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Lack of an association or an inverse association between low-density-lipoprotein cholesterol and mortality in the elderly: a systematic review

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ABSTRACT

Objective: It is well known that total cholesterol becomes less of a risk factor or not at all for all-cause and cardiovascular (CV) mortality with increasing age, but as little is known as to whether low-density lipoprotein cholesterol (LDL-C), one component of total cholesterol, is associated with mortality in the elderly, we decided to investigate this issue.

Setting, participants and outcome measures: We sought PubMed for cohort studies, where LDL-C had been investigated as a risk factor for all-cause and/or CV mortality in individuals ≥60 years from the general population.

Results: We identified 19 cohort studies including 30 cohorts with a total of 68 094 elderly people, where all-cause mortality was recorded in 28 cohorts and CV mortality in 9 cohorts. Inverse association between all-cause mortality and LDL-C was seen in 16 cohorts (in 14 with statistical significance) representing 92% of the number of participants, where this association was recorded. In the rest, no association was found. In two cohorts, CV mortality was highest in the lowest LDL-C quartile and with statistical significance; in seven cohorts, no association was found.

Conclusions: High LDL-C is inversely associated with mortality in most people over 60 years. This finding is inconsistent with the cholesterol hypothesis (ie, that cholesterol, particularly LDL-C, is inherently atherogenic). Since elderly people with high LDL-C live as long or longer than those with low LDL-C, our analysis provides reason to question the validity of the cholesterol hypothesis. Moreover, our study provides the rationale for a re-evaluation of guidelines recommending pharmacological reduction of LDL-C in the elderly as a component of cardiovascular disease prevention strategies.

Strengths and limitations of this study

- This is the first systematic review of cohort studies where low-density lipoprotein cholesterol (LDL-C) has been analysed as a risk factor for all-cause and/or cardiovascular mortality in elderly people.
- Lack of an association or an inverse association between LDL-C and mortality was present in all studies.
- We may not have included studies where an evaluation of LDL-C as a risk factor for mortality was performed but where it was not mentioned in the title or in the abstract.
- We may have overlooked relevant studies because we have only searched PubMed.
- Minor errors may be present because some of the authors may not have adjusted LDL-C by appropriate risk factors.
- Some of the participants with high LDL-C may have started statin treatment during the observation period and, in this way, may have added a longer life to the group with high LDL-C and some of them may have started with a diet able to influence the risk of mortality.
- We may have overlooked a small number of relevant studies because we only searched papers in English.

INTRODUCTION

Rationale

For decades, the mainstream view has been that an elevated level of total cholesterol (TC) is a primary cause of atherosclerosis and cardiovascular disease (CVD). There are several contradictions to this view, however. No study of unselected people has found an association between TC and degree of atherosclerosis.[1] Moreover, in most of the Japanese epidemiological studies, high TC is not a risk factor for stroke, and further, there is an inverse association between TC and all-cause mortality, irrespective of age and sex.[2]

In a recent meta-analysis performed by the Prospective Studies Collaboration, there was

an association between TC and CV mortality in all ages and in both sexes.[3] However, even in this analysis, the risk decreased with increasing age and became minimal after the age of 80 years. Since atherosclerosis and CVD are mainly diseases of the elderly, the cholesterol hypothesis predicts that the association between CV mortality and TC should be at least as strong in the elderly as in young people. There may be a confounding influence in these studies, however, because TC includes high-density lipoprotein cholestrol (HDL-C), and multiple studies have shown that a high level of HDL-C is associated with a lower risk of CVD.

Objectives

We examined the literature assessing low-density lipoprotein cholesterol (LDL-C) as a risk factor for mortality in elderly people. Since the definition of CVD varies considerably in the scientific literature, we have chosen to focus on the association between LDL-C and all-cause and CVD mortality, because mortality has the least risk of bias among all outcome measures. If Goldstein and Brown's recent statement that LDL-C is 'the essential causative agent' of CVD[4] is correct, then we should find that LDL-C is a strong risk factor for mortality in elderly people.

METHODS

Search strategy

UR and RS searched PubMed independently from initial to 17 December 2015. The following keywords were used: 'lipoprotein AND (old OR elderly) AND mortality NOT animal NOT trial'. We also retrieved the references in the publications so as not to miss any relevant studies. The search was limited to studies in English.

Inclusion and exclusion criteria

All included studies should meet the following criteria: the study should be a cohort study of people aged 60 years or older selected randomly from the general population, or a study where the authors had found no significant differences between the participants and the source population's demographic characteristics. The studies should include an initial assessment of LDL-C levels, the length of the observation time and information about all-cause and/or cardiovascular mortality at the end of follow-up. The studies should also include information about the association between LDL-C and all-cause and/or CVD mortality. We excluded studies that did not represent the general population (eg, case–control studies; case reports; studies that included patients only); studies where data about elderly people were not given separately, and studies without multivariate correction for the association between LDL-C and all-cause and/or CV mortality. We accepted studies where the authors had excluded patients with serious diseases or individuals who had died during the first year.

Study selection, data items and extraction

Studies where the title or abstract indicated that they might include LDL-C data of elderly people, were read in full, and the relevant data were extracted by at least three of the authors, for example, year of publication, total number of participants, sex, length of observation time, exclusion criteria, LDL-C measured at the start and the association between initial LDL-C and risk of all-cause and/or CV mortality at follow-up. When more than one adjusted HR was reported, the HR with the most fully adjusted model was selected.

Quality assessment

The design of the study satisfies almost all points of reliability and validity according to the Newcastle Ottawa Scale as regards selection, comparability and exposure.[5] Thus, all studies represented elderly people only; ascertainness of exposure (eg, measurement of LDL-C) was present in all studies, and outcome was unknown at the start. It can be questioned if all of the studies represented the general population because, as shown below, in some of them various types of disease groups were excluded.

RESULTS

Study selection

Our search gave 2894 hits. We excluded 160 studies, which were not in English, and 2452 studies because, judged from the abstract, it was obvious that they were irrelevant.

The rest of the papers were read in full; 263 of these studies were excluded for the following reasons: (1) the participants did not represent the general population; (2) LDL-C was not measured at the start; (3) follow-up information was not given for the elderly separately; or (4) no information was present about mortality during the observation period (figure 1). One of the studies[6] was excluded because it included the same individuals as in a previous study.[7]

Study characteristics

The remaining 19 studies including 30 cohorts with a total of 68 094 participants met the inclusion criteria (figure 1). All-cause mortality was recorded in 28 cohorts. In 16 of these cohorts (representing 92% of the individuals), the association was inverse and with statistical significance in 14; in 1 of the cohorts, the association was mirror-J-formed with the lowest risk in the highest quartile; in the rest of the papers, no association was found. CV mortality was recorded in nine cohorts; in one of them, the association was almost U-shaped with the lowest risk in the highest quartile (curvilinear fit: p=0.001); in one of them, the association was mirror-J-formed and also with the lowest risk in the highest quartile (curvilinear fit: p=0.03); in the other seven cohorts, no association was found (table 1).

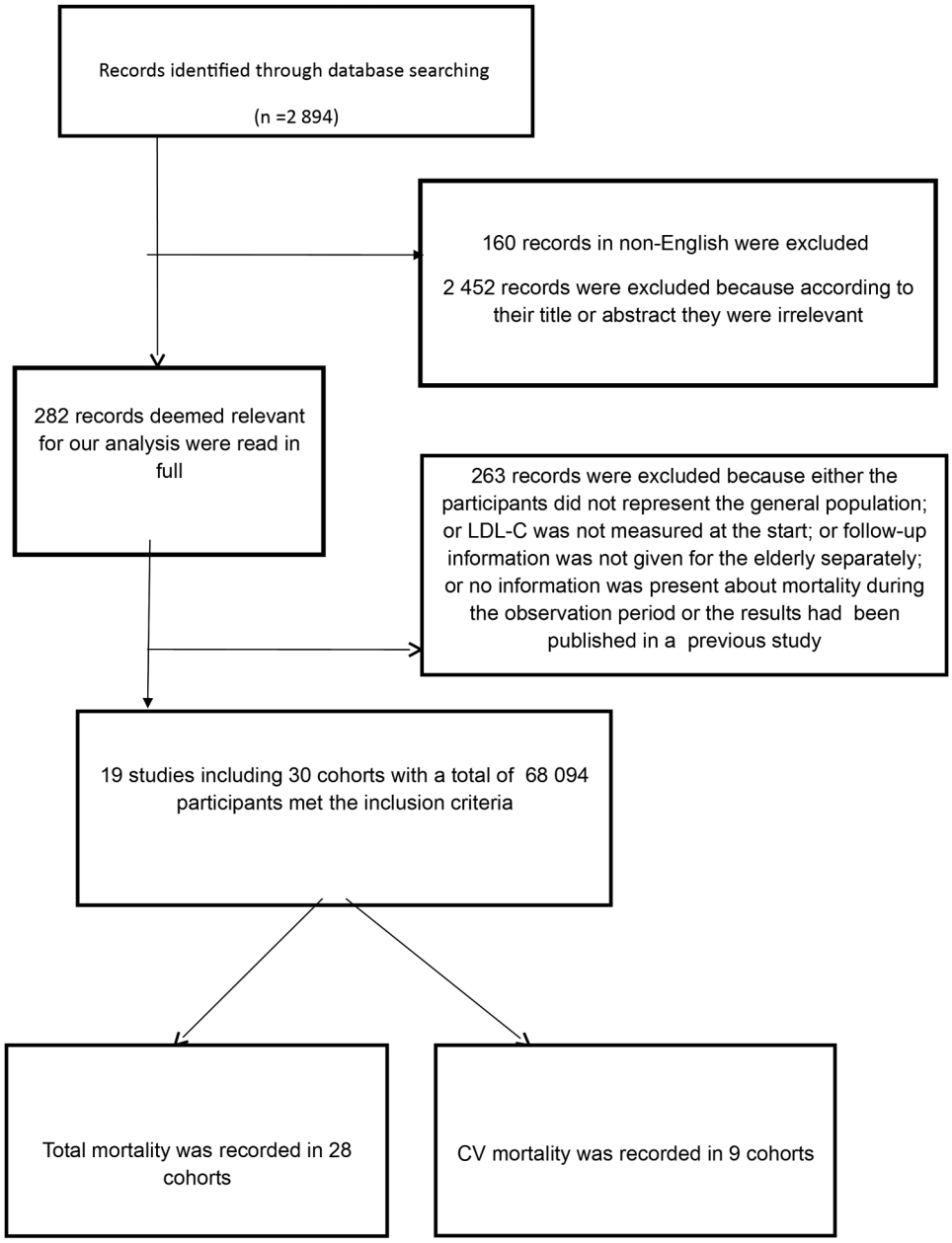

Figure 1 Flow Chart. CV, cardiovascular; LDL-C, low-density lipoprotein cholesterol.

Risk of bias across studies

One explanation for the increased risk of mortality among people with low cholesterol is that serious diseases may lower cholesterol soon before death occurs. Evidence to support this hypothesis may be obtained from 10 of the studies in which no exclusions were made for individuals with terminal illnesses. However, in four of the studies, participants with a terminal illness or who had died during the first observation year were excluded. In one of those studies,[8] LDL-C was not associated with all-cause mortality; in the three others,[16 20 24] which included more than 70% of the total number of participants in our review, LDL-C was inversely associated with all-cause mortality and with statistical significance. Thus, there is little support for the hypothesis that our analysis is biased by end of life changes in LDL-C levels.

It is also potentially relevant that all studies did not correct for the same risk factors, and some of them did not inform the reader about which risk factors they corrected for. However, taking all studies together, 50 different risk factors were corrected for in the Cox analyses (table 2).

It is worth considering that some of the participants with high LDL-C may have started statin treatment during the observation period. Such treatment may have increased the lifespan for the group with high LDL-C. However, any beneficial effects of statins on mortality would have been minimal because most statin trials have had little effect on CVD and all-cause mortality, with a maximum reduction of mortality of two percentage points. It is therefore relevant that the 4-year mortality among those with the highest LDL-C in the included cohorts was up to 36% lower than among those with the

Table 1 Association between LDL-C and all-cause mortality and CVD mortality, respectively, in 19 studies including 30 cohorts with 68 094 individuals from the general population above the age of 60 years

Authors	Race if indicated	Sex	N	Age; years	Obs. Years	All-cause mortality LDL-C tertiles or quartiles (HRs) I	II	III	IV	CV mortality	Exclusion criteria
Zimetbaum *et al*[8]		MF	350	75–85	6, 3	No association				NI	Terminal illness, dementia
Kronmal *et al*[9]		MF	747	66–75	10	No association				NI	None
			176	>75		No association				NI	
Räihä *et al*[10]		MF	347	≥65	11	NI				No association	Living in an institution
Fried *et al*[7]		MF	5201	≥65	4.8	1.0	?	?	0.66 (significant)	NI	Wheelchair user; cancer treatment
Chyou and Eaker[11]		M	367	≥65	8–10	No association				NI	None
		F	622			No association					
Weverling-Rijnsburger *et al*[12]		MF	599	≥85	4	1.0	0.57	0.71 p for trend*		No association	None
Schupf *et al*[13]		MF	2277	≥65	3	1.0	0.79	0.63	0.53 p for trend**	NI	Dementia
Tikhonoff *et al*[14]		M	1233	≥65	11, 1	Mirror-J-formed association with the highest risk in the lowest quartile				Almost U-formed association with the highest risk in the lowest quartile*	Dementia
		F	1887			Inverse association*				Mirror-J-formed association with the highest risk in the lowest quartile*	
Störk *et al*[15]		M	403	>70	4	No association				No association	None
Akerblom *et al*[16]											
	Caucasians	MF	705	≥65	3.5	1.0	0.89	0.72	0.56*	NI	Dementia, first year deaths
	African-Americans	MF	797			1.0	0.79	0.58	0.53*		
	Hispanics	MF	1054			1.0	0.79	0.79	0.71		
Upmeier *et al*[17]		MF	1032	70	12	No association				No association	None
Nilsson *et al*[18]		M	210	75	10	No association				NI	None
		F	222			No association					
Werle *et al*[19]		MF	187	≥80	8, 7	No association				No association	None
Bathum *et al*[20]†		M	13 733	60–70	1–9	1.0	0.67***	0.49***	0.45***	NI	Terminal disease, CVD, diabetes, patients with a prescription of statin during the last year before test date
			7493	≥70		1.0	0.71***	0.60***	0.52***		
		F	14 298	60–70		1.0	0.56***	0.45***	0.47***		
			9142	≥70		1.0	0.66***	0.52***	0.46***		
Linna *et al*[21]		MF	1260	≥64	10	Inverse association**				NI	None
Jacobs *et al*[22]		MF	512	78–85	8	No association				NI	None
			702	85–90	5	No association					
Takata *et al*[23]		MF	207	85	10	Survivors 3.2 mmol/L; non-survivors: 2.9 mmol/L**				No association	None
Lv *et al*[24]		M	266	≥80	3	1.0	0.67	0.43	0.41*	NI	First year deaths
		F	596			1.0	0.69*	0.57**	0.59*		
		MF				1.0	0.72*	0.59**	0.60*		
Blekkenhorst *et al*[25]		F	1469	≥70	10	NI				No association	None

In the study by Fried *et al*, degree of significance was not reported.
*p<0.05; **p<0.01; ***p<0.001.
†HR for 7415 men and 8314 women on statin treatment was 0.63 and 0.61, respectively.
F, females; M, males; NI, no information.

Table 2 Factors corrected for in the multifactorial analyses of each study

Authors	Factors adjusted for
Zimetbaum *et al*	Age, smoking, health self-rating, BMI, BP, diabetes, MI and IQ.
Kronmal *et al*	Age, sex, BP, BMI, BMI squared, smoking.
Räihä *et al*	Age, sex, smoking, alcohol use, BMI, CHD, BP and diabetes.
Fried *et al*	Race, height, hip and waist circumference, BMI, smoking, diastolic BP, antihypertensive and lipid-lowering treatment, TC, HDL-C, TG, diabetes, fasting insulin, factor VII and VIII, serum potassium and uric acid, asthma, emphysema, angina, MI, stroke, claudication, arthritis, renal disease, cancer, hearing and visual impairment, FEV, mitral stenosis and regurgitation, carotid stenosis
Chyou and Eaker	Age, sex, CHD, stroke, cancer, diabetes, BP, BMI, smoking, alcohol consumption
Weverling-Rijnsburger *et al*	Comorbidities, BMI, use of β-blocking agents, thyroid dysfunction
Schupf *et al*	Age, sex, ethnic group, BMI, level of education, APOE genotype, diabetes, heart disease, stroke, cancer, smoking
Tikhonoff *et al*	Age, BP, pulse rate, BMI, CV events, smoking, alcohol intake, diabetes, serum creatinine and uric acid.
Störk *et al*	Cox regression analysis. No details
Akerblom *et al*	Age, sex, education, BMI, APOE genotype, heart disease, BP, diabetes, stroke, dementia, smoking.
Upmeier *et al*	Gender, BMI, smoking, angina pectoris, stroke, diabetes, hypertension, cancer
Nilsson *et al*	BMI, smoking, non-HDL-C, TG, BP, diabetes, previous MI
Werle *et al*	Factors that showed a trend for association in the univariate analyses, well-known risk factors for total mortality or cardiovascular mortality.
Bathum *et al*	Cox regression analysis. No details
Linna *et al*	Age, sex, BMI, smoking, BP, diabetes
Jacobs *et al*	Statin treatment, sex, CHD, BP, neoplasm, self-rated health, smoking, albumin, BMI, triglycerides.
Takata *et al*	Sex, smoking, alcohol intake, stroke, heart disease, serum albumin, BMI, systolic BP
Lv *et al* 2015	Age, sex, marital status, smoking, alcohol drinking, tea drinking, central obesity, cognitive impairment, daily activity, blindness, anaemia, BT, diabetes, CKD, HDL-C
Blekkenhorst *et al*	Age, BMI, physical activity, renal function, smoking, diabetes, CVD, low-dose aspirin, antihypertensive and statin medication, energy intake, SFA

In studies not corrected for age, all participants were of the same age.
BMI, body mass index; BP, blood pressure; CHD, coronary heart disease; CKD, chronic kidney disease; CRP, C reactive protein; CVD, cardiovascular disease; FEV, forced expiratory volume; HDL-C, high-density lipoprotein cholesterol; IMT, intima-media thickness; MI, myocardial infarction; SFA, superficial femoral artery; TG, tryglicerides.

lowest LDL-C. Furthermore, in the largest study[20] that included about two-thirds of the total number of participants in our study, the risk was lower among those with the highest LDL-C than among those on statin treatment.

It is also possible that those with the highest LDL-C were put on a different diet than those with low LDL-C. However, this potential bias in mortality outcomes could have gone in both directions. Some of the individuals with high LDL-C may have followed the official dietary guidelines and exchanged saturated fat with vegetable oils rich in linoleic acid. In a recent study, the authors reported that among participants who were older than 65 at baseline, a 30 mg/dL decrease in serum cholesterol was associated with a higher risk of death (HR 1.35, 95% CI 1.18 to 1.54).[26] If applied to the general population, this finding suggests that the conventional dietary treatment for high cholesterol with vegetable oil replacing saturated fat may actually increase mortality in those individuals with high LDL-C. Thus, the lack of an association between LDL-C and mortality may have been even stronger than reported since the dietary intervention may have been counterproductive.

Finally, it is potentially relevant that we limited our literature search to PubMed. In preliminary searches with PubMed, OVID and EMBASE, we identified 17 relevant studies in PubMed, but only 2 in OVID and EMBASE, and these 2 studies were found in PubMed as well. Therefore, it is highly unlikely that there are studies with findings with divergent results from those we have reported here, as all of them reported either no association or an inverse association between LDL-C and mortality.

DISCUSSION

Assessments of the association between serum cholesterol and mortality have been studied for decades, and extensive research has shown a weak association between total cholesterol and mortality in the elderly; several studies have even shown an inverse association. It is therefore surprising that there is an absence of a review of the literature on mortality and levels of LDL-C, which is routinely

referred to as a causal agent in producing CVD[4] and is a target of pharmacological treatment of CVD.

Our literature review has revealed either a lack of an association or an inverse association between LDL-C and mortality among people older than 60 years. In almost 80% of the total number of individuals, LDL-C was inversely associated with all-cause mortality and with statistical significance.

These findings provide a paradoxical contradiction to the cholesterol hypothesis. As atherosclerosis starts mainly in middle-aged people and becomes more pronounced with increasing age, the cholesterol hypothesis would predict that there should be a cumulative atherosclerotic burden, which would be expressed as greater CVD and all-cause mortality, in elderly people with high LDL-C levels.

Our results raise several relevant questions for future research. Why is high TC a risk factor for CVD in the young and middle-aged, but not in elderly people? Why does a subset of elderly people with high LDL-C live longer than people with low LDL-C? If high LDL-C is potentially beneficial for the elderly, then why does cholesterol-lowering treatment lower the risk of cardiovascular mortality? In the following we have tried to address some of these questions.

