by the size and shape of lipoproteins. Four major subclasses are designated LDL I through LDL IV, and both LDL III and LDL IV are further divided into two subtypes [11,14,19,21,24,26–28] (Table 1-II-A). As in the case of UC, pattern types A and B have been used to determine two distinct electrophoresis-based phenotypes with peak LDL particle diameters > 25.5 nm and ≤ 25.5 nm, respectively [26,27] (Table 1-II-A). The particle size correlates well with particle density, although they are not completely identical. Some investigators adopted a simpler method to separate LDL subclasses using tube gel electrophoresis (TGE), and calculated LDL subfraction scores as a marker of LDL particle size [29,30] (Table 1-II-B). As this system uses 3% pre-cast polyacrylamide gels, we cannot completely eliminate the effects of LDL particle charge. Particle size can also be measured by nuclear magnetic resonance (NMR) [29,31,32] (Table 1-III).

2.2. Methods for separating LDL subclasses

There are different methodological techniques for measuring the size, number, distribution, and cholesterol content of LDL particles in clinical laboratories. In addition to conventional UC [15–25] and GGE [14,19,21,26–28] methods, previous studies have used TGE [29,30], NMR [31,32], high-performance liquid chromatography (HPLC) with gel filtration columns [33], homogenous assays [34–38], ion mobility analysis (IM) [39,40], and dynamic light scattering (DLS) [41,42] to evaluate LDL subclasses. A homogenous assay for sd-LDL-C was first described by Hirano et al. [34], and modified into a simpler and more convenient method. In the original method, large buoyant LDL and VLDL were precipitated with heparin and magnesium, and removed by centrifugation. Cholesterol concentration was then measured as sd-LDL-C using the reagents for the LDL-C homogeneous assay. The modified homogenous assay (direct assay) separates sd-LDL from large buoyant LDL with detergent and sphingomyelinase, and does not require any pretreatment (precipitation procedure) [37,38]. Thus, it is applicable to large-scale intervention studies, which may confirm the benefits of sd-LDL-C reduction in terms of CAD risk reduction. IM is a gas-phase differential electrophoretic macromolecular mobility-based method that was first reported by Caulfield et al. [39]. This novel technique, which employs the principles of aerosol science, has already been used in a large-scale cohort study [40]. DLS is another unique method to measure sd-LDL, and was developed in the late 1970s [41]. LDL particle size is determined by the intensity of scattered light fluctuations using two types of latex particle solutions as standards. This method requires highly specialized techniques and expensive instruments. In addition, the influence of noise and artifacts from large scattered effects prevent its widespread adoption [41]. Recently, Sakurai et al. modified this technique using relatively inexpensive instruments, which leaves some room for its application in point-of-care testing for sd-LDL [42]. Table 1 presents a summary of the principles, detection procedures, and other features of these methods with respect to their advantages and disadvantages.

GGE and TGE are semi-quantitative methods that detect the predominance of smaller particles among LDL subclasses. Therefore, it is not easy to compare the results of studies using different techniques due to substantial heterogeneity in separated LDL subclasses. In fact, the rate of complete matching of LDL subclass phenotyping was only 8% in 40 subjects classified by GGE, NMR, DGUC, and TGE [14]. Witte

et al. reported that the agreement rate of pattern B between GGE and NMR was less than 50% in 152 type 1 diabetes patients and 172 controls [32]. Lower rates of agreement were found in diabetic patients, women, and subjects with triglyceride levels less than 1.30 mmol/L. Care should also be taken regarding the definitions of LDL subclasses by NMR, which are different from those determined based on other methods (Table 1-III). Therefore, the standardization of LDL subclasses among different methods is not satisfactory. In addition, study subject characteristics seem to affect baseline sd-LDL-C concentrations and their responses to lipid-lowering agents [10,11] (Tables 2, 3).