Inverse causation

A common argument to explain why low lipid values are associated with an increased mortality is inverse causation, meaning that serious diseases cause low cholesterol. However, this is not a likely explanation, because in five of the studies in table 1 terminal disease and mortality during the first years of observation were excluded. In spite of that, three of them showed that the highest mortality was seen among those with the lowest initial LDL-C with statistical significance.[18 20 24]

Is high LDL-C beneficial?

One hypothesis to address the inverse association between LDL-C and mortality is that low LDL-C increases susceptibility to fatal diseases. Support for this hypothesis is provided by animal and laboratory experiments from more than a dozen research groups which have shown that LDL binds to and inactivates a broad range of microorganisms and their toxic products.[27] Diseases caused or aggravated by microorganisms may therefore occur more often in people with low cholesterol, as observed in many studies.[28] In a meta-analysis of 19 cohort studies, for instance, performed by the National Heart, Lung and Blood Institute and including 68 406 deaths, TC was inversely associated with mortality from respiratory and gastrointestinal diseases, most of which are of an infectious origin.[29] It is unlikely that these diseases caused the low TC, because the associations remained after the exclusion of deaths occurring during the first 5 years. In a study by Iribarren *et al*, more than 100 000 healthy individuals were followed for 15 years. At follow-up, those whose initial cholesterol level was lowest at the start had been hospitalised significantly more often because of an infectious disease that occurred later during the 15-year follow-up period.[30] This study provides strong evidence that low cholesterol, recorded at a time when these people were healthy, could not have been caused by a disease they had not yet encountered.

Another explanation for an inverse association between LDL-C and mortality is that high cholesterol, and therefore high LDL-C, may protect against cancer. The reason may be that many cancer types are caused by viruses.[31] Nine cohort studies including more than 140 000 individuals followed for 10–30 years have found an inverse association between cancer and TC measured at the start of the study, even after excluding deaths that occurred during the first 4 years.[32] Furthermore, cholesterol-lowering experiments on rodents have resulted in cancer,[33] and in several case–control studies of patients with cancer and controls matched for age and sex, significantly more patients with cancer have been on cholesterol-lowering treatment.[32] In agreement with these findings, cancer mortality is significantly lower in individuals with familial hypercholesterolaemia.[34]

That high LDL-C may be protective is in accordance with the finding that LDL-C is lower than normal in patients with acute myocardial infarction. This has been documented repeatedly without a reasonable explanation.[35–37] In one of the studies,[37] the authors concluded that LDL-C evidently should be lowered even more, but at a follow-up 3 years later mortality was twice as high among those whose LDL-C had been lowered the most compared with those whose cholesterol was unchanged or lowered only a little. If high LDL-C were the cause, the effect should have been the opposite.

CONCLUSIONS

Our review provides the first comprehensive analysis of the literature about the association between LDL-C and mortality in the elderly. Since the main goal of prevention of disease is prolongation of life, all-cause mortality is the most important outcome, and is also the most easily defined outcome and least subject to bias. The cholesterol hypothesis predicts that LDL-C will be associated with increased all-cause and CV mortality. Our review has shown either a lack of an association or an inverse association between LDL-C and both all-cause and CV mortality. The cholesterol hypothesis seems to be in conflict with most of Bradford Hill's criteria for causation, because of its lack of consistency, biological gradient and coherence. Our review provides the basis for more research about the cause of atherosclerosis and CVD and also for a re-evaluation of the guidelines for cardiovascular prevention, in particular because the benefits from statin treatment have been exaggerated.[38–40]

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35 EGGS PER DAY IN THE TREATMENT OF SEVERE BURNS

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IN order to maintain a positive nitrogen balance in extensive burns, the daily calorie requirements may be as much as 7,000 (Artz, 1968). Oral feeding is the pathway of choice and parenteral hyperalimentation should be avoided unless adequate oral feeding is impossible, for example with gastric dilatation or stress ulcers. It is, however, often difficult for the extensively burned patient to ingest large quantities of meat, fish and other calorie rich nutrients especially when the appetite is impaired. Over the past 2 decades we have been feeding our severely burned patients with between 30 and 40 eggs per day. They appear to be readily assimilable, easily ingested and without untoward effects.

Serum protein and lipoprotein levels have been measured in 8 burned patients ingesting 35 eggs per day. The patients were all in their twenties and had second and third degree burns covering 30 to 60 per cent of the body surface. As soon as their general condition permitted, usually between the 4th and 6th hospital day, the egg enriched diet was begun.

The total intake is shown in the Table. Additional vitamins and iron were also given. Twenty-five eggs were given during the day in a variety of forms only limited by the ingenuity of the nursing staff; some of the most popular ways were fried, raw in soups or wine, omelettes and egg-enriched ice cream. During the night 10 raw eggs mixed with ½-1 litre of milk plus ample sugar were fed through a nasogastric tube.

INVESTIGATIONS AND RESULTS

Serum samples were taken 1-2 hours after withdrawal of the nasogastric tube and before breakfast, prior to the egg diet starting and on the 2nd, 4th, 6th, 10th, 16th, 19th,

TABLE

Total Daily Intake from all Sources

Source	Protein (g)	Carbohydrates (g)	Fats (g)	Calories
Eggs (35)	215	—	215	2,795
Meat (300 g)	54	—	51	675
Milk and products (1,500 ml)	45	—	45	585
Beer Juice Bread Sugar Potatoes Chocolate	—	600	—	2,400
Soups Margerine Butter	—	—	25	225
Total	314	600	336	6,680

25th and 30th days thereafter. All the patients survived this period although one subsequently died of a pulmonary embolus on the 59th day.

Serum proteins. Albumin, globulin and total serum protein levels were determined in an auto-analyser. The average initial level of total serum proteins was 4·1 g/100 ml but this had risen to 6·25 g/100 ml by the 16th day and thereafter remained constant. The albumin and globulin figures showed the same trend; initially slightly below normal they subsequently rose into the normal range and thereafter remained constant.

Serum lipoproteins. The membrane filtration and nephelometric method of Stone and Thorpe (1966) was used. The results were reported in terms of the concentration of large, medium and small particle size. Small lipoprotein particles comprise mainly cholesterol with little triglyceride lipoprotein content. Medium-sized particles are those containing triglycerides in the main with smaller proportions of cholesterol protein. Large particles are a measure of chylo-micron content (Stone *et al.*, 1970, 1971). Serum cholesterol levels were also determined by the technique of Huang *et. al.* (1961).

All small, medium and large lipoprotein levels were within normal limits. Initially the serum cholesterol levels were subnormal but subsequently rose and remained within normal limits throughout.

Discussion

Over the past 20 years burn treatment in our service has undergone many changes, yet the dietary regime of high egg intake has remained the same. It reduces the need for bulk ingestion and provides the basic calorie requirement, all other food intake being supplementary. The calorie content of 1 egg averages 70 calories (Wohl *et al.*, 1971) so that a 35-egg intake would supply 2,450 calories. The additional ordinary ward diet allows the desired calorie intake of 6,000-7,000 to be achieved. Another important feature is that the egg regime provides most of the protein requirements. Each egg contains 6 g of protein and 35 eggs thus provide 210 g per day. According to Artz (1968) the daily requirement of a burn patient to maintain positive nitrogen balance is about 3 g protein per kg body weight and 30 calories for each gram of protein. Ingestion of a much smaller bulk is a further advantage; thus 100 g of meat contains only 18 g protein. The ingestion of 3 eggs is obviously far easier than that of 100 g of meat.

All of our patients over the years have tolerated this regime surprisingly well. In almost no instance could diarrhoea, vomiting and allergic reactions, if they appeared, be attributed to the high egg intake. This has been borne out in the 8 burned patients under investigation, none of whom developed any untoward reactions while on the diet. A further interesting feature was that no distaste for eggs was acquired. This could partly be explained by the fact that practically all the patients were unaware of the number of eggs they were ingesting. Absorption must also have been adequate since none of these patients developed steatorrhoea or diarrhoea. This is in contrast to the use of synthetic high calorie oral preparations, which besides being to a certain extent impalatable, often give rise to worrying diarrhoea.

Serum protein levels rapidly improved on the high egg regime and within days achieved and maintained normal limits. This is in marked contrast to the experience of Fox *et al.* (1970) who infused their seriously burned cases with albumin and plasma. The average serum albumin level in their cases fell to 2 g per 100 ml serum by the middle of the first week and remained in the subnormal range despite the plasma and albumin infusions.

The high egg diet certainly represents a significant cholesterol load. An egg

contains between 250 and 550 mg of cholesterol and thus our patients imbibed over 7,000 mg of cholesterol per day. Contrary to expectations, serum cholesterol and lipoprotein levels remained normal throughout the period of study. Dogs fed a diet rich in saturated fatty acids with added cholesterol develop significant increases in all fractions of serum lipid concentration within the first week of the diet (Butkus *et al.*, 1970). Similar findings have been obtained in rabbits and monkeys (Anitschkow, 1933; Blaton *et al.*, 1970). No similar situation whereby such a high cholesterol load, sustained for a period of weeks in humans, has been encountered in the literature. The closest human model to our situation would be that of the Eskimos whose daily cholesterol intake ranges from 420 to 1,650 mg/day (Ho *et al.*, 1972). Studies on the Masai Africans who eat primarily a diet consisting of milk, blood and meat, and who were supplemented with 2,000 mg of crystalline cholesterol, have been performed (Biss *et al.*, 1971; Ho *et al.*, 1971; Taylor and Ho, 1971). In both these groups serum cholesterol levels remained within normal limits.

Four homeostatic mechanisms are described for the control of cholesterol metabolism. These consist of limitation of absorption, suppression of cholesterol synthesis, an increase in the rate of cholesterol degradation to bile acids and consequently increased secretion and finally reversible tissue cholesterol storage. Limited capacity for absorption is usually quoted as the most important mechanism. In the Eskimo the efficiency of cholesterol absorption was 57 per cent of dietary cholesterol (Ho *et al.*). Other mechanisms must then operate as the serum cholesterol remained normal. The Masai Africans were found to absorb 33 per cent of dietary cholesterol and were able to suppress endogenous cholesterol synthesis by 50 per cent (Biss *et al.*; Ho *et al.*; Taylor and Ho). Caucasians in the USA were assumed to have a limited ability to absorb cholesterol. Kaplan *et al.* (1963) calculated this to be in the region of 300 mg/day and this figure was confirmed by Wilson and Lindsey (1965) and Grundy and Ahrens (1966). However, Ho (1972) and Kudchodker (1971) reported on similar, fixed, absorption efficiencies which approximated 37 ± 5 per cent of dietary cholesterol over a range of 300 mg-1,250 mg/day.

In the absence of a cholesterol metabolic study it is not possible to determine the homeostatic mechanisms that were operating in our burned patients. It may just be possible that in these severely burned patients the high demands of the body for utilisation of fats as calories may play a role in maintaining normal serum lipoprotein levels. In this context it is interesting to recall the work of Wilmore *et al.* (1973) who described a state of super-utilisation of fats in acutely burned patients. Fat clearance curves demonstrated an accelerated plasma disappearance of the emulsion.

An important consideration in instituting any high calorie therapy in mass burn casualties should also be the costs. In Israel, at present prices, 1 egg costs about 6 cents (USA) and egg protein is cheaper than proteins derived from almost every other source. One kilogram of red meat costs about $6 and contains 180 g of protein. Thirty eggs would provide this amount of protein and would cost only $1.80. Somewhat lower figures apply to fish and poultry.

It is realised that this study, as it applies to a positive nitrogen balance has certain shortcomings. Proof of this balance would best be established by bed weighing of the patients. This unfortunately was impossible because of lack of the necessary facilities. Clinically the patients appeared to thrive, without evidence of severe weight loss. A certain degree of negative nitrogen balance is inevitable in severe burns but our impression was that this was minimal. Further support for this view was their rapid recovery, manifested by good skin graft acceptance and without much troublesome infection.

In conclusion, the premise that high egg consumption in burn patients is helpful and not detrimental has been confirmed by our observations. The long-term effects of such a diet, however, are impossible to predict.

SUMMARY

A regime involving the dietary management of 8 severely burned patients has been described, the main feature of this diet being the high egg intake. The eggs were given in a variety of forms without any untoward effects and without any distaste being developed. No pathological levels of serum lipoproteins or cholesterol were attained. Serum proteins from an initial low level reached normal values within a relatively short time.

It is felt that a high egg diet is a valuable and safe addition in the management of severely burned patients.

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BMJ Open

Randomised trial of coconut oil, olive oil or butter on blood lipids and other cardiovascular risk factors in healthy men and women

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ABSTRACT

Introduction High dietary saturated fat intake is associated with higher blood concentrations of low-density lipoprotein cholesterol (LDL-C), an established risk factor for coronary heart disease. However, there is increasing interest in whether various dietary oils or fats with different fatty acid profiles such as extra virgin coconut oil may have different metabolic effects but trials have reported inconsistent results. We aimed to compare changes in blood lipid profile, weight, fat distribution and metabolic markers after four weeks consumption of 50 g daily of one of three different dietary fats, extra virgin coconut oil, butter or extra virgin olive oil, in healthy men and women in the general population.

Design Randomised clinical trial conducted over June and July 2017.

Setting General community in Cambridgeshire, UK.

Participants Volunteer adults were recruited by the British Broadcasting Corporation through their websites. Eligibility criteria were men and women aged 50–75 years, with no known history of cancer, cardiovascular disease or diabetes, not on lipid lowering medication, no contraindications to a high-fat diet and willingness to be randomised to consume one of the three dietary fats for 4 weeks. Of 160 individuals initially expressing an interest and assessed for eligibility, 96 were randomised to one of three interventions; 2 individuals subsequently withdrew and 94 men and women attended a baseline assessment. Their mean age was 60 years, 67% were women and 98% were European Caucasian. Of these, 91 men and women attended a follow-up assessment 4 weeks later.

Intervention Participants were randomised to extra virgin coconut oil, extra virgin olive oil or unsalted butter and asked to consume 50 g daily of one of these fats for 4 weeks, which they could incorporate into their usual diet or consume as a supplement.

Main outcomes and measures The primary outcome was change in serum LDL-C; secondary outcomes were change in total and high-density lipoprotein cholesterol (TC and HDL-C), TC/HDL-C ratio and non-HDL-C; change in weight, body mass index (BMI), waist circumference, per cent body fat, systolic and diastolic blood pressure, fasting plasma glucose and C reactive protein.

Results LDL-C concentrations were significantly increased on butter compared with coconut oil (+0.42, 95% CI 0.19 to 0.65 mmol/L, P<0.0001) and with olive oil (+0.38, 95% CI 0.16 to 0.60 mmol/L, P<0.0001), with no differences in change of LDL-C in coconut oil compared with olive oil (−0.04, 95% CI −0.27 to 0.19 mmol/L, P=0.74). Coconut oil significantly increased HDL-C compared with butter (+0.18, 95% CI 0.06 to 0.30 mmol/L) or olive oil (+0.16, 95% CI 0.03 to 0.28 mmol/L). Butter significantly increased TC/HDL-C ratio and non-HDL-C compared with coconut oil but coconut oil did not significantly differ from olive oil for TC/HDL-C and non-HDL-C. There were no significant differences in changes in weight, BMI, central adiposity, fasting blood glucose, systolic or diastolic blood pressure among any of the three intervention groups.

Conclusions and relevance Two different dietary fats (butter and coconut oil) which are predominantly saturated fats, appear to have different effects on blood lipids compared with olive oil, a predominantly monounsaturated fat with coconut oil more comparable to olive oil with respect to LDL-C. The effects of different dietary fats on lipid profiles, metabolic markers and health outcomes may vary not just according to the general classification of their main component fatty acids as saturated or unsaturated but possibly according to different profiles in individual fatty acids, processing methods as well as the foods in which they are consumed or dietary patterns. These findings do not alter current dietary recommendations to reduce saturated fat intake in general but highlight the need for further elucidation of the more nuanced relationships between different dietary fats and health.

Trial registration number NCT03105947; Results.

Strengths and limitations of this study

- The randomised trial design comparing three dietary fat interventions minimised confounding and bias.
- There was good compliance and participants were from the general community in a 'real life' setting.
- Objective measures of outcome—blood biochemistry and anthropometry—were used, minimising bias.
- Participants were not blinded as to the intervention, and the intervention was relatively short term over 4 weeks.

INTRODUCTION

This trial was conducted in the context of debate over longstanding dietary recommendations to reduce dietary fat intake for health. The Women's Health Initiative reported no differences in cardiovascular disease in women randomised to low fat and usual diets over 8 years[1] while an intervention comparing a low-fat diet with a Mediterranean diet with extra virgin olive oil or nuts (PREDIMED) reported approximately 30% lower cardiovascular events in both Mediterranean diet arms after 4.8 years[2]; meta-analyses of observational studies and trials report inconsistent findings in the relationship between dietary saturated fatty acids and cardiovascular disease[3 4] and the relationships of dairy fats including milk and butter with cardiovascular disease also being debated.[5–7] Part of the debate relates to the increasing evidence that different individual fatty acids, such as the odd chain or even chain saturated fatty acids, or short, medium and long chain saturated fatty acids, may have different metabolic pathways and subsequent potential health effects as well as the understanding that diet is more complex than individual nutrients or generic biochemical nutrient groups and that contextual factors such as foods and dietary patterns are important. The 2015–2020 US dietary guidelines[8] now focus on foods and dietary patterns and while they recommend limiting saturated and trans fats, they no longer explicitly recommend limiting total fat. In this context therefore, there is renewed interest in the health effects of different fats and oils.

Extra virgin coconut oil has recently been promoted as a healthy oil. Though high in saturated fat, the main saturated fatty acid, lauric acid (c12:0), has been suggested to have different metabolic and hence health effects compared with other saturated fatty acids such as palmitic acid (c16:0), predominant in butter, palm oil and animal fat. In particular, it has been suggested that coconut oil does not raise total cholesterol (TC) or low-density lipoprotein cholesterol (LDL-C) as much as butter. A recent review on coconut oil and cardiovascular risk factors in humans concluded that the evidence of an association between coconut oil consumption and blood lipids or cardiovascular risk was mostly poor quality.[9] While some small studies have been reported comparing coconut oil and butter, these have been small[10 11] and none conducted in the UK where overall dietary patterns are different from Asia, USA or New Zealand where most trials have been conducted. The 2017 American Heart Association Presidential advisory on dietary fats and cardiovascular disease highlighted the paucity of evidence over the long-term health effects of saturated fats such as coconut oil and reinforced strongly recommendations to lower dietary saturated fat and replacement with unsaturated fat to lower LDL-C and prevent cardiovascular disease.[12] In particular, they stated 'because coconut oil increases LDL-C, a cause of cardiovascular disease and has no known off setting favourable effects, we advise against the use of coconut oil'.[12]

Though the PREDIMED study reported lower cardiovascular disease events in those randomised to extra virgin olive oil or added nuts,[2] this trial reported no overall effects on LDL-C or TC for those on olive oil compared with the low-fat diet,[13] results consistent with a review of intervention trials of high phenolic olive oil.[14]

We therefore aimed to examine whether in free living healthy men and women in the UK, we could observe differences in blood lipids after 1 month's consumption of 50 g daily of one of three different fats within the context of their usual diet. Although this was a short-term trial that did not address cardiovascular disease events, blood lipids are a well established risk factor for coronary heart disease and the aim was to compare directly the effects of three different fats, extra virgin coconut oil, butter (both predominantly saturated fats) with extra virgin olive oil (monounsaturated fat) on blood lipid profiles and metabolic measures, in a pragmatic trial using amounts feasible in daily diets.

METHODS

Study population

Participants were volunteers living in the general community predominantly in the Cambridgeshire area, recruited through British Broadcasting Corporation (BBC) advertising in May and June 2017. Eligible participants were men or women aged between 50 and 75 years who did not have a known medical history of heart disease, stroke, cancer or diabetes and who were not taking medication for lowering blood lipids such as statins. They had to be willing to be randomised to consume 50 g daily of one of the designated fats for four weeks and not have any contraindications to eating a high-fat diet such as gall bladder or bowel problems. Of 160 individuals expressing an interest, 96 were eligible and randomised to the intervention, 2 withdrew prior to the start of the study and 94 attended a baseline assessment.

Allocation to intervention

Participants were assigned a unique study identification number (ID). These ID numbers were randomised by computer generated allocation conducted by an independent statistician separately in men and women, into one of three parallel intervention arms approximately equal in size: extra virgin coconut oil, butter or extra virgin olive oil.

Intervention

Participants attending the baseline assessment, at the end of their appointment, received 1 month's supply of one of the three different dietary fats to which they had been randomly allocated: extra virgin coconut oil or butter or extra virgin olive oil. The BBC study organiser was given an ID list with the random allocation to the fats/oils and was responsible for giving each participant their supply of fat/oils. They were asked to eat 50 g of these fats daily for 4 weeks and given measuring cups for the

50 mL fat and oils: butter was prepacked in 20 g and 30 g portions. They were asked to continue with their usual diet and either incorporate the fat or oil into their daily diet to substitute for other fats or oils or they could eat these fats as a supplement. They also had information sheets with suggestions for how the fats could be consumed including recipes. The fats selected were standard products available from supermarkets bought from suppliers; organic extra virgin coconut oil, organic unfiltered extra virgin olive oil and organic unsalted butter. Samples of the oils/fats used in the trial were sent to a reference laboratory: the West Yorkshire Analytic Services, a UKAS accredited testing service for food composition.

Assessments

Participants attended two assessments at a community centre in Cambridge: one at baseline before the start of the intervention in June 2017 and one at the end of 4 weeks in July 2017. Prior to their initial assessment, they were asked to fill in a short questionnaire about their health and lifestyle including physical activity and diet as well as complete an online 24 hours dietary assessment questionnaire with automated nutrient intake estimation, developed in Oxford, the DietWebQ.[15] All assessments were conducted between 08:00 and 12:30 hours. Participants were all fasted for a minimum of 4 hours prior to attending the assessment; the majority were fasted overnight. They had height and waist circumference measured to a standardised protocol in light clothing without shoes and blood pressure measured using an automated OMRON device after being seated resting for 5 min. The mean of two readings for blood pressure, height and waist was used for analysis. Weight and per cent body fat were measured using a Tanita body composition monitor. All measurements were conducted by two trained observers unaware of allocation to the oils/fats. Participants gave a 20 mL blood sample which was stored in a 4°C refrigerator and then sent to the laboratory by courier for same day sample processing and storage for later analysis.

After 4 weeks at the end of the intervention, they attended again for a follow-up assessment where the same measurements of height, waist circumference, blood pressure, weight and per cent body fat were conducted, and another fasting 20 mL blood sample taken. Measurements were recorded on new forms and observers and participants did not have access to the measurements taken at the baseline visit. Just prior to this visit, participants were asked to fill in again the online 24-hour DietWebQ. Participants also filled in short questionnaire about their experiences on the intervention fats. This included a question about their overall experience of consuming the assigned oil/fat in the study where they were asked on average, over the past 4 weeks whether they felt mostly the same as usual, mostly felt better than usual or mostly felt worse than usual with an open-ended section for comments including side effects and overall compliance with consuming the fats which they were asked to self-rate between 0% and 100%. They were also asked whether they changed their type, level or frequency of physical activity in the past month since being in the study and had three options, no overall change in activity, increase in activity or decrease in activity.

Blood samples were identified only by a study ID number and were processed using standard protocols and assayed in two batches at the end of the baseline and follow-up assessments in the Core Biochemical Assay Laboratory (CBAL) Cambridge University Hospitals which has UKAS Clinical Pathology Accreditation; blood samples from individuals on different interventions were thus all assayed in the same batch. The laboratory assays were conducted in a blinded fashion without any indication of the allocated intervention. Cholesterol (TC) and triglycerides were measured using enzymatic assays,[16 17] high-density lipoprotein cholesterol (HDL-C) was measured using a homogenous accelerator selective detergent assay automated on the Siemens Dimension RxL analyser and LDL-C was calculated from the triglyceride, HDL and cholesterol concentrations as described in the Friedewald formula [LDL = Cholesterol - HDL – (Triglycerides/2.2)].[18] Total to HDL-C ratio was computed, and non-HDL-C was computed as TC minus HDL-C.

Plasma glucose was measured using the hexokinase-glucose-6-phosphate dehydrogenase method, and high-sensitivity human C reactive protein was assayed using an automated colourimetric immunoassay: Siemens Dimension CCRP *Cardio*Phase high-sensitivity CRP.

Trial outcomes

The trial was registered in April 2017 with clinical trials registration: NCT03105947. The primary outcome of the trial was change in LDL-C from baseline to follow-up. Secondary outcomes were change in each of the following variables from baseline to follow-up: TC, HDL-C, triglycerides; ratio of TC/HDL-C, non-HDL-C, fasting blood glucose, C reactive protein, weight, body mass index (BMI), body fat %, waist circumference, systolic blood pressure and diastolic blood pressure.

Statistical analysis

The study aimed to recruit a total of 90 participants: 30 individuals per group would provide approximately 80% power to detect a difference in mean within-person change in LDL-C (baseline to follow-up) comparing pairs of randomised groups (butter vs coconut oil and butter vs olive oil) of approximately 0.5 mmol/L, assuming a SD of LDL-C of 1.04 mmol/L[19] and a correlation between baseline and follow-up values of 0.79[20] incorporated using the method described by Borm *et al*.[21] With 2 primary pairwise comparisons, the significance level for each comparison was set to 2.5%.

This magnitude of difference was what can be estimated from metabolic ward studies in which replacement of 10% dietary calories from saturated fat is associated with 0.52 mmol/L cholesterol difference[22] though

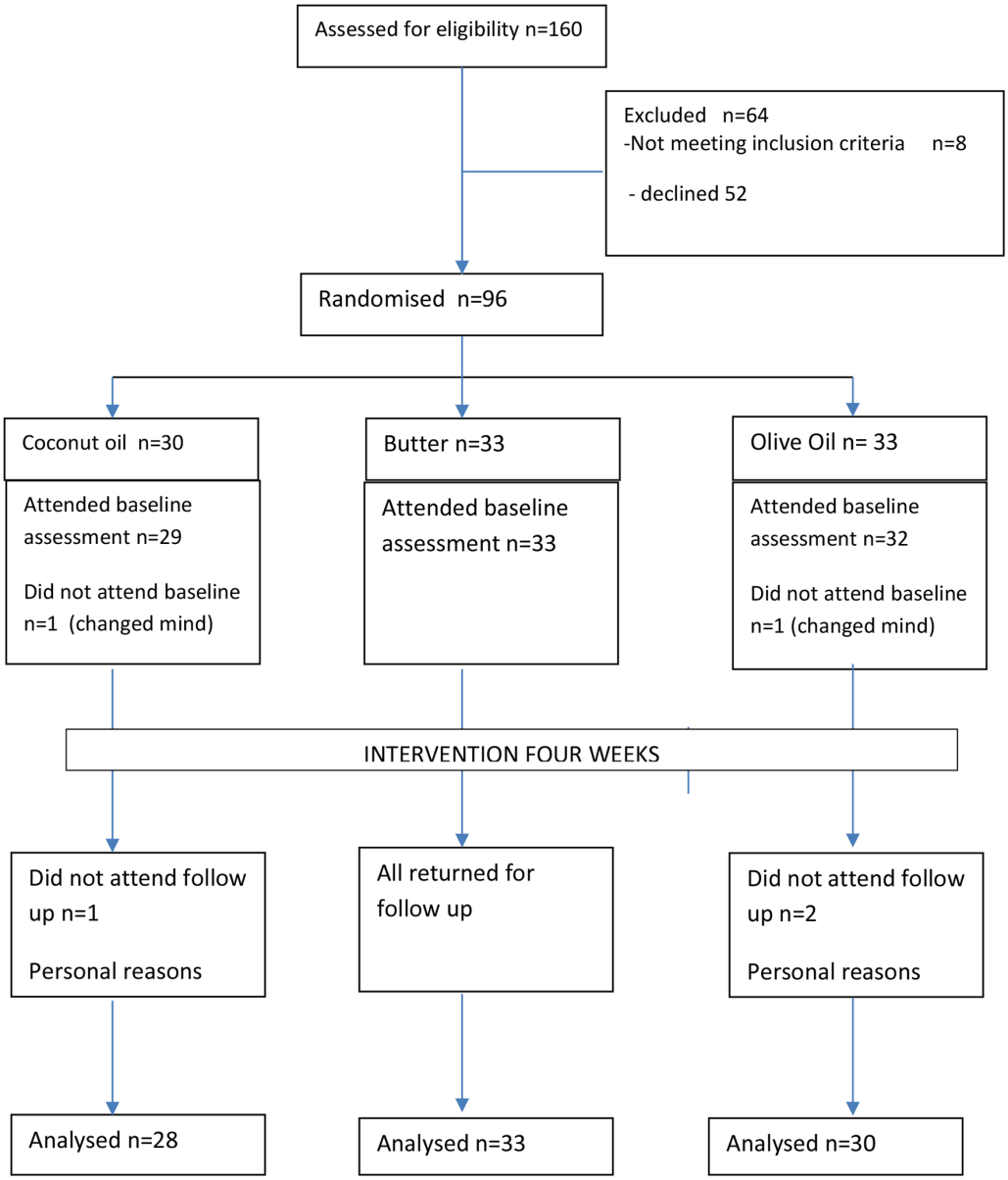

Figure 1 Recruitment and flow diagram (CONSORT) for coconut oil, olive oil or butter trial.

this did not specify the food sources of saturated fats, and a small intervention trial (n=28) comparing butter and coconut oil with sunflower oil.[10]

Baseline characteristics were summarised separately for each randomised group. As recommended by CONSORT, no P values were calculated for this table. The primary analysis used an intention-to-treat (ITT) population, which included all individuals in the group to which they were randomised, regardless of the extent to which they adhered to the intervention. A secondary analysis used a per protocol (PP) population. This was a subset of the ITT population consisting of those individuals who adhered to the intervention. Participants who reported >75% adherence when asked at the follow-up visit were included in the PP population.

For each outcome, a P value was calculated to compare the three randomised groups using a linear regression model, in which change from baseline was the outcome and including a dummy variable for randomised group and the baseline value of the outcome variable as covariates, that is, an analysis of covariance model. Differences between each pair of randomised groups and 95% CIs were also estimated from a similar model.

Patient and public involvement

The BBC originally proposed the idea of a study to examine claims about the health benefits of coconut oil in response to public interest; the study would be part of their 'Trust me, I'm a doctor' series. The study was designed as a randomised trial with participants from the general community in discussion with the BBC.

RESULTS

Figure 1 is the CONSORT diagram for the trial. The recruitment was conducted by the BBC coordinator

through BBC website advertising. From 160 individuals initially expressing an interest and after exclusion criteria, 96 individuals were randomised and invited to a baseline assessment session in June 2017. Two individuals subsequently withdrew and 94 individuals attended the baseline assessment session in June 2017. At the 4-weekfollow-up assessment in July 2017, 91 individuals attended; three individuals did not attend follow-up indicating personal circumstances.

Table 1 shows descriptive characteristics for the participants at the baseline assessment according to the allocation to dietary oils/fats. Two thirds of the participants were women and the mean age overall was 60 years.

Table 2 shows mean changes in the primary and secondary outcomes at the 4-week follow-up within each randomised group and comparisons between each pair of randomised groups. LDL-C concentrations were significantly increased on butter compared with coconut oil (+0.42, 95% CI 0.19 to 0.65 mmol/L, P<0.0001) and olive oil (+0.38 L, 95% CI 0.16 to 0.60 mmol/L, P<0.0001), with no differences in change of LDL-C in coconut oil compared with olive oil (−0.04, 95% CI −0.27 to 0.19 mmol/L, P=0.74). Coconut oil significantly increased HDL-C compared to butter (+0.18 95% CI 0.06 to 0.30 mmol/L) or olive oil (+0.16, 95% CI 0.03 to 0.28 mmol/L).

Butter significantly increased the cholesterol/HDL-C ratio compared with coconut oil (+0.36, 95% CI 0.18 to 0.54) and olive oil (+0.22, 95% CI 0.04 to 0.40) and also increased non-HDL-C compared with coconut oil (+0.39, 95% CI 0.16 to 0.62 mmol/L) and olive oil (+0.39 (95% CI 0.16 to 0.62) but coconut oil did not significantly differ from olive oil for change in cholesterol/HDL-C ratio (−0.14, 95% CI −0.33 to 0.05) or non-HDL-C (0.002, 95% CI −0.23 to 0.24 mmol/L).

Coconut oil also significantly lowered C reactive protein in comparison with olive oil (−0.58, 95% CI −1.12 to −0.04 mg/L) but not compared with butter. There were no significant differences in changes in weight, BMI, central adiposity, fasting blood glucose, systolic or diastolic blood pressure among any of the three intervention groups. For weight, for example, the estimated mean(SD) changes in weight were +0.27 (0.77) kg, 0.04 (1.00) kg and −0.04 (0.84) kg for coconut oil, butter and olive oil, respectively. Adjusting for age, sex and body mass index did not materially alter the results (online supplementary table 1).

Figure 2 shows the difference in the primary outcome (LDL-C) between each pair of randomised groups in the 91 individuals who attended baseline and follow-up. Figures 3–5 show the differences in secondary outcomes comparing butter versus coconut oil, coconut oil versus olive oil and butter versus olive oil, respectively. For comparability, the differences are reported in units of baseline SD for the different outcomes in figures 3–5.

Self-reported compliance was high: 87% of participants reported more than 75% compliance with the intervention over the 4 weeks which was similar among the groups (86% coconut oil, 88% butter and 85% olive oil). Secondary analyses on the 82 participants reporting more than 75% compliance showed similar results (not shown). Reported experience consuming the fats was similar between groups: 57%, 66% and 60% reported feeling no different, 18%, 6% and 13% reported feeling better and 25%, 27% and 23% reported feeling worse in the coconut oil, butter and olive oil groups, respectively. Comparison of dietary intake using the 24-hour DietWebQ showed similar levels of dietary intake across intervention groups at baseline. Following the intervention, total fat intake increased in all intervention groups but estimates for absolute intakes of carbohydrate, protein and alcohol did not differ between intervention groups (table 3). Most of the participants reported no changes in usual physical activity (79%, 73% and 89% no change; 14%, 15% and 4% increased usual physical activity and 7%, 12% and 7% decreased usual physical activity in the coconut oil, butter and olive oil groups, respectively). In a posthoc exploratory analysis, exclusion of individuals who reported increasing usual physical activity had little effect on significant differences between interventions for LDL-C and HDL-C and did not alter the findings for weight change (online supplementary table 2).

Online supplementary appendix 1 shows the fatty acid composition of the three oils/fats used in the intervention. Coconut oil was 94% saturated fatty acids, of which the main components were lauric acid C12:0 (48%), myristic acid C14:0 (19%) and palmitic acid C16:0 (9%). Butter was 66% saturated fatty acids, of which the main components were palmitic acid C16:0 (28%), stearic acid C18:0 (12%) and myristic acid C14:0 (11%). Olive oil was 19% saturated fatty acids, mainly palmitic acid C16 (15%) with stearic acid C18:0 (3%) and 68% monounsaturates with the main component being oleic acid C18:1n9 (64%). These profiles are very similar to those reported from other studies.[9]

DISCUSSION

In this trial, middle-aged men and women living in the general community were randomly allocated to consume 50 g extra virgin coconut oil or 50 g butter or 50 g extra virgin olive oil for 4 weeks. We observed at the end of the trial significantly different changes in LDL-C and HDL-C concentrations between the three intervention groups; in pairwise comparisons, coconut oil did not significantly raise LDL-C concentrations compared with olive oil while butter significantly raised LDL-C concentrations compared with both coconut oil and olive oil. Coconut oil significantly raised HDL-C concentrations compared with both butter and olive oil. Butter also significantly raised cholesterol/HDL-C ratio and non-HDL-C more than both coconut oil and olive oil but there were no differences between coconut oil and olive oil for changes in cholesterol/HDL-C and non-HDL-C.

There were no significant differences in weight or BMI change, change in central adiposity as measured

Table 1 Descriptive characteristics at baseline assessment of participants in the COB trial according to allocation (intention to treat)

	Coconut oil	Butter	Olive oil
	n=29	n=33	n=32
	Mean (SD)	Mean (SD)	Mean (SD)
Age (years)	59.1 (6.1)	61.5 (5.8)	59.1 (6.4)
LDL-cholesterol (mmol/L)	3.5 (0.9)	3.5 (0.9)	3.7 (1.0)
Total cholesterol (mmol/L)	5.9 (1.0)	5.9 (1.0)	6.0 (0.9)
HDL-cholesterol (mmol/L)	2.0 (0.5)	1.9 (0.5)	1.8 (0.5)
Cholesterol/HDL ratio	3.2 (0.9)	3.2 (0.8)	3.5 (1.2)
Non-HDL-cholesterol (mmol/L)	3.9 (1.0)	4.0 (0.9)	4.2 (1.1)
Glucose (mmol/L)	5.3 (0.4)	5.4 (0.5)	5.4 (0.5)
Weight (kg)	73.9 (15.1)	70.8 (11.7)	71.1 (14.5)
Waist (cm)	85.4 (11.9)	83.7 (8.1)	86.2 (11.5)
Body fat (%)	29.7 (10.2)	29.2 (9.0)	31.5 (9.6)
Body mass index (kg/m^2)	25.5 (4.5)	24.8 (3.5)	25.0 (4.5)
Systolic blood pressure (mm Hg)	131.4 (18.8)	136.5 (18.8)	133.1 (16.5)
Diastolic blood pressure (mm Hg)	79.8 (9.3)	81.0 (12.0)	78.1 (6.7)
DietWebQ intake/day			
Total energy (MJ)	9.00 (3.70)	8.23 (2.17)	9.51 (3.5)
Protein % energy	14.8 (4.4)	16.0 (3.7)	15.7 (3.0)
Carbohydrate % energy	43.6 (8.9)	41.4 (8.7)	42.7 (11.7)
Total fat % energy	37.3 (7.3)	36.7 (8.7)	36.4 (10.3)
Saturated fat % energy	14.1 (3.6)	13.3 (4.4)	13.4 (4.9)
Alcohol % energy	4.2 (5.4)	5.9 (7.5)	5.1 (6.1)
Hours of walking in past week	8.9 (9.5)	10.9 (12.3)	10.1 (8.7)
Hours of cycling in past week	1.8 (2.6)	2.0 (2.5)	2.7 (5.5)
Hours of other physical exercise in past week	3.4 (3.4)	2.3 (4.0)	1.8 (2.6)
	n=29	n=33	n=32
	Median (IQR)	Median (IQR)	Median (IQR)
Triglycerides (mmol/L)	0.89 (0.74 to 1.10)	0.92 (0.70 to 1.20)	0.94 (0.79 to 1.31)
C reactive protein (mg/L)	1.04 (0.47 to 2.15)	1.08 (0.64 to 2.13)	1.13 (0.58 to 2.67)
	% (N)	% (N)	% (N)
Sex			
Men	37.9 (11)	33.3 (11)	28.1 (9)
Women	62.1 (18)	66.7 (22)	71.9 (23)
Ethnicity			
White	96.6 (28)	97.0 (32)	93.8 (30)
Non-white	3.4 (1)	3.0 (1)	3.1 (1)
Smoking status			
Never	58.6 (17)	66.7 (22)	68.8 (22)
Former	34.5 (10)	33.3 (11)	25.0 (8)
Current	6.9 (2)	0.0 (0)	6.3 (2)
Alcohol consumption in past year			
Never or once per month	20.7 (6)	30.3 (10)	28.1 (9)
1–4 times per week	72.4 (21)	48.5 (16)	59.4 (19)
Almost every day or every day	6.9 (2)	21.2 (7)	12.5 (4)
Highest level of education			
School to age 16	13.8 (4)	12.1 (4)	15.6 (5)
School to age 18	27.6 (8)	9.1 (3)	9.4 (3)
University	58.6 (17)	78.8 (26)	75.0 (24)
Currently in paid job			

Continued

Table 1 Continued

	% (N)	% (N)	% (N)
No	20.7 (6)	45.5 (15)	25.0 (8)
Yes	75.9 (22)	54.5 (18)	75.0 (24)

HDL, high-density lipoprotein; LDL, low-density lipoprotein.

by waist circumference or per cent body fat. There were also no significant differences in change in fasting glucose or systolic and diastolic blood pressure among the three different fat interventions. In pairwise comparison, coconut oil significantly lowered C reactive protein compared to olive oil but there were no significant differences between coconut oil and butter for C reactive protein.

The results were somewhat surprising for a number of reasons. Coconut oil is predominantly (approximately 90%) saturated fat which is generally held to have an adverse effect on blood lipids by increasing blood LDL-C concentrations. However, the saturated fatty acid profiles of different dietary fats vary substantially; coconut oil is predominantly (around 48%) lauric acid (12:0) compared with butter (66% saturated fat) which is about 40% palmitic (16:0) and stearic (18:0) acids, leading to suggestions that coconut oil may not have the same health effects as other foods high in saturated fat.[9] Nevertheless, though reviews on coconut oil and cardiovascular disease risk factors have concluded that the evidence of an association between coconut oil consumption and blood lipids or cardiovascular risk was mostly poor quality,[9] trials have generally reported that coconut oil consumption raises LDL-C in comparison to polyunsaturated oil such as safflower oil, though not as much in comparison to butter.[10 11]

Based on three randomised crossover trials of good scientific quality, one trial reported butter increased LDL-C more than coconut oil which raised LDL-C more compared with safflower oil[10]; a second reported that coconut oil raised LDL-C more than beef fat which raised LDL-C more than safflower oil[23] and a third reported that coconut oil raised LDL-C more than palm oil which raised LDL-C more than olive oil.[24] The current study observed that butter raised LDL-C more than coconut oil but that coconut oil did not differ from olive oil. Two studies showed higher HDL-C with coconut oil compared with other fats whether beef fat, safflower oil or olive oil.[23 24] Thus far, the current results are consistent with previous studies indicating that butter raises LDL-C more than coconut oil and also that coconut oil also raises HDL-C. However, the present study is an exception in not finding any increase in LDL-C compared with an unsaturated oil, in this case, olive oil. In this trial, the difference of 0.33 mmol/L in LDL-C on butter compared with olive oil is consistent with previous studies.[25]

This is the largest trial reported to date on coconut oil and lipids apart from a recent study of 200 individuals with established coronary heart disease comparing coconut oil with sunflower oil over 2 years that reported no differences in blood lipids but virtually all the participants were on statin therapy[26] which makes findings difficult to interpret.