3. Metabolic origins of LDL subclasses

There is convincing evidence that precursor lipoproteins of sd-LDL are secreted from the liver and generated in the circulation during lipolytic modifications [9,10,13]. Berneis et al. proposed two distinct pathways dependent on hepatic triglyceride (TG) availability [9,10]. The liver secretes two types of precursors: TG-rich and TG-poor apolipoprotein B (apoB)-containing lipoproteins (Lp) (TG-rich Lp and TG-poor Lp) (Fig. 1). Under conditions of low TG availability (pathway 1), the liver secretes VLDL 1 as TG-rich Lp and IDL 2 as TG-poor Lp. Under conditions of high TG availability (pathway 2), the liver secretes greater amounts of larger particles, i.e., larger VLDL 1 as TG-rich Lp and VLDL 2 as TG-poor Lp. In either pathway, TG-rich Lp is delipidated mainly by lipoprotein lipase (LPL) and partly by hepatic lipase (HL), and converted to small dense LDL subclasses (LDL III from VLDL 1 and LDL IV from larger VLDL 1). If TG is transferred to LDL III and LDL VI by cholesteryl ester transfer protein (CETP), these particles will be further delipidated by HL, resulting in the generation of much smaller particles [9,10]. On the other hand, TG-poor Lp (IDL 2 and VLDL 2) is a precursor of larger LDL subclasses (LDL I and LDL II).

This hypothetical model was supported by the results of a kinetic in vivo rat experiment [43] and interventional human study [9,10,44] reported by the same group (Krauss et al.). In 501 healthy non-smoking subjects, LDL subclasses were determined at baseline and 4–6 weeks after they were fed high or low fat diets [9]. The results indicated that LDL I was inversely correlated with LDL III, while LDL II was inversely correlated with LDL IV. Similar relationships between respective LDL subclasses were reported in 81 healthy men with a sedentary lifestyle who continued to exercise for one year [44]. These results strongly suggest that sd-LDL has a distinct precursor-product pathway from the liver as proposed above.

Recent genome-wide association studies (GWASs) suggest that genetic factors are also involved in the production of sd-LDL. Musunuru et al. reported that a causative single nucleotide polymorphism (SNP) at the 1p13 locus (minor haplotype), with common variants in various races showing occurrence rates of about 30%, alters hepatic sortilin synthesis by modulating a transcription factor binding site of the sortilin promoter region [45]. Sortilin is a sorting receptor with multiple ligands, including apoB, and is involved in hepatic VLDL secretion and LDL uptake [46]. Hepatic modulation of sortilin results in alteration of hepatic VLDL secretion, and hence 20% increases in very small LDL were seen in major allele homozygotes compared to minor allele homozygotes [45]. In mouse models, overexpression of sortilin results in an 88% reduction in medium small LDL particles and a 73% reduction in very small LDL particles. As a result, LDL peak particle size increases from 20.9 nm to 22.0 nm ($P=0.05$) [45]. Thus, modulation of sortilin expression may be a new therapeutic target for patients with high sd-LDL concentrations. Other SNPs in CETP, LIPC, GALNT2, MLXIPL, APOA1/A5, and LPL loci were also associated with increased small and medium LDL levels [40]. Therefore, these genetic factors may be involved in the alteration of LDL subclasses.

Table 2
sd-LDL and various disease groups.