Direct comparisons between studies are problematic because of different oils used; we used extra virgin olive oil as a comparison group rather than a polyunsaturated oil such as safflower or sunflower oil, for feasibility reasons of likely participant compliance with the requirement for 50 g intake daily. The PREDIMED study reported no significant difference in change in LDL-C or TC but significant lowering of the cholesterol/HDL-C ratio in the Mediterranean diet supplemented with extra virgin olive oil compared with a low-fat diet.[2 13] A recent review reported that high phenolic olive oil does not modify the lipid profile compared with its low phenolic counterpart[14] though other studies have reported that extra virgin olive oil decreases LDL-C directly measured as concentrations of apoB-100 and the total number of LDL particles as assessed by NMR spectroscopy.[27 28] We therefore expected coconut oil would raise LDL-C compared with olive oil, but in the current study, we observed no evidence of an overall average increase in LDL-C in individuals allocated either to the coconut oil or olive oil intervention.

Lack of compliance with consuming the dietary fat would lead to no differences between groups and hence explain the lack of differences in LDL-C between coconut oil and olive oil groups. However, in this group of volunteers, reported compliance was high and did not differ between groups; in addition, those in the coconut oil group had significantly greater increases in HDL-C compared with those allocated to olive oil or butter, so lack of compliance is unlikely to be an explanation.

The predominant fatty acid in coconut oil, lauric acid (C12:0) as well as myristic acid (C14:0) are medium chain fatty acids that are rapidly absorbed, taken up by the liver and oxidised to increase energy expenditure which is a possible explanation for why coconut oil may have different effects compared with other saturated fats[29]. It is also possible that differences could be attributed to the use of extra virgin preparations of coconut oil rather than standard coconut oil; different methods of preparation such as the chilling method for virgin coconut oil compared with refined, bleached and deodorised coconut oil may influence phenolic compounds and antioxidant activity,[30] thus, processing of oils changes their composition, biological properties and consequent potential metabolic effects. The variations in possible health effects resulting from variations in processing of different fats is well documented in the

Table 2 Mean change in variables between baseline and follow-up after dietary interventions and pairwise comparisons between fats in 91 participants

	Change from baseline				Pairwise comparisons		
	Coconut oil	Butter	Olive Oil	P value Comparison between groups	Coconut oil vs olive oil	Butter vs Coconut oil	Butter vs olive oil
	n=28	n=33	n=30				
	Mean (SD)	Mean (SD)	Mean (SD)		Difference (95% CI)	Difference (95% CI)	Difference (95% CI)
LDL-cholesterol (mmol/L)	–0.09 (0.49)	0.33 (0.48)	–0.06 (0.39)	<0.001	–0.04 (–0.27 to 0.19)	0.42 (0.19 to 0.65)	0.38 (0.16 to 0.60)
Total cholesterol (mmol/L)	0.22 (0.55)	0.42 (0.59)	0.03 (0.43)	0.022	0.19 (–0.08 to 0.46)	0.19 (–0.08 to 0.45)	0.38 (0.11 to 0.64)
HDL-cholesterol (mmol/L)	0.28 (0.29)	0.09 (0.27)	0.10 (0.15)	0.009	0.16 (0.03 to 0.28)	–0.18 (– 0.30 to –0.06)	–0.02 (–0.14 to 0.09)
Triglycerides (mmol/L)	0.07 (0.58)	–0.001 (0.36)	–0.03 (0.27)	0.65	0.10 (–0.12 to 0.32)	–0.08 (–0.29 to 0.13)	0.02 (–0.19 to 0.23)
Cholesterol/HDL ratio	–0.26 (0.36)	0.10 (0.41)	–0.13 (0.32)	<0.001	–0.14 (–0.33 to 0.05)	0.36 (0.18 to 0.54)	0.22 (0.04 to 0.40)
Non HDL-cholesterol (mmol/L)	–0.06 (0.44)	0.33 (0.51)	–0.07 (0.42)	0.001	0.002 (–0.23 to 0.24)	0.39 (0.16 to 0.62)	0.39 (0.16 to 0.62)
Glucose (mmol/L)	–0.05 (0.49)	0.02 (0.48)	–0.06 (0.49)	0.68	0.01 (–0.23 to 0.25)	0.08 (–0.15 to 0.32)	0.09 (–0.14 to 0.33)
C reactive protein (mg/L)	–0.31 (1.09)	–0.04 (0.93)	0.23 (1.40)	0.11	–0.58 (–1.12 to –0.04)	0.29 (–0.24 to 0.82)	–0.29 (–0.80 to 0.23)
Weight (kg)	0.27 (0.77)	0.04 (1.00)	–0.04 (0.84)	0.42	0.30 (–0.16 to 0.76)	–0.22 (-0.67 to 0.23)	0.08 (–0.36 to 0.52)
Waist (cm)	1.29 (3.31)	0.26 (3.43)	0.59 (3.25)	0.52	0.71 (–1.00 to 2.42)	–0.95 (–2.63 to 0.72)	–0.24 (–1.89 to 1.41)
Body fat (%)	0.24 (1.03)	0.34 (1.31)	0.13 (1.30)	0.82	0.09 (–0.54 to 0.73)	0.10 (–0.52 to 0.72)	0.19 (– 0.42 to 0.81)
Body mass index (kg/m^2)	0.09 (0.27)	0.02 (0.35)	–0.01 (0.29)	0.13	0.10 (–0.06 to 0.26)	–0.07 (– 0.22 to 0.09)	0.03 (–0.12 to 0.18)
Systolic blood pressure (mm Hg)	0.18 (11.46)	–3.79 (11.11)	–3.67 (8.23)	0.29	3.91 (–1.22 to 9.04)	–3.22 (–8.26 to 1.82)	0.69 (– 4.26 to 5.64)
Diastolic blood pressure (mm Hg)	–2.02 (5.71)	–1.33 (6.24)	–0.45 (8.48)	0.81	–0.73 (– 3.88 to 2.42)	0.99 (–2.08 to 4.05)	0.26 (–2.78 to 3.30)

HDL, high-density lipoprotein; LDL, low-density lipoprotein.

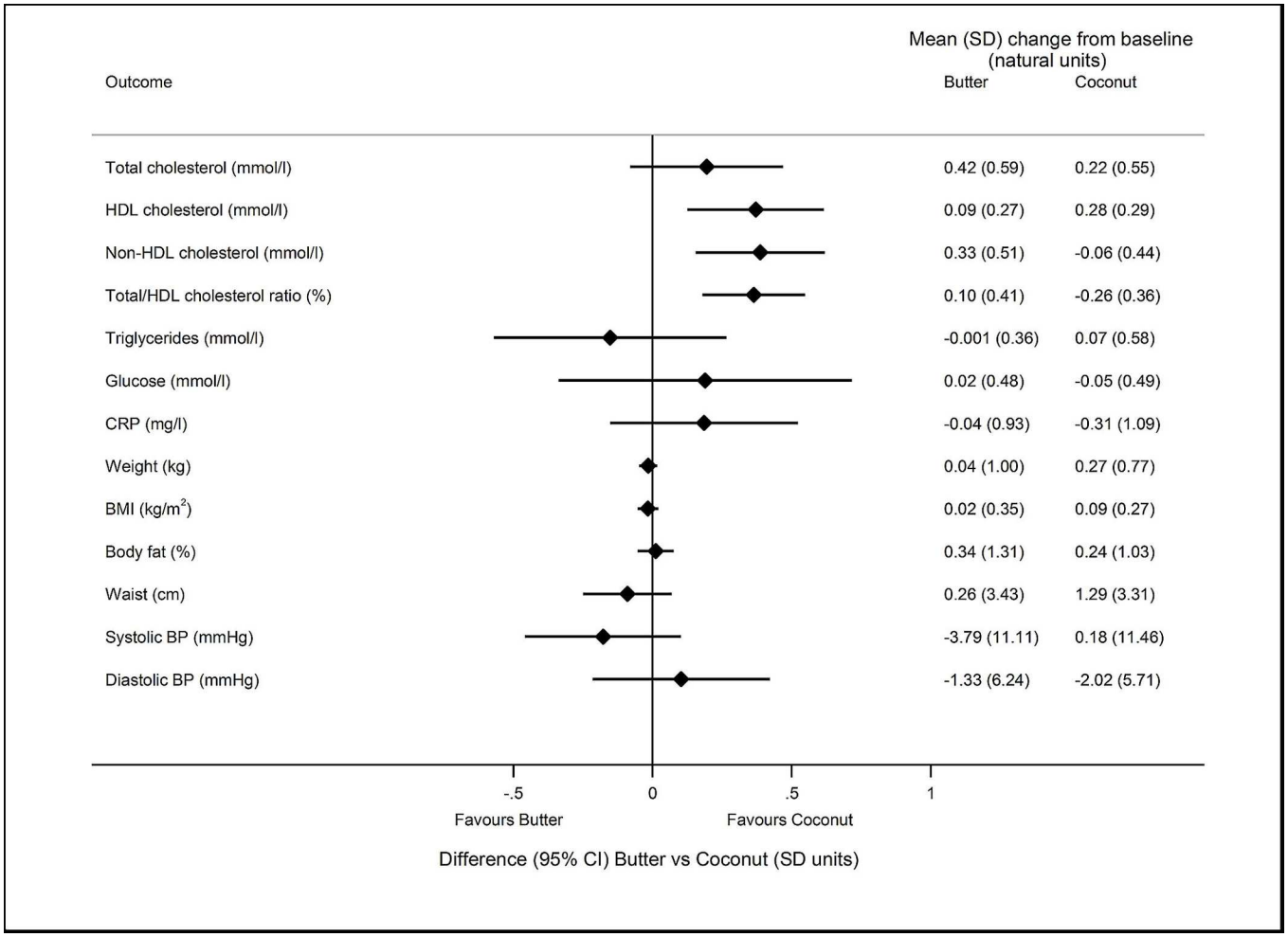

Figure 2 Difference (95% CI) in the primary outcome (LDL cholesterol) between each pair of randomised groups, reported in units of baseline SD. Mean (SD) change from baseline is also presented for each group in mmol/L. COB study, intention-to-treat population, n=91. BMI, body mass index; BP, blood pressure; CRP, C reactive protein; HDL, high-density lipoprotein; LDL, low-density lipoprotein.

large literature on hydrogenation of polyunsaturated oils to make solid margarines which may increase harmful trans fats.[31]. In this context, it is notable that the major trial (PREDIMED) reporting reduction in cardiovascular risk with a Mediterranean diet used extra virgin olive oil,[2] while other studies which reported null findings with olive oil may not have always specified the product used.[14]

There was no evidence of difference between groups in mean weight, BMI, per cent body fat or central adiposity at the end of this trial; however, these were secondary endpoints for which the trial was not specifically powered. Nevertheless, the estimated 95% CI around mean weight differences at the end for the trial were not large. The participants were asked to consume 50 g of fat or oils daily. They could do this in the context of their usual diet by substituting for their usual fats or by consuming these as a supplement. In practice, most participants reported finding it difficult to substitute the different fats or oils for cooking in their usual diet and usually consumed these as a supplement. These fats if taken in addition to their usual diet would have been approximately 450 additional calories daily, which if consistently taken over 4 weeks might be expected to be nearly 13 000 additional calories resulting in likely weight gain of 1–2 kg. This information was provided in the information sheet with the informed consent for participants. While it is possible that participants may have consciously changed behaviours to maintain body weight such as reducing their other dietary intake because of the additional fat or being more physically active, many participants reported that the high-fat diet resulted in feeling full and eating less.

It is also possible that even though this was a randomised trial, in an unblinded study, participants may have changed behaviours differentially in the different intervention groups resulting in differences in lipids or lack of differences in weight observed rather than being attributed to the dietary fat interventions. The majority of the participants reported no change in usual physical activity though slightly more participants in the coconut oil and butter groups reported increasing usual physical activity (14% and 15%, respectively) compared with 4% in the olive oil group. Nevertheless exclusion of all individuals reporting increased usual physical activity from the analyses did not change the findings. Dietary factors

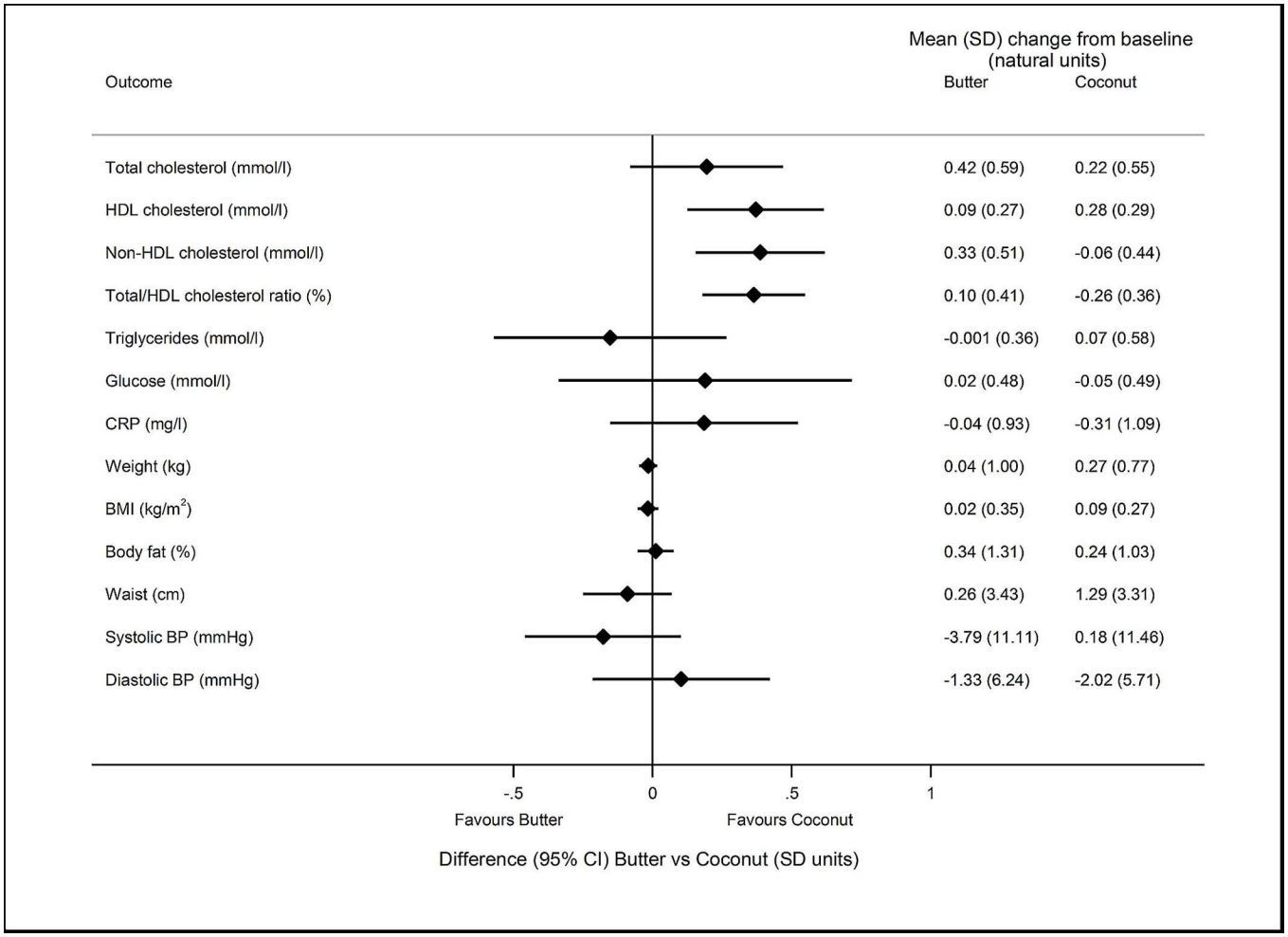

Figure 3 Difference (95% CI) in secondary outcomes comparing butter vs coconut oil groups, reported in units of baseline SD. Mean (SD) change from baseline is also presented for each group in the natural units of the outcome. COB study, intention-to-treat population, n=91. For HDL cholesterol, sign of difference and 95% CI is the opposite of that reported in table 2, on the assumption that higher HDL is better, so the negative estimated difference (butter vs coconut) reported in table 2 is presented on the side of the graph which favours the coconut group. BMI, body mass index; BP, blood pressure; CRP, C reactive protein; HDL, high-density lipoprotein; LDL, low-density lipoprotein.

apart from fat most likely to influence HDL-C, total alcohol intake or change in alcohol intake did not differ significantly between intervention groups and in fact alcohol intake decreased slightly during the trial which would not explain any increases in HDL-C observed. There is therefore no evidence to suggest that differences in lipids or lack of differences in weight change were likely to be attributed to differential changes in behaviour.

The main strengths of this study are the randomised design with high completion rate (91/94 individuals returned to follow-up) and self-reported dietary compliance (nearly 90% participants with over 75% adherence) over 4 weeks. This is also larger than most trials reported with the exception of the trial in India in individuals with heart disease most of whom were taking statins[26]. The current trial by contrast was conducted in individuals in the general population.

This trial has limitations. It was a short-term trial of 4 weeks intervention, so we are unable to know what would have happened if the intervention had continued for a longer period. Moreover, the current findings only apply to the intermediate metabolic (lipid) risk markers and cannot be extended to findings for clinical endpoints.

It was designed as a pragmatic trial in free living individuals rather than a controlled metabolic ward trial such that individuals were asked only to consume the 50 g of allocated fat or oil daily. As this was a 'real-world' study, we made no attempt to control other aspects of their usual diet in particular, total energy intake. For this reason, our results cannot be taken to reflect what would happen when the only change to a diet is the substitution of one fat with another (eg, replacing butter with coconut oil or replacing butter with olive oil). Individuals may have changed their behaviours in different ways to accommodate this additional fat, whether by modifying other aspects of their diet for instance, increasing foods such as bread and potatoes or salads to eat with the fats or consciously reducing other food intake or changing physical activity patterns to control energy balance. Nevertheless, this trial is more reflective of real-life situations.

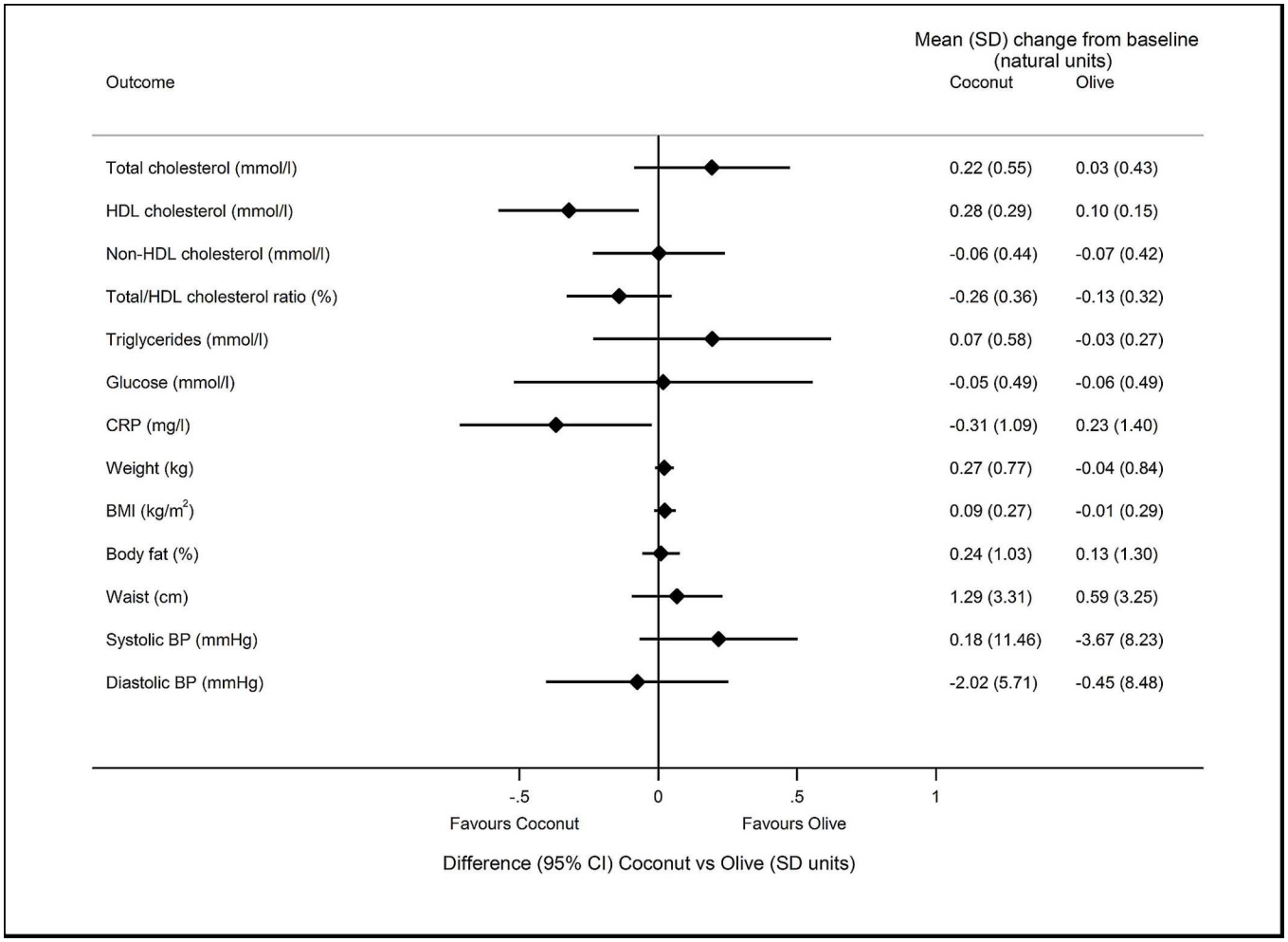

Figure 4 Difference (95% CI) in secondary outcomes comparing coconut oil vs olive oil groups, reported in units of baseline SD. Mean (SD) change from baseline is also presented for each group in the natural units of the outcome. COB study, intention-to-treat population, n=91. For HDL-cholesterol, sign of difference and 95% CI is the opposite of that reported in table 2, on the assumption that higher HDL is better, so the positive estimated difference (coconut vs olive) reported in table 2 is presented on the side of the graph which favours the coconut group. BMI, body mass index; BP, blood pressure; CRP, C reactive protein; HDL, high-density lipoprotein; LDL, low-density lipoprotein.

While self-reported compliance was high, this was subjective and we did not measure the blood fatty acid profile in participants following the intervention for an objective biomarker of compliance. Nevertheless, we did observe differential changes in blood lipids during the intervention.

The generalisability of the findings to the wider population is also unclear. The volunteers were clearly highly selected to be willing to participate in such a study and also likely to be healthier than the general population, as for ethical reasons we excluded those with known prevalent cardiovascular disease, cancer or diabetes and also those on any lipid lowering medication or other contraindications to a high-fat diet. Nevertheless, it is unlikely that the effect of these dietary fats in this group of individuals recruited from the general population would be biologically different from the general population.

Implications

We focused on LDL-C for the primary endpoint as the causal relationship between LDL-C concentrations and coronary heart disease risk is well established, with about a 15% increase in coronary heart disease risk per 1 mmol/L increase in LDL-C concentrations and reduction of LDL-C cholesterol lowers coronary heart disease risk.[32] Increase in LDL-C concentrations has been the main mechanism through which dietary saturated fat is believed to increase heart disease risk, though other pathways have been postulated. However, it is notable that some Mediterranean diet interventions such as the Lyon heart study (alpha linolenic acid)[33] or PREDIMED (extra virgin olive oil)[2] which have been reported to reduce cardiovascular risk in secondary and primary prevention may have effects through other pathways such as inflammation or endothelial function.[34 35] Whatever the mechanisms, the evidence from prospective studies is consistent and strong that substitution of saturated fats by unsaturated fats is beneficial for cardiovascular risk.[36]

The results of this study indicate that two different dietary fats (coconut oil and butter), which are predominantly saturated fats, appear to have different effects

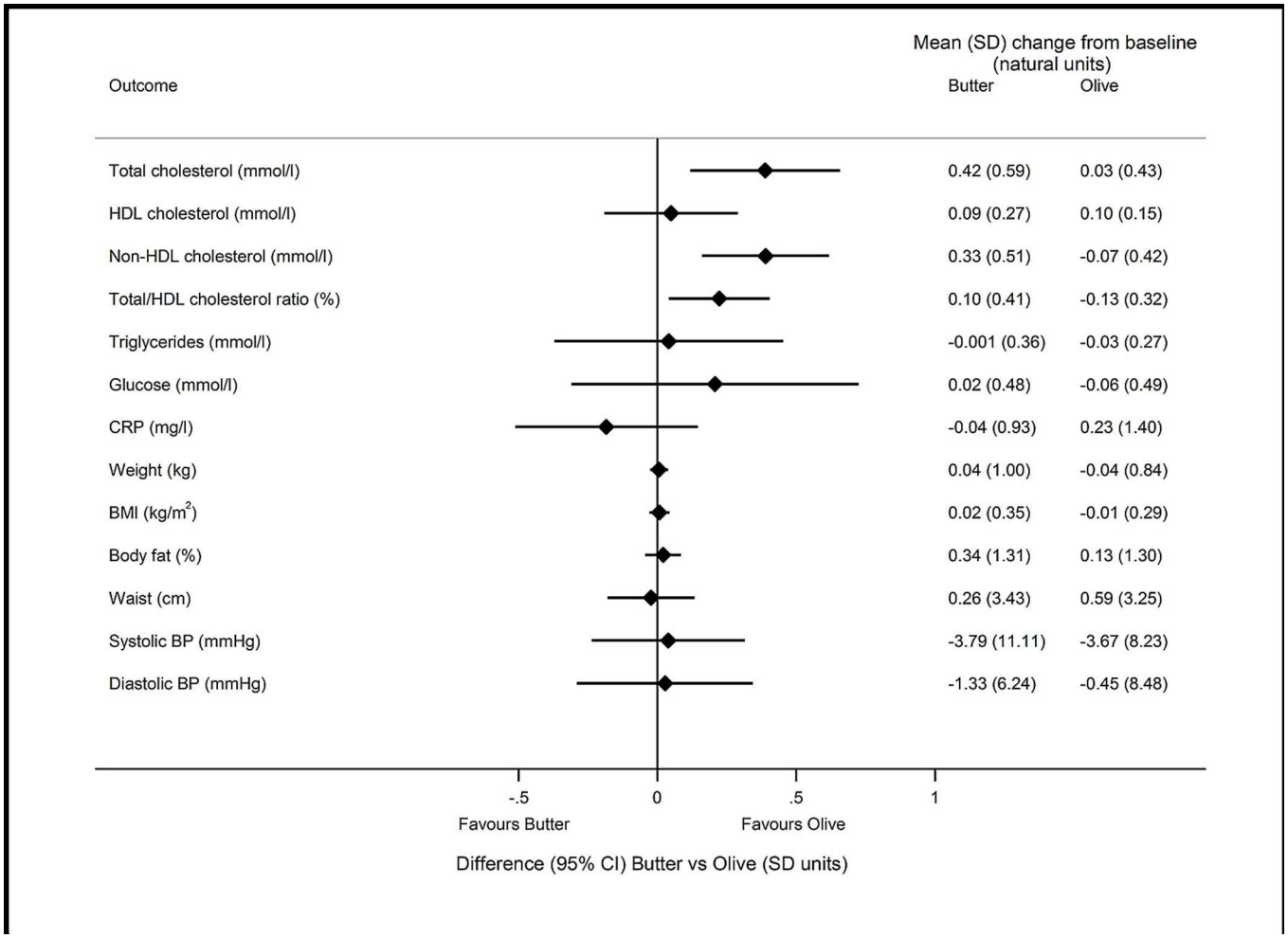

Figure 5 Difference (95% CI) in secondary outcomes comparing butter vs olive oil groups, reported in units of baseline SD. Mean(SD) change from baseline is also presented for each group in the natural units of the outcome. COB study, intention-to-treat population, n=91. For HDL-cholesterol, sign of difference and 95% CI is the opposite of that reported in table 2, on the assumption that higher HDL is better, so the negative estimated difference (butter vs olive) reported in table 2 is presented on the side of the graph which favours the olive group. BMI, body mass index; BP, blood pressure; CRP, C reactive protein; HDL, high-density lipoprotein; LDL, low-density lipoprotein.

on blood lipids compared with olive oil, a predominantly monounsaturated fat. The effects of different dietary fats on lipid profiles, metabolic markers and health outcomes may vary not just according to the general classification of their main component fatty acids as saturated or unsaturated but possibly according to different profiles in individual fatty acids, processing methods as well as the foods in which they are consumed or dietary patterns. There is increasing evidence that associations of saturated fatty acids with health outcomes may vary according to whether they are odd or even chain saturated fatty acids or their chain length.[37–39] Indeed, while overall the evidence indicates the substitution of dietary saturated fats with polyunsaturated fats is beneficial for coronary heart disease risk[40] heterogeneity in findings from observational studies and trials may reflect different dietary sources of fats.[4 41] As the Joint FAO/WHO 2008 Expert Consultation on Fats and Fatty Acids in Human Nutrition comments:

'There are inherent limitations with the convention of grouping fatty acids based only on number of double bonds…major groups of fatty acids are associated with different health effects…individual fatty acids within each broad classification may have unique biological properties or effects…Intakes of individual fatty acids differ across world depending on predominant food sources of total fats and oils.' The associations with health endpoints may well vary depending on the food sources.

In this trial, extra virgin coconut oil was similar to olive oil and did not raise LDL-C in comparison with butter. The current short-term trial on an intermediate cardiovascular disease risk factor, LDL-C, does not provide evidence to modify existing prudent recommendations to reduce saturated fat in the diet as emphasised in most consensus recommendations[8 12] and dietary guidelines should be based on a range of criteria.[42] However, the findings highlight the need for further elucidation of the more nuanced relationships between different dietary fats and health. There is increasing evidence that to understand

Table 3 Baseline and follow-up dietary intake by allocation to coconut oil, butter or olive oil* estimated using 24-hour DietWebQ

DietWebQ intake/day	Coconut oil	Butter	Olive oil
Baseline prior to start of intervention	n=27	n=33	n=32
Energy (MJ/day)	9.0 (3.7)	8.2 (2.2)	9.5 (3.5)
Total fat (g/day)	94 (47)	81 (26)	98 (50)
Protein (g/day)	74 (29)	75 (19)	87 (34)
Carbohydrate (g/day)	238 (95)	215 (75)	243 (95)
Alcohol (g/day)	16 (22)	17 (23)	18 (22)
At 4 weeks of intervention	n=24	n=32	n=27
Energy (MJ/day)	9.6 (3.2)	8.6 (2.4)	9.6 (3.1)
Total fat (g/day)	127 (47)	94 (37)	138 (38)
Protein (g/day)	71 (25)	77 (29)	78 (31)
Carbohydrate (g/day)	215 (84)	214 (64)	197 (101)
Alcohol (g/day)	9 (15)	13(15)	8 (18)
Change from baseline	n=24	n=32	n=27
Energy (MJ/day)	0.3 (2.9)	0.5 (2.0)	−0.4 (2.8)
Total fat (g/day)	29 (43)	14 (36)	28 (40)
Protein (g/day)	−7 (33)	3 (30)	−12 (26)
Carbohydrate (g/day)	−31 (74)	4 (69)	−55(81)
Alcohol (g/day)	−8 (22)	−5(23)	−11 (27)

*Numbers do not total 94 as not all participants completed the baseline and follow-up DietWebQ.

the relationship between diet and health, we need to go beyond simplistic associations between individual nutrients and health outcomes and examine foods and dietary patterns as a whole. In particular, present day diets with high intakes of processed foods now incorporate many fats and oils such as soya bean oil, palm oil and coconut oil which have not been previously widely used in Western societies and not well studied. The relationships between different dietary fats, particularly some of the now more commonly used fats, and health endpoints such as cardiovascular disease events need to be better established.

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Contributors K-TK had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: KT-K, NGF, LF. Acquisition of data: KT-K, NGF, LF, IA, RL, ML. Analysis and interpretation of the data: KT-K, NGF, LF. Drafting of the manuscript: KT-K. Critical revision of the manuscript for important intellectual content: NGF, SJS, IA, LF, RL, ML. Obtaining funding: KT-K, NGF, LF. Administrative, technical or material support: KT-K, NGF, LF, IA, RL, SJS, ML.

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Disclaimer The lead author and guarantor K-TK affirms that the manuscript is an honest, accurate and transparent account of the study being reported; that no important aspects of the study have been omitted and that any discrepancies from the study as planned have been explained.

Competing interests None declared.

Patient consent Obtained.

Ethics approval Ethics approval was given for the study by the University of Cambridge Human Biology Research Ethics committee HBREC 2017.05.

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Circulation

ORIGINAL RESEARCH ARTICLE

Lipoprotein Particle Profiles, Standard Lipids, and Peripheral Artery Disease Incidence

Prospective Data From the Women's Health Study

Editorial, see p 2342

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BACKGROUND: Despite strong and consistent prospective associations of elevated low-density lipoprotein (LDL) cholesterol concentration with incident coronary and cerebrovascular disease, data for incident peripheral artery disease (PAD) are less robust. Atherogenic dyslipidemia characterized by increased small LDL particle (LDL-P) concentration, rather than total LDL cholesterol content, along with elevated triglyceride-rich lipoproteins and low high-density lipoprotein (HDL) cholesterol (HDL-C), may be the primary lipid driver of PAD risk.

METHODS: The study population was a prospective cohort study of 27 888 women ≥45 years old free of cardiovascular disease at baseline and followed for a median of 15.1 years. We tested whether standard lipid concentrations, as well as nuclear magnetic resonance spectroscopy–derived lipoprotein measures, were associated with incident symptomatic PAD (n=110) defined as claudication and/or revascularization.

RESULTS: In age-adjusted analyses, while LDL cholesterol was not associated with incident PAD, we found significant associations for increased total and small LDL-P concentrations, triglycerides, and concentrations of very LDL (VLDL) particle (VLDL-P) subclasses, increased total cholesterol (TC):HDL-C, low HDL-C, and low HDL particle (HDL-P) concentration (all *P* for extreme tertile comparisons <0.05). Findings persisted in multivariable-adjusted models comparing extreme tertiles for elevated total LDL-P (adjusted hazard ratio [HR_{adj}] 2.03; 95% CI, 1.14–3.59), small LDL-P (HR_{adj} 2.17; 95% CI, 1.10–4.27), very large VLDL-P (HR_{adj} 1.68; 95% CI, 1.06–2.66), medium VLDL-P (HR_{adj} 1.98; 95% CI, 1.15–3.41), and TC:HDL-C (HR_{adj}, 3.11; 95% CI, 1.67–5.81). HDL was inversely associated with risk; HR_{adj} for extreme tertiles of HDL-C and HDL-P concentration were 0.30 (*P* trend < 0.0001) and 0.29 (*P* trend < 0.0001), respectively. These components of atherogenic dyslipidemia, including small LDL-P, medium and very large VLDL-P, TC:HDL-C, HDL-C, and HDL-P, were more strongly associated with incident PAD than incident coronary and cerebrovascular disease. Finally, the addition of LDL-P and HDL-P concentration to TC:HDL-C measures identified women at heightened PAD risk.

CONCLUSIONS: In this prospective study, nuclear magnetic resonance–derived measures of LDL-P, but not LDL cholesterol, were associated with incident PAD. Other features of atherogenic dyslipidemia, including elevations in TC:HDL-C, elevations in triglyceride-rich lipoproteins, and low standard and nuclear magnetic resonance–derived measures of HDL, were significant risk determinants. These data help clarify prior inconsistencies and may elucidate a unique lipoprotein signature for PAD compared to coronary and cerebrovascular disease.

CLINICAL TRIAL REGISTRATION: URL: https://www.clinicaltrials.gov/. Unique Identifier: NCT00000479.

Key Words: coronary artery disease ■ lipoproteins ■ magnetic resonance spectroscopy ■ peripheral artery disease

Sources of Funding, see page 2340

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Clinical Perspective

What Is New?

- Among women aged 45 years and older without cardiovascular disease at baseline, elevated levels of low-density lipoprotein cholesterol were not associated with future peripheral artery disease (PAD).
- Using both standard lipids and nuclear magnetic resonance-derived lipoprotein measures, we found strong associations of an atherogenic dyslipidemia profile, including small, dense low-density lipoprotein particle concentration, triglyceride-rich lipoproteins, high-density lipoprotein cholesterol and particle concentration, and total cholesterol: high-density lipoprotein cholesterol with incident PAD.
- These same components of atherogenic dyslipidemia were more strongly associated with PAD than with a composite of cardiovascular and cerebrovascular disease, suggesting a unique lipoprotein profile for incident PAD.

What Are the Clinical Implications?

- Focus on low-density lipoprotein cholesterol in terms of atherosclerotic risk prediction underestimates the risk of PAD among middle-aged, low-risk women.
- The addition of nuclear magnetic resonance-derived lipoprotein measures to traditional lipid measures may improve risk assessment for PAD, and importantly may elucidate a novel therapeutic strategy for PAD prevention.
- Ongoing clinical trials are investigating whether treating atherogenic dyslipidemia, rather than elevations in low-density lipoprotein cholesterol alone, is beneficial in preventing PAD.

Atherogenic dyslipidemia, which comprises a triad of increased blood concentrations of small, dense low-density lipoprotein (LDL) particles (LDL-P), decreased high-density lipoprotein (HDL) particles (HDL-P), and increased triglyceride-rich lipoproteins, has been linked to a composite of coronary artery disease and cerebrovascular disease (CCVD).[1] However, the specific lipoprotein components that contribute to peripheral artery disease (PAD) risk are less clear. In contrast to CCVD, the epidemiological data supporting a link between LDL cholesterol (LDL-C) and incident PAD are limited, especially among women.[2,3] Additionally, individuals with heterozygous familial hypercholesterolemia and genetically elevated levels of LDL-C have notably *lower* rates of PAD compared to coronary artery disease (CAD).[4] Instead, studies suggest that dyslipidemia parameters, such as an elevated ratio of total cholesterol (TC):HDL cholesterol (HDL-C), mixed dyslipidemia, and hypertriglyceridemia, may be the strongest lipid risk factors for incident PAD and PAD progression.[2,5–8]

One means of delineating the lipid-related risk in PAD is by using more detailed lipoprotein measures derived from proton nuclear magnetic resonance (NMR) spectroscopy. Standard lipid panels measure the entire plasma cholesterol or triglyceride content in concentration per deciliter of each lipoprotein class. In contrast, NMR spectroscopy quantifies both the number and size of lipoprotein particles.[9] Plasma cholesterol concentration can differ among individuals due to both variations in particle size as well as metabolic processes that regulate the cholesterol and triglyceride content of the lipoprotein particle core, and these differences often lead to discrepant risk estimates based on traditional versus NMR-derived lipoprotein measures.[10] NMR-derived lipoprotein measures are associated with future myocardial infarction (MI),[11–16] stroke,[11,14,16] diabetes,[17,18] and hypertension.[19] To our knowledge, this methodology has not yet been applied to PAD.

Given the lack of robust data showing a link between LDL-C and PAD, we hypothesized that NMR-derived lipoprotein subclass abnormalities associate with incident PAD and would be distinct from those previously described in other cardiovascular disorders.[11] Therefore, in the current study, we evaluated baseline NMR lipoprotein particles and conventional lipid concentrations in a prospective cohort of middle-aged and older American women free of PAD, MI, and stroke at baseline and measured the association of these lipid measures with incident PAD.

METHODS

Data Availability

The data will not be made available to other researchers for purposes of reproducing the results. However, the methods used in the analysis are available on request.

Study Population

Participants were identified from the WHS (Women's Health Study), a previously completed randomized, double-blind, placebo-controlled trial of low-dose aspirin and vitamin E in the primary prevention of cardiovascular disease.[20] From 1992 to 1995, the study enrolled a total of 39 876 female healthcare professionals in the United States without a history of cancer, MI, stroke, coronary revascularization, or peripheral artery revascularization. At the time of enrollment, women completed questionnaires on baseline demographics, anthropometrics, medical history, and lifestyle factors. Following completion of the trial, willing individuals consented to participate in a longitudinal observational component of the WHS. All participants provided written informed consent, and the study was approved by the institutional review board at Brigham and Women's Hospital.

Before randomization, 28 345 of the participants consented to provide blood samples, and 98.9% (n = 28 024) of these samples underwent NMR lipoprotein profiling. Individuals missing baseline demographic data on body mass

index, as well as history of smoking, hypertension, or hormonal therapy, were excluded from the analysis. In addition, subjects with confirmed prerandomization PAD (n = 30) were excluded from the present analysis. The final study population (n = 27 888) was followed for a median of 15.1 years.