Diseases	No of		Subgroup	LDL-C (mmol/l or mg/dl)		sd-LDL-C (μmol/l or mg/dl)		Mean LDL size or proportion		Methods	Ref
	Cont	Pt		Cont	Pt	Cont	Pt (% change)	Cont	Pt (% change)		
								[Mean LDL size (nm)]			
Hepatic steatosis	22	23	Moderate steatosis	3.25±0.16	3.38±0.17	0.65±0.08	0.81±0.11 (+25)†	21.23±0.18	20.95±0.26	NMR	[58]
With type 2 DM[a]		22	Severe steatosis		3.25±0.16		1.12±0.12 (+72)‡		20.35±0.20†		
With obese NGT[a]	–	37	HFF <5.5%	–	2.24±0.16	–	0.57±0.04	–	–	NMR	[59]
		12	>5.5%		2.00±0.24		0.78±0.06 (+37)‡		–		
Obesity	33	34	GH sufficient (GHS)	101±5	116±4 †	0.38±0.04	0.72±0.08 (+89)#	21.6±0.1	21.0±0.1 (−2.9)#	NMR	[60]
With GH deficient[a]		35	GH deficient (GHD)		(GHS+GHD)		0.89±0.07 (+134)#		20.6±0.1 (−4.9)#		
OSAS[b]	–	30	MetS (−)	–	3.35±0.73†	–	–	–	27.2±1.1	GGE	[65]
		28	MetS (+)		2.95±0.76		–		26.5±0.9 (−2.6)‡		
HIV infection	–	392	CAD (−)	–	77.5	–	5.4	–	–	Sequential UC	[67]
		98	CAD (+)		87		8.2 (+52)		–		
	14	10	Uninterrupted therapy	3.18±0.13	2.84±0.23	–	–	25.93±0.09	25.25±0.16 (−2.7)§	GGE	[68]
		14	Interrupted therapy		2.43±0.29†		–		25.09±0.10 (−3.3)§		
								[LDL III proportion (%)]			
PCOS[c]	14	52	–	3.0	3.2	25	38 (+52)†	8.2[c]	12.8[c,‡]	DGUC	[61]
								[Pattern B proportion (%)]			
[b,c]	70	70	–	2.48±0.68[b]	2.50±0.70[b]	–	–	2.9 [c]	12.9[c,†]	TGE	[63]
Renal transplant recipients[b]	32	21	–	2.85±0.81[b]	2.87±0.89[b]	0.39[d]	0.59[d,§]	–	–	Homogeneous	[72]

Cont, controls; Pt, patients; LDL-C, low-density lipoprotein cholesterol; sd-LDL-C, small dense LDL cholesterol; DM, diabetes mellitus; NGT, normal glucose tolerance; HFF, hepatic fat fraction; GH, growth hormone; GHS, GH sufficient; GHD, GH deficient; OSAS, obstructive sleep apnea syndrome; MetS, metabolic syndrome; HIV, human immunodeficiency virus; CAD, coronary artery disease; PCOS, polycystic ovary syndrome.
The abbreviations for the methods are defined in Table 1. The data are expressed as the mean±SE.
[a] sd-LDL-C concentrations are expressed as particle concentrations (μmol/l).
[b] Data are expressed as the mean±SD.
[c] sd-LDL data are shown as proportion (%).
[d] Raw data not shown. Data were estimated from figures in the reference and expressed as the median (mmol/l).
† $P<0.05$, vs. control or other subgroups.
‡ $P<0.01$, vs. control or other subgroups.
§ $P<0.001$, vs. control or other subgroups.
# $P<0.0001$ vs. control or other subgroups.

Table 3
Effects of lipid-lowering agents on the LDL-C and sd-LDL-C levels.