Outcome Ascertainment

Health outcomes of WHS participants were ascertained using annual questionnaires. The primary outcome of interest for the present study was symptomatic lower extremity PAD defined as intermittent claudication and/or peripheral artery revascularization (surgical or percutaneous). To validate reported events, PAD outcomes were initially identified through annual questionnaires, and then confirmed through physician interview and medical records review. For cases of claudication, confirmation was performed using the Edinburgh Claudication Questionnaire, which was administered during telephone interviews conducted by a physician adjudicator. The Edinburgh Claudication Questionnaire is an accepted tool for the detection of PAD that is commonly used in clinical research, and has been validated against in-office physician-diagnosed intermittent claudication with a sensitivity of 91.3% and a specificity of 99.3%.[21] These values for sensitivity and specificity are similar to—and in some cases, higher than—those for reported resting ankle-brachial index.[22] If patients reported lower extremity revascularization on their questionnaire, these events were confirmed by cardiologist review of primary medical records. CCVD was defined as nonfatal MI, percutaneous coronary intervention, coronary artery bypass grafting, nonfatal stroke, or coronary-related death, and these end points were adjudicated as previously described.[23] Utilizing these criteria, we confirmed 130 cases of incident PAD. The most common causes for nonischemic leg pain in disconfirmed cases were venous disease, lower extremity arthritis, lumbar disk disease, and peripheral neuropathy.

Laboratory Analysis

Blood samples were stored in liquid nitrogen (–150°C to –180°C) until analysis. Samples were thawed, aliquoted, and shipped in 200-µL frozen aliquots to LipoScience (now LabCorp, Raleigh, NC) for analysis. The lipoprotein analysis used in the present study is the NMR LipoProfile 4 panel. In this panel, the concentration of each lipoprotein particle subclass is calculated from the NMR signal of terminal methyl groups, and these same NMR signals are used to help calculate weighted-average lipoprotein particle sizes.[10] Particles are classified based on size into the following categories: LDL-P, HDL-P, and very LDL (VLDL-P). Table I in the online-only Data Supplement lists lipoprotein particle diameters.

A core laboratory certified by the National Heart, Lung, and Blood Institute/Centers for Disease Control and Prevention Lipid Standardization Program measured standard lipids and apolipoproteins. LDL-C was measured using a homogeneous direct method with a Hitachi 917 analyzer using reagents from Roche Diagnostics (Indianapolis, IN). HDL-C was measured using a direct enzymatic colorimetric assay, and triglycerides were measured enzymatically with correction for endogenous glycerol. Coefficients of variation were <3% for all standard lipids. Non–HDL-C was calculated by subtracting HDL-C from TC. Apolipoproteins B_{100} and A-1 were measured using immunoturbidometric assays (DiaSorin, Stillwater, MN) with coefficients of variation of 5% and 3%, respectively. High-sensitivity C-reactive protein was measured by a high-sensitivity immunoturbidimetric assay (Denka Seiken, Niigata, Japan).

Statistical Analysis

Continuous data are summarized as either mean±SD or median with interquartile range depending on normality of the distributions. Categorical data are listed as percentages. Between-group differences were assessed by the Wilcoxon rank-sum test for continuous data and the χ^2 test for categorical data. Lipid biomarkers were divided into tertiles. Cox proportional-hazards models were used to estimate the hazard ratio (HR) and 95% CI for each biomarker tertile, and results are presented as top tertile compared to bottom tertile; similar analyses were performed per SD increase of each biomarker. Tests of linear trend across tertiles were performed using the median value from each tertile. We also calculated Spearman rank correlation coefficients to test the relationships between standard and NMR-derived lipoprotein measures.

As preventive therapies instituted at diagnosis of MI or stroke may dramatically alter the subsequent risk of vascular events, we censored women having non-PAD vascular events (e.g., CCVD) at the time of diagnosis. Thus, within these models, follow-up time was censored at the time of the PAD event except in situations in which a CCVD event occurred first—in which case, censoring occurred at the time of the CCVD event. There were a total of 130 confirmed PAD cases, and in 20 of these cases, a CCVD event occurred before the PAD event. Thus, the final population in the current analysis was 110 cases of incident PAD.

Regression models were sequentially adjusted for age followed by smoking pack-years (Model 1). Fully adjusted models (Model 2) were adjusted for age, smoking pack-years, metabolic syndrome, hypertension, postmenopausal hormone therapy, high-sensitivity C-reactive protein, lipid-lowering therapy, and body mass index. Data on smoking pack-years was collected as a categorical variable within WHS, and this variable was divided into the following categories for the purposes of regression modeling: 0, 1 to 10, 11 to 29, and ≥30. All regression results in the text are presented for Model 2 unless otherwise noted. Additionally, all models were adjusted for randomized treatment within the WHS trial. Measures of serum triglycerides were log-transformed for *P* trend analysis due to a right-skewed distribution. Models of LDL particle size were also adjusted for total LDL particle concentration as previously described.[24] Given the inverse correlation of LDL-P subclasses,[24] models assessing each LDL-P subclass were adjusted additionally for the remaining LDL-P subclasses to delineate independent risk associations. The likelihood ratio χ^2 statistic was used to assess model fit. To evaluate the joint effects of LDL-C concentration with LDL-P as well as TC:HDL-C with LDL-P, HDL-P, and VLDL-P, individuals were classified into 4 groups based on the values of each biomarker relative to the population median. Kaplan-Meier survival curves were plotted based on these strata and analyzed using a log-rank test for trend with 3 degrees of freedom. All statistical analyses were performed using SAS statistical software version 9.4 (SAS Institute, Cary, NC). All 95% CIs are 2-tailed, and the *P* value cutoff for all analyses was 0.05.

RESULTS

As shown in Table 1, women with incident PAD were more likely to be older, be current smokers, and have higher rates of baseline hypertension. In this population with a low prevalence of baseline diabetes, there was no significant difference in diagnosed diabetes, although individuals with incident PAD were more likely to have a history of metabolic syndrome. Baseline levels of triglycerides, apolipoprotein B_{100}, non–HDL-C, TC:HDL-C, and high-sensitivity C-reactive protein were all higher in individuals who developed PAD. The baseline levels of HDL-C and apolipoprotein A-1 were lower in individuals with incident PAD. There was no statistically significant difference in TC or LDL-C.

Table 2 shows median concentrations of NMR-derived lipoprotein particles according to case status. Total LDL-P and small LDL-P subclass concentrations were

Table 1. Baseline Characteristics of the Study Population

	Women Remaining Free of PAD Events (n=27 778)*	Women Developing PAD Events (n=110)†	*P* Value
Age, mean (SD), y	54.7 (7.1)	59.2 (7.5)	<0.0001
BMI, mean (SD), kg/m^2	25.9 (5.0)	25.7 (4.5)	0.75
Non-Hispanic white, %	95.3	98.2	0.25
Current smoking, %	11.5	47.3	<0.0001
Prior smoking, %	36.6	36.4	1.00
Pack-years, %			
0	52.3	16.5	<0.0001
1–10	15.3	6.4	
11–29	22.6	32.1	
≥30	9.8	45.0	
Diabetes, %	2.5	3.6	0.35
Metabolic syndrome, %	24.6	33.6	0.03
Hypertension, %	25.1	39.1	0.001
Treatment for hypercholesterolemia, %	3.2	5.5	0.17
Family history of premature CAD, %	14.4	18.5	0.22
Exercise ≥1 time/wk, %	43.2	38.2	0.33
Current HT use, %	42.6	36.4	0.21
WHS trial assignment to vitamin E, %	50.1	49.1	0.85
WHS trial assignment to aspirin, %	50.1	50.9	0.92
hsCRP, mg/L	2.0 (0.8–4.4)	2.8 (1.6–6.6)	<0.0001
Standard chemical lipids, mg/dL			
Total cholesterol	208 (184–235)	214 (185–246)	0.13
LDL cholesterol	121 (101–144)	130 (103–153)	0.05
HDL cholesterol	52 (43–62)	44 (37–55)	<0.0001
Triglycerides	118 (84–175)	146 (104–218)	0.0001
Apolipoproteins, mg/dL			
Apolipoprotein B_{100}	100 (84–121)	115 (91–133)	<0.0001
Apolipoprotein A-1	149 (132–168)	138 (124–152)	<0.0001
Non–HDL cholesterol, mg/dL	154 (129–182)	172 (137–200)	0.0007
Total cholesterol:HDL cholesterol	3.97 (3.23–4.92)	4.66 (3.87–6.02)	<0.0001

Values are median (25th to 75th percentile) unless otherwise indicated. *P* values for continuous variables were obtained from the Wilcoxon rank-sum test. *P* values for categorical variables were obtained using the chi-square test. BMI indicates body mass index; CAD, coronary artery disease; hsCRP, high-sensitivity C-reactive protein; HDL, high-density lipoprotein; HT, hormonal therapy; LDL, low-density lipoprotein; PAD, peripheral artery disease; and WHS, Women's Health Study.

*Number missing: 234 for race; 227 for pack-years; 15 for diabetes, 49 for metabolic syndrome; 20 for treatment for hypercholesterolemia; 462 for family history of premature CAD; 10 for exercise; 86 for hsCRP; 87 each for total cholesterol and HDL cholesterol; 86 each for LDL cholesterol and triglycerides; 224 for apolipoprotein B_{100}; 220 for apolipoprotein A-1; and 88 each for non-HDL cholesterol and total cholesterol: HDL cholesterol.

†Number missing: 1 for race; 1 for pack-years; 2 for family history of premature CAD; and 4 each for apolipoprotein B_{100} and apolipoprotein A-1.

Table 2. Baseline NMR Lipoprotein Profile

	Women Remaining Free of PAD Events (n=27 778)*	Women Developing PAD Events (n=110)†	*P* Value
NMR lipoprotein particle concentrations, nmol/L			
LDL particles			
Total	1567 (1329–1839)	1723 (1495–1989)	<0.0001
Large	306 (160–467)	233 (69–474)	0.02
Medium	156 (0–347)	128 (0–285)	0.03
Small	953 (685–1336)	1208 (875–1599)	<0.0001
VLDL particles			
Total	166.5 (130.3–208.4)	180.1 (145.3–221.9)	0.005
Very large	0.1 (0.1–0.2)	0.2 (0.1–0.5)	0.003
Large	1.6 (0.3–4.3)	2.9 (0.6–5.5)	0.004
Medium	15.8 (8.5–25.5)	20.8 (13.2–31.9)	0.0009
Small	55.4 (34.0–81.8)	62.2 (36.3–85.2)	0.22
Very small	84.3 (58.9–114.8)	87.8 (64.9–118.9)	0.18
HDL particles			
Total	24 400 (22 000–27 000)	23 150 (20 950–25 250)	0.0009
Large	2100 (1300–3300)	1600 (1050–2500)	0.0002
Medium	5300 (3700–7200)	3850 (2600–6200)	<0.0001
Small	16 300 (14 100–18 700)	16 750 (15 150–18 650)	0.11
NMR average particle size, nm			
LDL particles	20.9 (20.6–21.2)	20.7 (20.4–21.1)	0.003
VLDL particles	42.5 (38.6–47.9)	44.0 (39.1–50.8)	0.04
HDL particles	8.9 (8.7–9.2)	8.7 (8.6–9.1)	0.0001

Values are median (25th to 75th percentile). *P* values were obtained from the Wilcoxon rank-sum test. HDL indicates high-density lipoprotein; LDL, low-density lipoprotein; NMR, nuclear magnetic resonance; PAD refers to peripheral artery disease; and VLDL, very low-density lipoprotein.

*Number missing: 581.

†Number missing: 6.

higher in women with PAD, whereas large LDL-P, medium LDL-P, and total HDL-P were significantly lower. Among HDL-P subclasses, all but small HDL-P were lower in individuals with PAD. Total very LDL (VLDL) particles (VLDL-P), very large VLDL-P, large VLDL-P, and medium VLDL-P concentrations were higher in those with PAD. Differences in small and very small VLDL-P concentrations did not reach statistical significance. Consistent with data for particle subclass concentrations, women developing PAD had smaller average LDL-P and HDL-P size and a larger average VLDL-P size.

Table II in the online-only Data Supplement shows Spearman correlation coefficients for NMR lipoproteins with standard lipid and apolipoprotein measures in the total sample. Total LDL-P concentration correlated strongly with LDL-C (r=0.71), as well as apolipoprotein B-100 (r=0.86), non–HDL-C (r=0.78), and TC:HDL-C (r=0.66). Large and small LDL-P concentration correlated modestly with LDL-C (r=0.27 and 0.25, respectively). Large HDL-P correlated strongly with HDL-C (r=0.75), but total HDL-P (r=0.52) and the medium HDL-P subclass (r=0.50) showed more modest correlations. Table III in the online-only Data Supplement lists Spearman correlation coefficients for NMR lipoproteins with themselves. Total LDL-P strongly correlated with small LDL-P (r=0.63), and total VLDL-P most strongly correlated with very small VLDL-P (r=0.69). HDL-P had similar positive correlations with large, medium, and small HDL-P subclasses (r=0.35, 0.49, and 0.51, respectively).

Figure 1 and Table IV in the online-only Data Supplement show the results from Cox regression analyses adjusted for both age and nonlipid risk factors in women classified based on *standard* lipid and apolipoprotein tertiles. The strongest positive risk association was with TC:HDL-C (multivariable-adjusted HR, 3.11; 95% CI, 1.67 to 5.81; *P* trend=0.0005). Serum triglyceride concentration was strongly associated with incident PAD in age-adjusted, but not multivariable-adjusted, models (adjusted HR, 1.46; 95% CI, 0.82 to 2.61; *P* trend=0.22). Significant findings were also seen for both apolipoprotein B_{100} and non–HDL-C in age-adjusted but not multivariable-adjusted models. Importantly, no significant associations were seen for TC or LDL-C. In both age-adjusted and multivariable-adjusted mod-

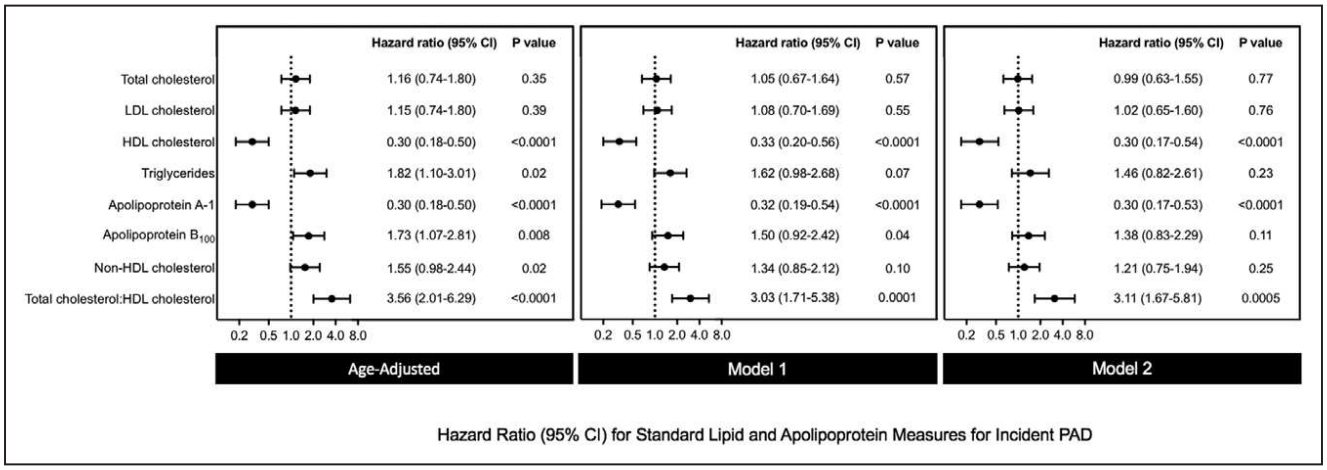

Figure 1. Risk associations between standard lipid and apolipoprotein measures and incident peripheral artery disease.
Hazard ratios and 95% CIs for the top versus bottom tertile of standard lipid and apolipoprotein measures. Model 1 adjusted for age and smoking pack-years. Model 2 adjusted for age, smoking pack-years, metabolic syndrome, hypertension, hormonal therapy, high-sensitivity C-reactive protein, lipid-lowering therapy, randomized treatment assignment, and body mass index. HDL indicates high-density lipoprotein; and LDL, low-density lipoprotein.

els, HDL-C concentration was inversely associated with PAD with a 70% lower relative risk for the lowest tertile versus the highest (adjusted HR, 0.30; 95% CI, 0.17 to 0.54; *P* trend<0.0001). There was a similar strong inverse association seen with apolipoprotein A-1 in fully adjusted models (HR, 0.30; 95% CI, 0.17 to 0.53; *P* trend<0.0001). Overall, similar results were seen for incident PAD per SD increase of each biomarker (Table V in the online-only Data Supplement), although the association for apolipoprotein B_{100} reached statistical significance in the fully adjusted model (HR, 1.23; 95% CI, 1.02 to 1.49; *P* trend=0.03).

Similarly, age-adjusted and multivariable-adjusted analyses for *total NMR-derived* lipoprotein particle concentrations are displayed in Figure 2 and Table VI in the online-only Data Supplement. In contrast to the null association of LDL-C with PAD, total LDL-P was the strongest positive lipoprotein risk factor (adjusted extreme tertile HR, 2.03; 95% CI, 1.14 to 3.59; *P* trend=0.02). Both LDL-P size (adjusted extreme tertile HR, 0.60; 95% CI, 0.36 to 1.02; *P* trend=0.02) and HDL-P size (adjusted extreme tertile HR, 0.39; 95% CI, 0.21 to 0.70; *P* trend=0.002) were inversely associated with incident PAD. No significant association was seen for total VLDL-P concentration or VLDL-P size. Total HDL-P was inversely associated with PAD (adjusted HR, 0.29; 95% CI, 0.16 to 0.52; *P* trend<0.0001). The associations between each biomarker analyzed per SD and incident PAD are displayed in Table VII in the online-only Data Supplement.

In multivariable models that evaluated *lipoprotein particle subclass concentrations*, small LDL-P remained significantly associated with incident PAD (adjusted HR, 2.17; 95% CI, 1.10 to 4.27; *P* trend=0.02) (Figure 3; Table VIII in the online-only Data Supplement). No residual association was seen for large or medium LDL-P. Both large and medium HDL-P were associated with protection

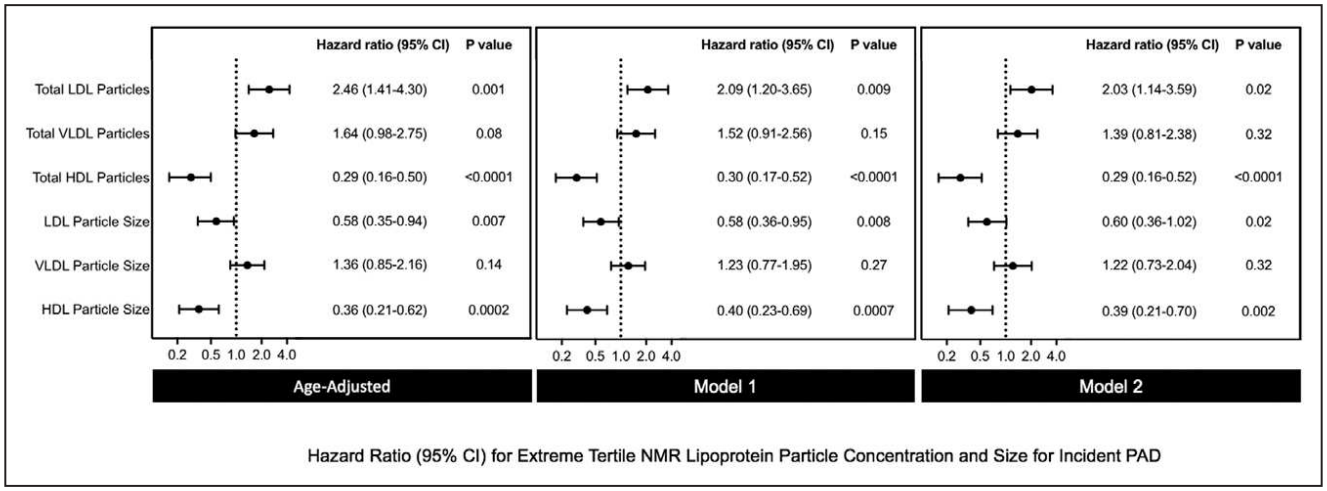

Figure 2. Risk associations between nuclear magnetic resonance lipoprotein particle concentrations and size and incident peripheral artery disease.
Hazard ratios and 95% CIs for the top versus bottom tertile of nuclear magnetic resonance lipoprotein particle concentrations and sizes. Model 1 adjusted for age and smoking pack-years. Model 2 adjusted for age, smoking pack-years, metabolic syndrome, hypertension, hormonal therapy, high-sensitivity C-reactive protein, lipid-lowering therapy, randomized treatment assignment, and body mass index. HDL indicates high-density lipoprotein; LDL, low-density lipoprotein; and VLDL, very LDL.

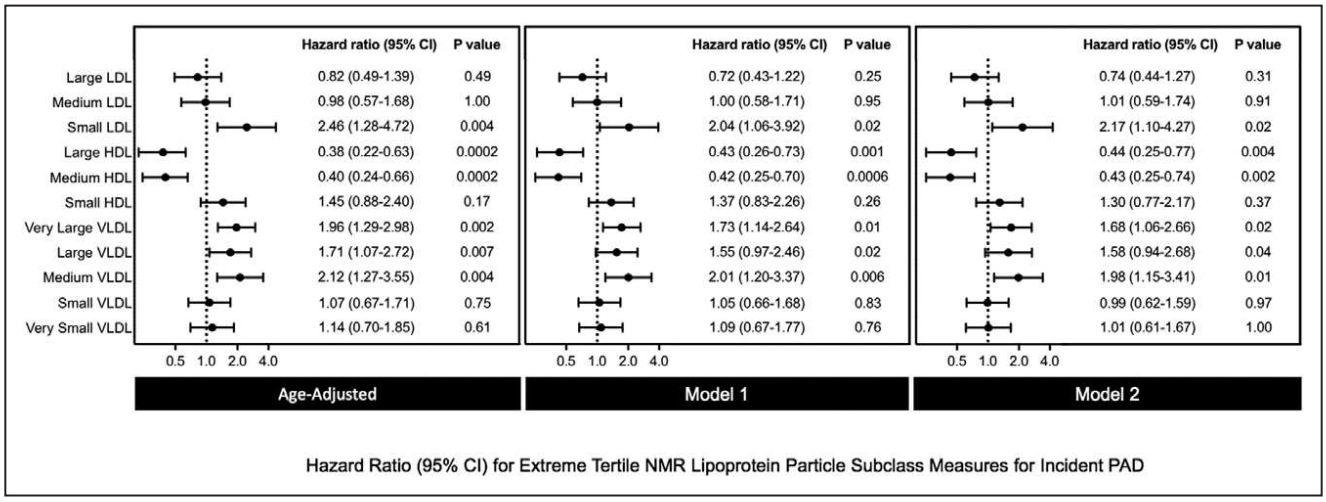

Figure 3. Risk associations between nuclear magnetic resonance lipoprotein particle subclasses and incident peripheral artery disease.
Hazard ratios and 95% CIs for the top versus bottom tertile of nuclear magnetic resonance lipoprotein particle subclasses. Model 1 adjusted for age and smoking pack-years. Model 2 adjusted for age, smoking pack-years, metabolic syndrome, hypertension, hormonal therapy, high-sensitivity C-reactive protein, lipid-lowering therapy, randomized treatment assignment, and body mass index. HDL indicates high-density lipoprotein; LDL, low-density lipoprotein; and VLDL, very LDL.

against PAD (adjusted HR, 0.44; 95% CI, 0.25 to 0.77; *P* trend=0.004; and adjusted HR, 0.43; 95% CI, 0.25 to 0.74; *P* trend=0.002, respectively). Of VLDL-P subclasses, very large (size range: 90 to 240 nm), large (size range: 50 to 89 nm), and medium VLDL-P (size range: 37 to 49 nm) were significantly associated with incident PAD (adjusted HR, 1.68; 95% CI, 1.06 to 2.66; *P* trend=0.02; adjusted HR, 1.58; 95% CI, 0.94 to 2.68; *P* trend=0.04; and adjusted HR, 1.98; 95% CI, 1.15 to 3.41; *P* trend=0.01, respectively). Table IX in the online-only Data Supplement shows the association per SD increase in each biomarker.

Figure 4 (Figure I and Table X in the online-only Data Supplement) displays multivariable-adjusted risk associations for standard lipid, apolipoprotein, and NMR lipoprotein measures for both incident PAD and CCVD ranked by magnitude of the risk estimate. Results are

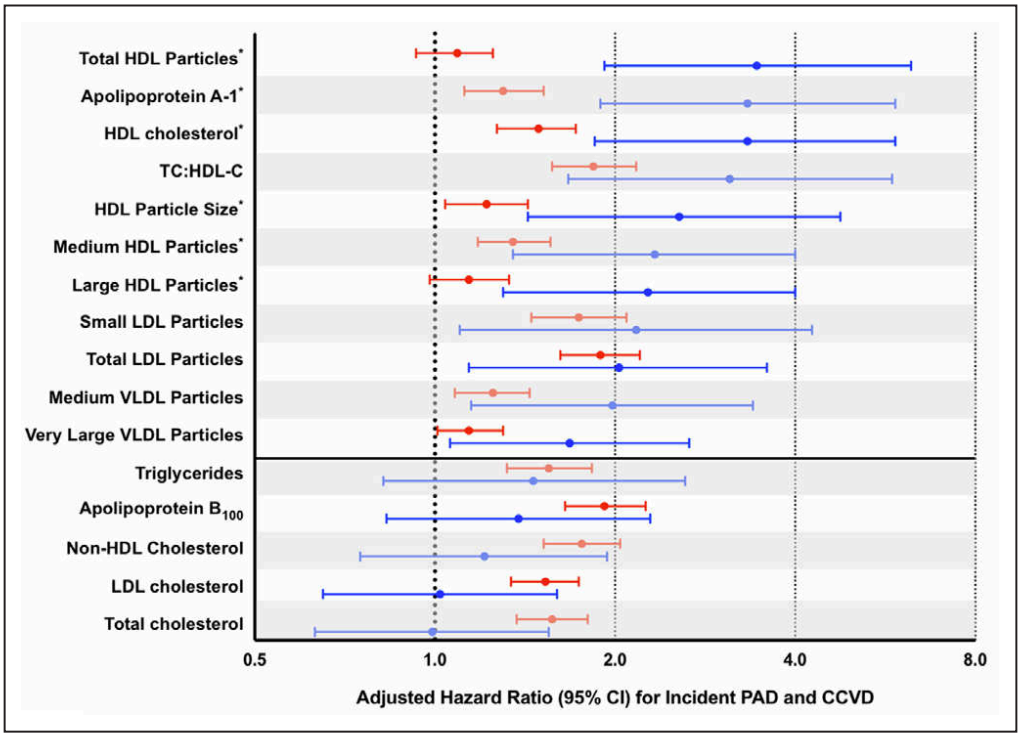

Figure 4. Risk associations between nuclear magnetic resonance lipoprotein and standard lipid measures with incident peripheral artery disease (PAD) versus incident coronary and cerebrovascular disease (CCVD).
Hazard ratios and 95% CIs for the top versus bottom tertile of incident PAD (blue) and CCVD (red), adjusted for age, smoking pack-years, metabolic syndrome, hypertension, hormonal therapy, high-sensitivity C-reactive protein, lipid-lowering therapy, randomized treatment assignment, and body mass index. Measures displayed include all standard lipid and apolipoprotein assays, as well as nuclear magnetic resonance–derived measures with a statistically significant association for incident PAD. Horizontal line separates markers of atherogenic dyslipidemia from other measures without statistical significance for incident PAD. HDL indicates high-density lipoprotein; LDL, low-density lipoprotein; TC:HDL-C, total cholesterol:HDL cholesterol; and VLDL, very LDL.

displayed comparing highest versus lowest tertile, except for biomarkers associated with protection against incident disease, in which case results are presented as lowest versus highest tertile to facilitate comparisons. Of all measures analyzed, HDL-P, apolipoprotein A-1, HDL-C, and TC:HDL-C had the largest HRs for incident PAD (Figure 4). Statistically significant associations were also seen for HDL-P size, medium and large HDL-P, small and total LDL-P, and medium, large, and very large VLDL-P.

Although TC and LDL-C were not associated with incident PAD, both were associated with incident CCVD (adjusted HR, 1.57; 95% CI, 1.37 to 1.80; *P* trend<0.0001; and adjusted HR, 1.53; 95% CI, 1.34 to 1.74; *P* trend<0.0001, respectively [Figure 4; Figure II and Table X in the online-only Data Supplement]). Among standard lipid and apolipoprotein measures, adjusted HRs for apolipoprotein B_{100} and non–HDL-C were nominally larger and statistically significant only for incident CCVD, while HDL-C, apolipoprotein A-1, and TC:HDL-C were more strongly associated with incident PAD. Of the NMR-derived lipoprotein measures, total LDL-P, small LDL-P, large and medium subclasses of VLDL-P, and total HDL-P appeared more strongly associated with incident PAD than incident CCVD.

Women were categorized based on both LDL-C and total LDL-P concentration (above or below median) to evaluate the joint role of these biomarkers in PAD risk prediction (Figure 5A). Overall, women with total LDL-P values above the population median were at highest risk of incident PAD, irrespective of their LDL-C measure. Additionally, we reclassified women based on total LDL-P, HDL-P, and VLDL-P concentrations and TC:HDL-C (above or below median; Figure 5B through 5D). Even among those with elevated TC:HDL-C, the addition of total LDL-P or total HDL-P concentration further differentiated individuals based on their risk of incident PAD. VLDL-P levels above the population median did not increase the incidence of PAD beyond the risk of an elevated TC:HDL-C.

DISCUSSION

In this prospective evaluation comparing standard lipid and NMR-derived lipoprotein measures, we found that TC:HDL-C, as well as total and small LDL-P concentration, had strong positive associations for incident PAD, particularly in comparison with LDL-C, non–HDL-C, and $apoB_{100}$, which had no significant associations in multivariable models. Medium, large, and very large VLDL-P were also significantly associated with PAD, while plasma triglycerides were a significant risk predictor in age-adjusted models only. In aggregate, these findings provide evidence that the atherogenic dyslipidemia profile is an important determinant of PAD risk in women, and this profile appears more strongly linked to incident PAD than to CCVD. Although small, retrospective stud-

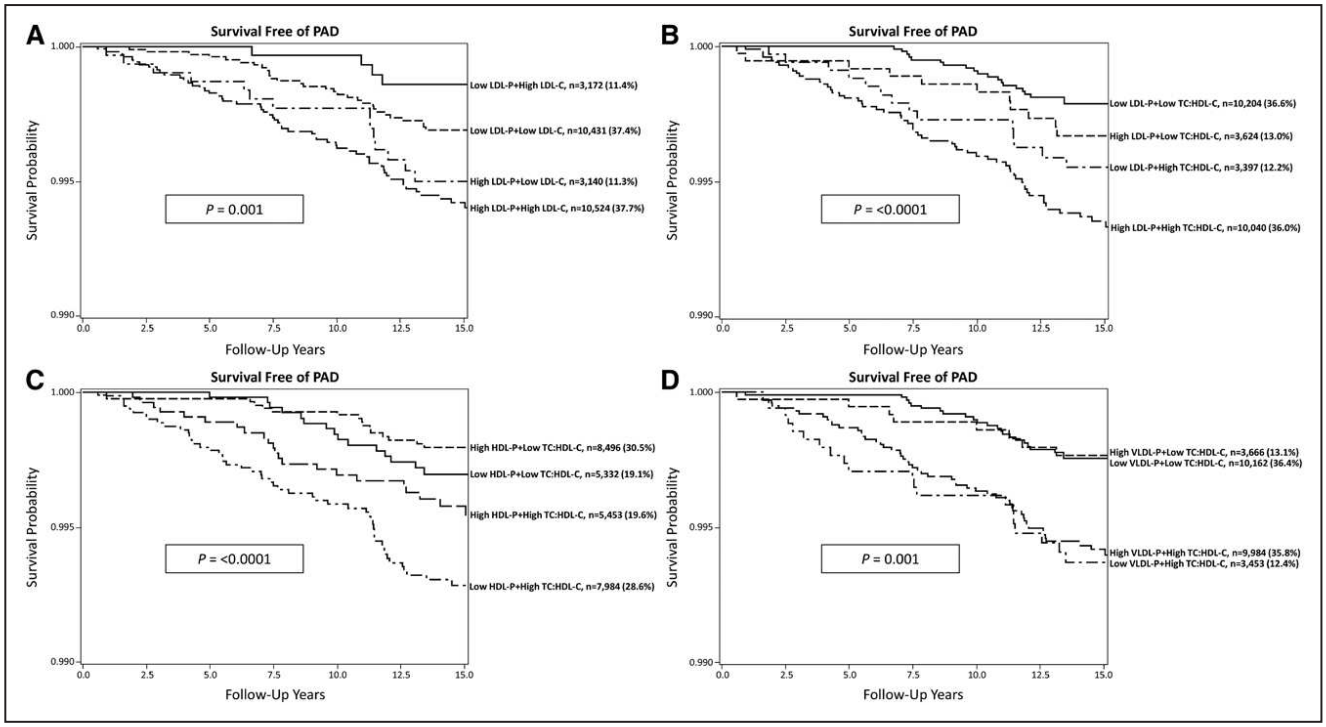

Figure 5. Joint effects of nuclear magnetic resonance lipoprotein and standard lipid measures with incident peripheral artery disease (PAD). **A**, PAD survival curve according to LDL-C and LDL-P particle concentration (above or below population median). **B**, PAD survival curve according to LDL-P particle concentration and TC:HDL-C (above or below population median). **C**, PAD survival curve according to HDL-P particle concentration and TC:HDL-C (above or below population median). **D**, PAD survival curve according to VLDL-P particle concentration and TC:HDL-C (above or below population median). HDL-C indicates high-density lipoprotein cholesterol; HDL-P, high-density lipoprotein particle concentration; LDL-C, low-density lipoprotein cholesterol; LDL-P, low-density lipoprotein particle concentration; TC:HDL-C, total cholesterol:HDL-C; and VLDL-P, very low-density lipoprotein particle concentration.

ies have shown an association between atherogenic dyslipidemia and PAD,[25,26] the current data provide a more robust evaluation of this important issue, including lipid subclassification, which may explain prior inconsistencies. Our data also show a joint association of total LDL-P concentration with measures of both LDL-C and TC:HDL-C, such that women with elevations of total LDL-P were at higher risk of PAD irrespective of these traditional lipid measures alone. Thus, if confirmed in other cohorts, our data suggest that LDL particle number may provide important prognostic information for PAD incidence in women, among whom few prospective data currently exist.

In contrast to well established associations for CCVD, the link between LDL-C and PAD is not robust. Few published studies have demonstrated that elevations of LDL-C are associated with incident PAD.[2,3] In the Physicians' Health Study, which was restricted to men, elevated LDL-C was a risk factor for developing PAD, but had no added value beyond the association with TC:HDL-C in models adjusting for both.[2] Data from the Cardiovascular Health Study showed that LDL-C measures within the highest quartile were associated with incident PAD in both men and women.[3] Of note, this cohort comprised older individuals (aged >65 years, mean baseline age ≈74 years) and included both subjects with prior cardiovascular disease, as well as men. Risk associations were not provided separately for women. Indeed, in contrast to CAD, published data on the link between LDL-C and PAD are notably absent from several large prospective cohorts having measured lipid levels and follow-up for PAD, including the Framingham Offspring Study,[27] the MESA study (Multi-Ethnic Study of Atherosclerosis),[5] and the Edinburgh Artery Study.[28]

Studies of patients with familial hypercholesterolemia and, thus, hereditary elevations in LDL-C, may also be informative. In these studies, prevalence of clinical coronary and cerebrovascular disease is higher than clinical PAD. In a large cohort of 2752 individuals with molecularly confirmed heterozygous familial hypercholesterolemia, 0.5% had a history of peripheral revascularization, while 9.0% had undergone coronary revascularization.[4] Other cohorts have also noted a lower prevalence of PAD compared to CAD in individuals with heterozygous familial hypercholesterolemia.[29,30] LDL particle concentrations were not reported in any of these investigations.

In addition to a risk association with TC:HDL-C, the present study found that both total LDL-P and small LDL-P concentrations were linked to PAD in women, and LDL particle size was inversely associated with incident PAD. Even among women with TC:HDL-C measures above the median, the addition of total LDL-P concentration values identified women at even greater risk of PAD. There are several potential explanations for these findings. Importantly, with even modestly elevated levels of serum triglycerides, triglyceride-rich lipoproteins (such as VLDL) exchange triglyceride molecules with cholesterol from large LDL particles with cholesterol-rich cores.[10] As a result, large LDL particles become enriched for triglycerides and undergo subsequent hydrolysis and conversion to small LDL. Individuals with smaller LDL particles also tend to have greater concentrations of LDL particles, which may further explain the risk association seen in our analysis.[24] Although prospective data for PAD are sparse, it is interesting to note that metabolic syndrome has been linked to a heightened risk of PAD,[31] and the predominant dyslipidemia pattern in these individuals is elevated small LDL-P and relatively normal LDL-C.[32]

Our findings pertaining to triglyceride-rich lipoproteins, such as VLDL, are of particular interest. These lipoproteins can cause increased inflammation, monocyte activation, and endothelial dysfunction.[33] In addition, the size of triglyceride-rich lipoproteins may be important. As previously discussed, large VLDL particles serve as a reservoir for triglyceride exchange with cholesterol-rich large LDL particles, thus facilitating their transition from large to highly atherogenic small LDL particles.[10] As a potential second mechanism, partially hydrolyzed VLDL particles in the ≤70 nm range (which includes large and medium VLDL particles in the current analysis) are small enough to traverse the endothelial barrier.[34] These cholesterol ester-enriched VLDL remnants may bind to and be retained by the connective tissue matrix, where uptake by arterial macrophages leads to foam cell formation. Previous studies have also shown triglyceride levels to be associated with PAD risk,[7,8] and trial data suggest both triglyceride lowering and raising of HDL-C with fibrate therapy may reduce claudication severity.[35] We found stronger risk associations for triglyceride-rich very large (90 to 240 nm), large (50 to 89 nm), and medium (37 to 49 nm) VLDL particles than for triglyceride level alone or even non–HDL-C concentration, suggesting that packaging in these triglyceride-rich lipoprotein particles may be the more potent driver of PAD risk.

Our findings with regard to HDL-C are not new, and several previous studies have found a negative correlation between HDL-C concentration and PAD.[36,37] Indeed, given the null risk association between TC and incident PAD, HDL-C (along with triglyceride-rich VLDL) is the primary driver of risk for TC:HDL-C in our study. However, we also note that concentrations of apolipoprotein A-1, HDL-C, and total HDL-P concentration, as well as HDL-P size were inversely associated with PAD. Additionally, low levels of HDL-C identified women at heightened risk for PAD beyond that of TC:HDL-C. HDL particle subclasses vary in terms of both cholesterol and apolipoprotein A-1 composition,[38] and some data suggest that large HDL particles are protective against CAD.[39] Increased concentration of small HDL-P is associated with prediabetes,[40] insulin-resistance,[41] and ab-

dominal obesity,[42] again suggesting a potential mechanistic link between atherogenic dyslipidemia and PAD.

These findings have several important clinical implications. First, they add to the growing body of evidence that the atherogenic dyslipidemia phenotype is a precursor to PAD. Second, our findings suggest that focus on LDL-C as a clinical risk factor for PAD, at least in women, may be insufficient, and that further characterization of LDL and VLDL particle concentrations may identify women at heightened risk of PAD who would otherwise remain undetected. Our data also suggest there may be important differences in the development of atherosclerosis and thrombosis in different arterial beds. Indeed, in clinical practice, many patients develop severe manifestations of PAD, but never exhibit overt evidence of CCVD, such as angina or MI. This was also seen in our analysis, in which 95 of 110 total individuals with incident PAD never suffered a CCVD event (data not shown). Although LDL-C may be an important risk factor for subclinical atherosclerosis in PAD as it is in CAD, our findings suggest that a lipoprotein profile of elevated triglyceride-rich lipoproteins, increased LDL particle (in particular small particle) concentration, and low HDL particle concentration may be more important in the pathogenesis of symptomatic PAD.

Our findings may be relevant for therapeutic trials in PAD patients. Observational studies have suggested a benefit of statin therapy on limb outcomes.[43,44] Arya et al. found that among 155 647 patients with incident PAD in the Veterans Affairs health system, statin utilization within the first year was associated with large and significant reductions in lower extremity amputation compared to individuals prescribed antiplatelet therapy alone.[44] However, because of statin pleiotropy,[45–47] it remains unclear whether this benefit was due to LDL-C reduction, inflammation reduction, or improvement in atherogenic dyslipidemia. Finally, it remains possible that individuals receiving statins in this observational study were also benefitting from more guideline-directed therapy overall. Data from the FOURIER (Further Cardiovascular Outcomes Research With PCSK9 Inhibition in Patients With Elevated Risk) randomized clinical trial of evolocumab, a PCSK9 inhibitor with potent LDL-C–lowering effects, and modest improvements in triglyceride and HDL-C levels, but no substantive high-sensitivity C-reactive protein reduction, add some clarity in this regard.[48] A 42% reduction in major adverse limb events was observed.[49] However, treatment effects on LDL particle number and other components of atherogenic dyslipidemia are currently unavailable and may yet explain these findings. Whether treatment of atherogenic dyslipidemia per se improves limb-related vascular outcomes will be assessed in the recently initiated PROMINENT trial (Pemafibrate to Reduce Cardiovascular Outcomes by Reducing Triglycerides In Patients With Diabetes) (NCT03071692) of pemafibrate to reduce cardiovascular events in patients with elevated triglycerides and low HDL-C.