Subjects	Medication		Duration (w)	No of Pt	LDL-C (mg/dl)			sd-LDL-C (mg/dl)			Difference in efficacy[a]	Methods	Ref
(Country)	Dose	(mg)			Before	After	(% change)	Before	After	(% change)			
Dyslipidemia													
Hyper TG[b,c] (USA)	Ator	10	8	40	181±8	117±4	(−34)[#]	Not shown	Not shown	(−26)[c,#]			
	Ator	20		56	174±6	101±4	(−41)[#]			(−31)[c,#]			
	Ator	40		47	173±7	88±4	(−49)[#]			(−41)[c,#]	Yes[§]	DGUC, GGE	[73]
	Ator	80		48	179±6	86±5	(−52)[#]			(−41)[c,#]			
HC (USA)	Ator	80	6	136	198±27	98[d]	(−50)	63±25	31[d]	(−46)	Yes[‡]	Homogenous	[74]
	Rosu	40		135	204±27	96[d]	(−52)	71±30	28[d]	(−53)			
FH hetero (Japan)	Pita	2	12	8	201±27	111±11	(−45)[#]	43±24	16±10	(−63)[‡]	No	Homogenous	[75]
	Ator	10		9	234±57	142±52	(−40)[‡]	44±17	19±10	(−55)[§]			
HL+DM (Japan)	Pita	1	12	total	171±40	125±34	(−27)[#]	37±20	25±15	(−26)[‡]	N/A	Homogenous	[76]
	Feno	100		72	135±36	132±35	(−2)	45±21	33±19	(−23)[‡]			
HC+MetS (Turkey)	Rosu	10	8	17	179±51	96±35	(−46)[#]	29.6±24.8	8.9±8.5	(−52)[§]			
	Ator	20		12	161±26	87±22	(−46)[#]	26.2±15.0	14.8±9.6	(−45)[†]	No	TGE	[77]
	Others			23	174±36	92±25	(−46)[#]	29.1±16.5	14.7±11.2	(−44)[#]			
CVD													
ACS+MetS (Japan)	Ator	20	24	35	102±23	70±28	(−31)[#]	29±15	22±13	(−24)[§]			
		(MetS −)		(20)	(98±24)	(71±30)	(−28)[§]	(24±10)	(22±13)	(−8)			
		(MetS +)		(15)	(107±22)	(70±25)	(−35)[#]	(35±18)	(22±13)	(−37)[§]	Yes[§]	Homogenous	[38]
	Diet only			36	104±28	104±25	(−0)	32±14	35±15	(+9)			
CVD and/or CVD risk[e] (Germany)	Sim 20→40		12	30	5.34±0.92	4.30±0.96	(−19)[‡]	0.34±0.21	0.31±0.12	(−9)			
	Pio 30→45			28	5.42±0.90	5.38±0.97	(−1)	0.38±0.22	0.31±0.15	(−18)[‡]	No	Sequential UC	[78]
	Combination			30	5.52±0.94	4.28±0.78	(−22)[‡]	0.38±0.15	0.29±0.11	(−24)[‡]			
CHD or CHD risk[b] (Japan)	Rosu, 2.5		12	81	132±4	105±3	(−18)[f,†]	61±2	46±2	(−26)[f,†]	N/A	Sequential UC	[79]
CAD (Lebanon)	Ator	40	8	50	99±21	86±14	(−13)[§]	5±7	2±4	(−60)[§]	No	TGE	[80]
	Ator 40+Eze 10				102±29	77±10	(−25)[§]	4±4	2±2	(−50)[§]			
Type 2 DM (Germany)	Sim	20	6	14	106±19	72±17	(−32)[§]	34.6±14.3	25.0±9.3	(−28)[‡]			
	Eze	10		12	123±36	105±33	(−15)[‡]	46.2±18.2	32.7±12.0	(−23)[†]	No	DGUC	[81]
	Combination			14	110±37	62±17	(−44)[§]	36.9±11.4	23.1±5.6	(−37)[‡]			

Hyper TG, hypertriglyceridemia; HC, hypercholesterolemia; FH hetero, familial hypercholesterolemia heterozygote; HL, hyperlipidemia; CVD, cardiovascular disease; ACS, acute coronary syndrome; CHD, coronary heart disease; Ator, atorvastatin; Rosu, rosuvastatin; Pita, pitavastatin; Feno, fenofibrate; Sim, simvastatin; Pio, pioglitazone; Eze, ezetimibe.; N/A, not analyzed. The other abbreviations are defined in Tables 1 and 2. Values are expressed as the mean±SD.

[a] Treatment difference on the sd-LDL-C levels was expressed as efficacy.
[b] Values are expressed as the mean±SE.
[c] Data were analyzed using the cholesterol concentrations of LDL IIIA and IIIB instead of sd-LDL-C.
[d] Raw data not shown. Data were estimated from tables in the reference.
[e] Data are described as the cholesterol concentrations (mmol/L) in plasma and LDL III (d 1.040–1.066 g/ml) instead of LDL-C and sd-LDL-C.
[f] % changes are expressed as the median.
[†] $P<0.05$, in mean % changes.
[‡] $P<0.01$, in mean % changes.
[§] $P<0.001$, in mean % changes.
[#] $P<0.0001$ in mean % changes.

4. Atherogenic properties of sd-LDL particles

Clinical studies strongly suggest that a predominance of sd-LDL is associated with CAD risk [9–12,22,26]. Austin et al. determined LDL subclasses using GGE in 109 patients with CAD and 121 controls. They found that subjects with pattern type B had a three-fold higher MI risk compared to those with pattern type A, and this relationship was independent of age, sex, and relative weight [26].

The atherogenic properties of sd-LDL particles can be explained by several metabolic features that have been revealed by experimental studies [9–12]. First, sd-LDL particles penetrate easily into the arterial wall because of their small size. Second, sd-LDL has a high affinity for proteoglycans in the arterial wall, which results in a prolonged residence time in the subendothelial space [47]. This greater affinity of sd-LDL to proteoglycans may be related to the sialic acid content of LDL particles. Sialic acid content was decreased in LDL particles in subjects with LDL pattern type B [11]. This observation is reasonable because the negative charge of sialic acid on the LDL surface is helpful for preventing the association of LDL particles with proteoglycans. Third, sd-LDL has a lower affinity for LDL receptors than larger LDL particles and is not easily cleared from plasma [11,12,48]. Lastly, sd-LDL particles lack vitamin E and are highly susceptible to oxidization [49,50]. These features all contribute to increased atherogenicity of small LDL subclasses.

Interestingly, recent findings suggest that apoB in sd-LDL is preferentially glycated compared to that in larger particles both in vitro and in vivo [51,52]. The percentage of glycated apoB was ten-fold higher in sd-LDL (LDL III: 1.044–1.063 g/ml) than in more buoyant LDL (LDL I and II: 1.019–1.044 g/ml) in 44 non-diabetic subjects (17.4% vs. 1.8%, $P<0.001$) [51]. Matsui et al. reported that serum glycated apoB levels were inversely correlated with LDL particle size as determined by NMR in subjects without medication [53]. Younis et al. reported that glycated apoB concentrations were higher in sd-LDL as compared with more buoyant LDL in metabolic syndrome (MetS) and type 2 diabetes mellitus (DM). In addition, glycated apoB was positively correlated with sd-LDL apoB in type 2 DM [54]. Both glycation and oxidation of LDL appear to increase the atherogenicity of LDL particles. During the oxidation of LDL, oxidation-specific epitopes (OSE) are generated on the LDL particles. Oxidized lipids mediate a variety of immune responses and are recognized by several receptors, such as CD36, toll-like receptor-4 [55]. Consequently, OSE are thought to be involved in the pathogenesis of inflammation and atherosclerosis. In fact, oxidized phospholipids on apoB-100 particles predict the presence and progression of CVD events independently of established risk factors [56]. Some other possible mechanisms have been proposed for the atherogenic potency of sd-LDL. For example, sd-LDL may induce the stimulation of plasminogen-activator-inhibitor 1 and accelerate thromboxane A2 synthesis [11,57].

5. sd-LDL in metabolic and other pathological diseases

Many studies have shown that sd-LDL levels are elevated in atherosclerotic disorders, such as dyslipidemia, diabetes, MetS, and cardiovascular disease [1,9–12,22,26,35,36,38]. In addition, there have been reports describing the associations of sd-LDL with a wide variety of metabolic disorders, including liver diseases [58,59], endocrine diseases, such as growth hormone (GH) deficiency [60] and polycystic ovary syndrome (PCOS) [61–64], obstructive sleep apnea syndrome (OSAS) [65,66], human immunodeficiency virus (HIV) infection [67–69], HT [70], and chronic kidney disease (CKD) (renal transplant recipients with immunosuppressive therapy) [71,72] (Table 2).

Non-alcoholic fatty liver disease (NAFLD) is a typical clinical feature of obese patients, especially MetS and type 2 DM. As the prevalence of these diseases is increasing, that of NAFLD has also been increasing in not only industrialized, but also developing countries. The most prominent cause of death is CAD, and elevated sd-LDL may partly account for poor clinical outcomes in these patients. For example, Toledo et al. reported that type 2 diabetic patients with moderate and severe hepatic steatosis had 25% and 72% higher sd-LDL concentrations than those without hepatic steatosis, respectively (0.81 ± 0.11 and 1.12 ± 0.12 vs. 0.65 ± 0.08 μmol/L, $P<0.05$, $P<0.01$) [58] (Table 2). Cali et al. reported that obese subjects with normal glucose tolerance and a higher hepatic fat fraction (HFF) (>5.5%) have a 37% higher sd-LDL concentration than those with lower HFF (<5.5%) (781.4 ± 63.0 vs. 570.2 ± 41.0 nmol/L, $P=0.007$) [59] (Table 2).

Several recent studies have indicated elevated sd-LDL levels in endocrine diseases. GH deficiency and the presence of PCOS are accepted as metabolic risk factors for CAD because they exhibit metabolic abnormalities, such as insulin resistance, hypertension, and dyslipidemia. Makimura et al. reported that sd-LDL-C concentrations were increased by 89% and 134% and mean LDL particle sizes were 2.9% and 4.9% smaller in obese patients with sufficient or deficient secretion of GH as compared with

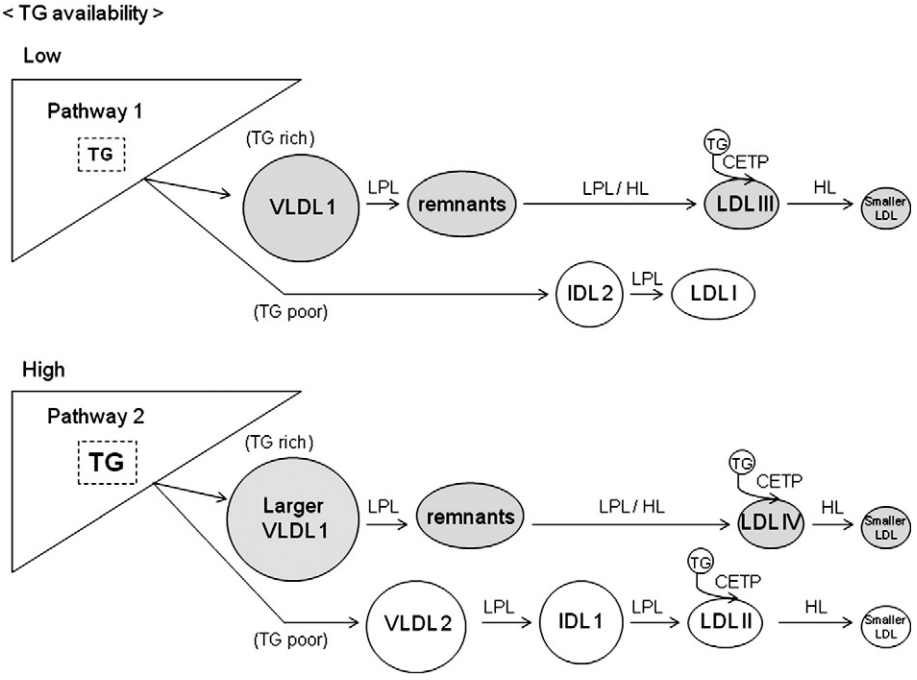

Fig. 1. Hypothetical metabolic scheme of proposed pathways dependent on TG availability for the production of LDL subclasses. Distinct pathways for the production of LDL subclasses are shown with slight modification from Ref [9]. Two pathways depend on TG availability. TG-rich lipoproteins are delipidated mainly by LPL and partly by HL, and converted to small dense LDL subclasses (from VLDL 1 to LDL III, and from larger VLDL 1 to LDL IV). LDL, low-density lipoprotein; TG, triglyceride; LP, lipoprotein lipase; HL, hepatic lipase; VLDL, very low-density lipoprotein.

normal weight subjects, respectively (0.72 ± 0.08, 0.89 ± 0.07 vs. 0.38 ± 0.04 μmol/L, $P<0.0001$, $P<0.0001$; 21.0 ± 0.1, 20.6 ± 0.1 vs. 21.6 ± 0.1 nm, $P<0.0001$, $P<0.0001$) [60]. Pirwany et al. reported that patients with PCOS had higher concentrations and proportions of sd-LDL (LDL III) measured by DGUC than controls (38.0 vs. 25.0 mg/dl, $P=0.026$; 12.8 vs. 8.2%, $P=0.006$) [61]. Vinaixa et al. reported that the radii of sd-LDL in PCOS were increased after 30-month combination therapy with low-dose flutamide, metformin, pioglitazone, and estroprogestagen (ethinylestradiol plus drospirenone) [64].

Furthermore, OSAS, HIV infection, HT, and CKD with renal transplantation also showed increased sd-LDL-C concentrations or decreased mean LDL particle sizes [65–72] (Table 2). These pathological diseases appear to be independent risk factors for CAD.

6. Measurement of sd-LDL-C: clinical significance as a marker for metabolic disorders with CAD risk

Among the techniques currently available, the recently developed homogenous assay is a simple and convenient method for evaluating sd-LDL-C concentrations. sd-LDL-C concentrations have been measured directly or semi-quantitatively in dyslipidemia [73–77], patients with CVD or CVD risk [38,78–80], and diabetes [35,36,76,81] (Table 3). In these reports, sd-LDL-C levels were closely associated with CAD risk. Ai et al. quantified sd-LDL-C in 1680 female participants of the Framingham Offspring Study with or without coronary heart disease (CHD). Mean sd-LDL-C levels were higher in those with CHD compared to those without CHD (0.83 vs. 0.68 mmol/L, $P=0.0015$), although they had the similar mean LDL-C levels (3.53 vs. 3.46 mmol/L, $P=0.543$) [82]. Thus, sd-LDL-C measurements seem to provide additional important information for the management of high-risk patients with CAD whose total LDL-C levels are not elevated. Unlike women, 1508 men with or without CHD in the same study had similar levels of mean sd-LDL-C (0.83 vs. 0.84 mmol/L, $P=0.609$). However, the percentage of sd-LDL-C to total LDL-C was higher in those with than in those without CHD (26.1% vs. 23.7%, $P=0.0019$) [82]. Since men generally had more confounding factors than women, the ratio of sd-LDL-C to total LDL-C may be more informative than the absolute concentration of sd-LDL-C.

Although total LDL-C remains relatively stable during the day, more attention should be paid to the sampling time for sd-LDL-C measurement to avoid misleading data due to pre-analytical errors. In our previous study, sd-LDL-C levels exhibited circadian changes in both diabetic patients and controls. In both groups, sd-LDL-C concentrations were highest early in the morning, and continued to decrease during the day. The mean maximum percent reduction was 35.9% in type 2 DM and 37.6% in controls [36]. In patients with acute coronary syndrome (ACS), sampling time varied from earlier morning to midnight [38]. As heparin injection markedly alters lipoprotein profiles [83] and a bolus of high-dose heparin is given to ACS patients at coronary intervention, fasting samples obtained on the day after admission are not likely to reflect baseline sd-LDL-C concentrations. Furthermore, it should be noted that there are no standardized reference values for sd-LDL-C levels. In fact, the value of sd-LDL-C seems to be slightly different in each study described in Section 7 (Table 3).

Table 4
Proposed recommendations for measuring the LDL subclasses in a clinical setting.

LDL subclasses	Diseases
Small dense LDL (LDL III, LDL IV, L1) Or Small dense LDL-cholesterol Or Pattern B	Cardiovascular disease Refs [1,22,26,38,78-80,82] Familial combined hyperlipidemia Ref [27] Diabetes mellitus Refs [35,36,58,76,81] Metabolic syndrome Refs [38,65,77] Chronic kidney disease Refs [71,72] Hypertriglyceridemia Ref [73]

7. Effects of statins and other hypolipidemic agents on sd-LDL-C

Although there is considerable consensus regarding the atherogenicity of sd-LDL, some authors have argued over the efficacy of lipid-lowering therapy on sd-LDL. Statins are the most intensively investigated of all hypolipidemic agents. As described in the previous section, statins successfully modulated LDL particle size in some studies, but failed to do so in other studies [10–12]. A recent meta-analysis concluded that it is uncertain whether measuring LDL subclasses provides additional benefits for classical risk factor assessment [84]. The guidelines of the National Academy of Clinical Biochemistry list sd-LDL as an emerging risk factor for CVD. However, the guidelines do not recommend measuring sd-LDL for risk assessment in primary prevention, or evaluating the effects of therapeutic intervention [85]. The guidelines also mention the need for standardized measuring methods. Some statins failed to alter the distribution of LDL particles, because they markedly decreased large LDL fractions. Therefore, in these studies the ratio of sd-LDL-C to buoyant LDL-C was unchanged [38] or pattern B frequency was increased [86]. Niacin and fibrates may be preferable to statins because they reduce sd-LDL-C rather than large buoyant LDL-C [11,12].

It is necessary to be aware of the fact that statins often similarly reduce both sd-LDL-C and large buoyant LDL-C. The relative concentration of sd-LDL or size distribution of LDL particles may not be improved even if the absolute concentration of sd-LDL-C is markedly decreased. Therefore, the effects of statins on sd-LDL should be evaluated with their absolute concentrations, and not their relative concentrations or size distributions. Phenotyping of the LDL size distribution using GGE may be especially misleading in studies of strong statins. In this section, we mainly summarized studies that determined sd-LDL-C levels by homogeneous assay and sequential UC to avoid this possibility.

Table 3 shows the effects of lipid-lowering agents, including statins, on absolute concentrations and % change of LDL-C and sd-LDL-C in patients with different pathological conditions [38,73–81]. Most studies showed significant reductions in LDL-C and sd-LDL-C concentrations regardless of race, sex, and patient characteristics. Dose-dependent reductions in sd-LDL were also confident [73]. As statins show a wide range of TG-lowering effects, they may also have different effects on sd-LDL-C [10–12]. Some authors suggested that strong statins, such as atorvastatin and rosuvastatin, are more promising for the reduction of sd-LDL-C than mild statins, while others argued against this position. As described in Section 2, confounding factors including differential methods and clinical characteristics may be responsible for these differences in conclusions. Thus, future intervention studies with statins are necessary to confirm whether the increase in LDL size or decrease in sd-LDL-C significantly contributes to CAD risk reduction. Considering the results of recent clinical studies (Sections 6 and 7), we propose tentative guidelines for measuring the LDL subclasses in a clinical setting (Table 4).

8. Conclusions

Although sd-LDL-C is closely associated with coronary atherosclerosis, there have been few convincing studies showing a reduction in coronary artery disease risk by lowering sd-LDL-C. This question remains to be clarified in future clinical studies using reliable and reproducible methods for measuring sd-LDL-C. The standardized methods for sd-LDL-C and intervention studies using strong statins are expected to resolve these questions in the near future.

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