Strengths of the present study include the prospective design, large sample size, long-term follow-up, and homogeneity of our study population, which may reduce confounding. However, several potential limitations should be considered. First, the WHS has no male enrollees, and the majority of participants were white and healthy at baseline. Thus, our conclusions may not be generalizable to other groups. It is unclear from our data whether NMR lipoprotein profiling would be beneficial in high-risk individuals or in those receiving lipid-lowering therapy, since the study population was comprised of relatively healthy women enrolled in 1992 to 1995. Second, because our study is observational, residual unmeasured confounding may be present. However, data collected on a broad range of established cardiovascular risk factors were available for multivariable adjustment. Third, the use of symptomatic PAD as the primary end point by definition excludes subclinical disease that might otherwise have been detected with the use of ankle-brachial index or abnormal pulse examination; however, we believe that our data are not only mechanistically relevant but also clinically important because claudication and ischemia requiring limb revascularization are the principal clinical manifestations of PAD. Importantly, each case included in this analysis was confirmed through rigorous methods with the use of a validated claudication questionnaire, cardiovascular physician interview, and medical record review. In addition, women enrolled in the WHS are female health professionals and are therefore less likely to encounter barriers to medical care, which may otherwise have led to underdiagnosis. Furthermore, although potential misclassification resulting from atypical or occult disease may have occurred, this, if anything, would have biased our results toward the null by inclusion of potentially misclassified cases in the event-free group. In terms of the traditional lipid measures used in the present study, more refined methods of calculating LDL-C have been developed.[50] However, the performance of the calculated LDL-C variable in this study was likely adequate given that LDL-C was associated with incident CCVD, as expected. Finally, given our relatively small sample size, the study may be underpowered to detect risk associations for some biomarkers, although numerous statistically significant associations were identified.

In summary, our data show that both standard lipid as well as NMR-derived lipoprotein measures indicative of atherogenic dyslipidemia are associated with PAD in women, whereas LDL-C, non–HDL-C, and apolipoprotein B_{100} were not. Measures of total and small LDL-P concentration further identified women at heightened risk of PAD beyond standard lipid measures. Importantly, our data also indicate that this lipoprotein signature may be unique to PAD in comparison to coronary

or cerebrovascular atherosclerosis. Our results require confirmation; the biological construct is not only plausible, but raises the intriguing possibility that therapeutic modulation of these lipoprotein abnormalities may have clinical benefits in patients at risk for PAD for whom few medical treatment options currently exist.

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Disclosures

Dr Ridker is listed as a coinventor on patents held by the Brigham and Women's Hospital that relate to the use of inflammatory biomarkers in cardiovascular disease, which have been licensed to AstraZeneca and Siemens; has received investigator research support from Kowa Research Institute, Novartis, Pfizer, and Astra-Zeneca; has served as a consultant to Jannsen, Novartis, and Sanofi-Regenerson; and serves as Co-Principal Investigator of the PROMINENT trial (NCT03071692). Dr Mora receives research grant support from Atherotech Diagnostics for research outside the current work; served as a consultant to Amgen, Lilly, Pfizer, and Quest Diagnostics; and is coinventor on a patent on the use of nuclear magnetic resonance–measured GlycA for predicting risk of colorectal cancer. Dr Pradhan receives investigator-initiated research support from Kowa Research Institute and serves as Co-Principal Investigator of the PROMINENT trial (NCT03071692). The other authors report no conflicts.

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Small Dense Low-Density Lipoprotein-Cholesterol Concentrations Predict Risk for Coronary Heart Disease

The Atherosclerosis Risk in Communities (ARIC) Study

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Objective—To investigate the relationship between plasma levels of small dense low-density lipoprotein-cholesterol (sdLDL-C) and risk for incident coronary heart disease (CHD) in a prospective study among Atherosclerosis Risk in Communities (ARIC) study participants.

Approach and Results—Plasma sdLDL-C was measured in 11 419 men and women of the biracial ARIC study using a newly developed homogeneous assay. A proportional hazards model was used to examine the relationship among sdLDL-C, vascular risk factors, and risk for CHD events (n=1158) for a period of ≈11 years. Plasma sdLDL-C levels were strongly correlated with an atherogenic lipid profile and were higher in patients with diabetes mellitus than non–diabetes mellitus (49.6 versus 42.3 mg/dL; $P<0.0001$). In a model that included established risk factors, sdLDL-C was associated with incident CHD with a hazard ratio of 1.51 (95% confidence interval, 1.21–1.88) for the highest versus the lowest quartile, respectively. Even in individuals considered to be at low cardiovascular risk based on their LDL-C levels, sdLDL-C predicted risk for incident CHD (hazard ratio, 1.61; 95% confidence interval, 1.04–2.49). Genome-wide association analyses identified genetic variants in 8 loci associated with sdLDL-C levels. These loci were in or close to genes previously associated with risk for CHD. We discovered 1 novel locus, *PCSK7*, for which genetic variation was significantly associated with sdLDL-C and other lipid factors.

Conclusions—sdLDL-C was associated with incident CHD in ARIC study participants. The novel association of genetic variants in *PCSK7* with sdLDL-C and other lipid traits may provide new insights into the role of this gene in lipid metabolism. **(*Arterioscler Thromb Vasc Biol*. 2014;34:1069-1077.)**

Key Words: coronary disease ■ genome-wide association study ■ triglycerides

Low-density lipoprotein-cholesterol (LDL-C) is considered one of the most important risk factors for cardiovascular disease and remains the primary target for current cardiovascular risk reduction strategies.[1,2] However, many individuals with LDL-C within the normal range still develop cardiovascular disease.[3,4] LDL particles are a heterogeneous population,[5] and it has long been hypothesized that a subfraction of LDL, particularly small dense LDL (sdLDL), possesses increased atherogenic potential and thus contributes to this observation. A number of mechanisms have been proposed to explain the enhanced atherogenicity of sdLDL,[6–10] including (1) a lower affinity for the LDL receptor, (2) facilitated entry into the arterial wall, (3) greater arterial retention because of increased binding to proteoglycans, and (4) greater susceptibility to oxidation. Because sdLDL particles are smaller and contain less cholesterol, increased levels of sdLDL also represent an increased number of atherogenic particles, which may not be reflected by the levels of LDL-C.

See accompanying editorial on page 959

Two of the earliest and most widely used methods for LDL classification involved density and size determinations based on ultracentrifugal and nondenaturing gradient density gel electrophoresis procedures. These resulted in the division of LDL particles somewhat arbitrarily into 2 categories for clinical assessment: sdLDL and large buoyant LDL (lbLDL). These also led to the development of a 2 phenotype classification system, with phenotype A (or pattern A) characterized as individuals with a predominance of lbLDL particles and phenotype B (or pattern B) as individuals with a predominance of

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Nonstandard Abbreviations and Acronyms	
apo	apolipoprotein
ARIC	Atherosclerosis Risk in Communities
CHD	coronary heart disease
CI	confidence interval
GWAS	genome-wide association study
HDL-C	high-density lipoprotein-cholesterol
HR	hazard ratio
hs-CRP	high-sensitivity C-reactive protein
lbLDL	large buoyant low-density lipoprotein
lbLDL-C	large buoyant low-density lipoprotein-cholesterol
LDL-C	low-density lipoprotein-cholesterol
sdLDL	small dense low-density lipoprotein
sdLDL-C	small dense low-density lipoprotein-cholesterol
SNP	single-nucleotide polymorphism

sdLDL particles.[11] This classification schema has been widely used, and pattern B has been recognized as a risk marker for cardiovascular disease. More recently, the efficacy of nuclear magnetic resonance methodology to determine both particle numbers and sizes of various lipoprotein fractions, including LDL, has been demonstrated.[12]

The distribution of LDL subfractions is determined by both genetic and environmental factors.[13,14] Furthermore, the concentration of sdLDL is highly correlated with triglyceride level and is increased in individuals with diabetes mellitus or the metabolic syndrome.[15] Therefore, it is plausible that genetic variants that affect circulating levels of sdLDL-C may also influence other lipid traits (eg, triglycerides and high-density lipoprotein-cholesterol [HDL-C]) and may aid in the identification of genes involved in the causal pathway linking atherogenic dyslipidemia characterized by sdLDL-C and coronary heart disease (CHD).

sdLDL has been found to be associated with increased risk for cardiovascular disease in cross-sectional studies[16–18] and prospective observational studies.[19–21] However, in most of these studies, sdLDL did not remain an independent risk predictor when adjusted for other lipid risk factor traits. Until recently, the methods available for the measurement of sdLDL were generally limited to nonquantitative, laborious, and highly complex techniques and, therefore, not readily adaptable to a large number of samples in a routine clinical laboratory environment. Recently, Ito et al[22] developed a simple homogeneous assay adaptable to autoanalyzers for the quantification of sdLDL-C. To date, few epidemiological studies have examined whether the cholesterol content of sdLDL can predict future cardiovascular events. In the present study, we measured sdLDL-C in 11 419 men and women of the Atherosclerosis Risk in Communities (ARIC) study. These participants were followed up for a period of 11 years during which the incidence of CHD was measured. The purpose of this study was to evaluate whether sdLDL-C is a better predictor of risk for CHD than that of LDL-C and other traditional or nontraditional cardiovascular risk factors. To understand the genetic determinants of sdLDL-C and lbLDL-C better, we investigated the association of both sdLDL-C and lbLDL-C levels with genetic markers spanning the genome.

Materials and Methods

Materials and Methods are available in the online-only Supplement.

Results

Baseline Characteristics

Key baseline (visit 4) demographics of the 11 419 ARIC participants are described in Table 1. The mean age of the study cohort was 62.8 years; 78% of study participants were white, and 56% were women. Of the study participants, 58% had a history of smoking cigarettes, 16.9% had diabetes mellitus, and 44.6% were classified with metabolic syndrome according to Adult Treatment Panel III criteria.[23] Prevalent CHD was present in 972 individuals at the baseline visit, and these individuals were excluded from analyses for incident events. In the remaining 10 225 individuals (excluding 222 who had missing data on incident CHD), incident CHD developed in 1158 participants for an average of 11 years of follow-up. The mean plasma sdLDL-C level was 43.5 mg/dL, and the mean sdLDL-C/LDL-C ratio was 0.35. Table I in the online-only Data Supplement shows the race- and sex-specific plasma lipid levels. Mean sdLDL-C levels were higher in whites than that in blacks and in men than women, whereas LDL-C was slightly higher in women than that in men but was not different between races. The proportion of LDL-C that was sdLDL-C was higher in whites than that in blacks and in men than women. Total cholesterol and HDL-C levels were higher in women than that in men, whereas triglyceride levels were higher in whites than that in blacks.

Association of sdLDL-C With Cardiovascular Risk Factors

Table 2 shows the means or proportions of baseline traditional risk factors and other characteristics by sdLDL-C quartiles. In general, individuals with sdLDL-C levels in the highest quartile had proatherogenic lipid profiles, were more likely to have diabetes mellitus, hypertension, and metabolic syndrome, and had higher body mass index and plasma high-sensitivity C-reactive protein (hs-CRP) levels. Statin use was higher in those individuals with sdLDL-C levels in the third and fourth quartile. sdLDL-C levels were not associated with smoking status.

The correlation between sdLDL-C levels and various traditional and nontraditional cardiovascular risk factors is shown in Table 3. Strong positive correlations with sdLDL-C ($|r|>0.50$) were found for the lipid risk factors, such as non–HDL-C, apolipoprotein (apo) B, LDL-C, total cholesterol, and log triglycerides. Circulating levels of lipoprotein-associated phospholipase A_2 activity and lactate showed moderate positive correlations, and HDL-C showed a moderate negative correlation with sdLDL-C ($0.25<|r|<0.50$). Weaker correlations with sdLDL-C were found for fasting plasma glucose, apoAI, lbLDL-C, and log hs-CRP. High-sensitivity cardiac troponin T was not significantly correlated with sdLDL-C.

sdLDL-C Levels and Incident CHD Events

The cumulative incidence curves for CHD risk by sdLDL-C and lbLDL-C quartiles, adjusted by age, race, and sex, are

Table 1. Description of 11 419 ARIC Participants at Baseline (ARIC Visit 4)

Characteristic	n=11 419
Age, y	62.83±5.67
Race	
Blacks	2515 (22)
Whites	8904 (78)
Sex	
Women	6385 (56)
Men	5034 (44)
Ever smoked cigarettes	6635 (58)
Ever drank alcohol	8989 (79)
Statin use	1308 (11.5)
Prevalent CHD	972 (8.7)
Diabetes mellitus	1943 (16.9)
Metabolic syndrome	5127 (44.6)
sdLDL-C, mg/dL	43.48±20.76
lbLDL-C, mg/dL	79.22±28.52
sdLDL-C/LDL-C	0.35±0.1496

Data are presented as mean±SD or n (%). ARIC indicates Atherosclerosis Risk in Communities; CHD, coronary heart disease; lbLDL-C, large buoyant low-density lipoprotein-cholesterol; and sdLDL-C, small dense low-density lipoprotein-cholesterol.

shown in Figure 1. The incidence of CHD events increased proportionately during the follow-up years for participants in each consecutive quartile of sdLDL-C. In contrast, lbLDL-C did not exhibit a concentration-dependent relationship with future incident CHD events. sdLDL-C and LDL-C levels were moderately correlated (Figure IA in the online-only Data Supplement; r=0.54) but often discordant. Figure IB in the online-only Data Supplement displays the prevalence and magnitude of this discordance. We examined concordant and discordant subgroups separately using a similar analysis approach as previously described by Otvos et al.[24] We defined discordance as a difference of >24 percentile units (points outside the dashed lines in Figure IB in the online-only Data Supplement). Figure II in the online-only Data Supplement shows the cumulative incidence curves for CHD risk for the subgroup with sdLDL-C>LDL-C discordance when compared with the concordant and the discordant sdLDL-C<LDL-C subgroups. The sdLDL-C>LDL-C discordant subgroup showed the highest CHD risk when compared with the concordant and discordant sdLDL-C<LDL-C subgroups.

We used proportional hazards regression analyses to investigate the association of incident CHD with baseline levels of sdLDL-C and LDL-C modeled in quartiles, using quartile 1 as the referent group (Table 4). In the basic model adjusted for age, race, and sex (model 1), individuals with sdLDL-C levels in the highest quartile had a 2-fold higher risk for incident CHD when compared with those in the lowest quartile (hazard ratio [HR], 2.00; 95% confidence interval [CI], 1.69–2.37). After additional adjustment for smoking, body mass index, hypertension, HDL-C, log triglycerides, lipid-lowering medications, diabetes mellitus, diabetes mellitus medications, and log hs-CRP (model 2), risk for incident CHD was attenuated but remained significant (HR, 1.51; 95% CI, 1.21–1.88). sdLDL-C was not significantly associated with risk for incident CHD after further adjustment for other lipid risk factors, such as LDL-C, apo B, and total cholesterol. This may be, in part, because of over adjustment of the multivariable model and is not surprising given the strong correlations of sdLDL-C with these lipid risk factors. In comparison, individuals with LDL-C levels in the highest quartile had a 56% and 68% higher risk for incident CHD (HR, 1.56; 95% CI, 1.32–1.83 and HR, 1.68; 95% CI, 1.42–1.99) in the basic model (model 1) and more fully adjusted model (model 2), respectively.

We further investigated the association of sdLDL-C with risk for incident CHD in participants stratified by LDL-C risk

Table 2. Adjusted Mean±SE or Proportions±SE of Cardiovascular Risk Factors by Quartiles of Small Dense LDL-C and *P* Value for Trend Across Quartiles

Characteristic	Q1	Q2	Q3	Q4	*P* Trend
Age, y*	62.77±0.106	62.95±0.106	62.80±0.106	62.79±0.106	0.8500
Men, %*	0.40±0.038	0.42±0.038	0.49±0.038	0.45±0.038	<0.0001
Black, %*	0.30±0.041	0.23±0.044	0.19±0.048	0.14±0.055	<0.0001
Total cholesterol, mg/dL	172.08±0.557	194.61±0.556	207.09±0.558	229.94±0.559	<0.0001
Triglycerides, mg/dL	91.70±1.370	116.20±1.368	154.69±1.373	213.49±1.374	<0.0001
LDL-C, mg/dL	97.55±0.537	119.30±0.537	129.36±0.542	145.64±0.551	<0.0001
HDL-C, mg/dL	56.16±0.271	52.37±0.271	47.85±0.272	43.47±0.272	<0.0001
BMI, kg/m^2	27.80±0.103	28.46±0.103	29.20±0.103	29.79±0.103	<0.0001
Smoking, %	59±4.0	62±4.0	57±3.9	58±4.0	0.1125
Diabetes mellitus, %	11.0±5.8	13.0±5.6	17.0±5.1	23.0±4.6	<0.0001
Hypertensive, %	40.0±4.0	46.0±3.9	51.0±3.9	54.0±3.9	<0.0001
Statin use, %	9.0±6.7	11.0±6.0	12.0±5.8	12.0±5.7	<0.0001
Metabolic syndrome, %	23.0±4.5	33.0±4.0	50.0±3.8	73.0±4.3	<0.0001
log hs-CRP	0.75±0.020	0.84±0.020	0.94±0.020	1.05±0.021	<0.0001

BMI indicates body mass index; HDL-C, high-density lipoprotein-cholesterol; hs-CRP, high-sensitivity C-reactive protein; and LDL-C, low-density lipoprotein-cholesterol. *Unadjusted mean or proportion; all others were adjusted for age, race, and sex.

Table 3. Correlation of Small Dense LDL-C With Traditional and Novel Cardiovascular Risk Factors

Risk Factor	n	Pearson *R*	*P* Value
Non–HDL-C	11 419	0.721	<0.0001
ApoB	10 720	0.706	<0.0001
Log triglycerides	11 419	0.641	<0.0001
Total cholesterol	11 419	0.608	<0.0001
LDL-C	11 234	0.543	<0.0001
Lp-PLA_2 activity	11 108	0.319	<0.0001
HDL-C	11 419	–0.291	<0.0001
Lactate	11 417	0.253	<0.0001
Fasting plasma glucose	10 902	0.169	<0.0001
ApoAI	10 720	–0.086	<0.0001
lbLDL-C	11 234	–0.076	<0.0001
Log hs-CRP	11 202	0.072	<0.0001
hs-cTroponin T	11 130	–0.013	0.1549

Apo indicates apolipoprotein; HDL-C, high-density lipoprotein-cholesterol; hs-CRP, high-sensitivity C-reactive protein; hs-cTroponin T, high-sensitivity cardiac troponin T; lbLDL-C, large buoyant low-density lipoprotein-cholesterol; and Lp-PLA_2, lipoprotein-associated phospholipase A_2.

categories (ie, LDL-C<100 mg/dL and LDL-C≥100 mg/dL). In these analyses, we used a multivariable model (adjusting for age, sex, race, ever smoking, body mass index, hypertension, diabetes mellitus, diabetes mellitus medication, and log hs-CRP), with quartile 1 for sdLDL-C in the LDL-C<100 mg/dL risk category as the referent group. Even in individuals with LDL-C levels <100 mg/dL, sdLDL-C was predictive of CHD risk across increasing sdLDL-C quartiles (Figure 2). Participants with LDL-C<100 mg/dL and sdLDL-C levels in the fourth quartile had a 61% increase in risk for incident CHD (HR, 1.61; 95% CI, 1.04–2.49) when compared with individuals with sdLDL-C levels in the first quartile. In comparison, participants with LDL-C≥100 mg/dL and sdLDL-C levels in the fourth quartile had an 86% increase in risk for incident CHD (HR, 1.86; 95% CI, 1.48–2.33) when compared with those in the same referent group.

In additional analyses, we examined the effects on CHD risk of sdLDL-C discordance among ARIC participants with low LDL-C (<100 mg/dL; <25th percentile) or equivalently low sdLDL-C (<27.8 mg/dL; <25th percentile; Figure 3). The cumulative incidence of CHD events was higher among individuals with low LDL-C but discordantly higher sdLDL-C (10.9%) when compared with individuals with low sdLDL-C but discordantly higher LDL-C (7.9%). Not surprisingly, the cumulative incidence of CHD events was highest among individuals with concordantly higher levels of LDL-C and sdLDL-C (12.7%) and lowest among individuals with concordantly lower levels of LDL-C and sdLDL-C (7.6%).

Genome-Wide Association Study of sdLDL-C

We performed genome-wide association study (GWAS) analyses of sdLDL-C, and Table 5 summarizes our primary findings. In total, 127 single-nucleotide polymorphisms (SNPs) were significantly associated with sdLDL-C ($P<5\times10^{-8}$). These SNPs were clustered at 8 different loci on chromosomes 1, 2, 7, 8, 11, and 19 and were located within 14 different genes (or gene clusters). With the exception of *PCSK7*, genetic variants within all of these genes have previously been found to be related to pathways involved in lipid metabolism and vascular inflammation (www.genome.gov).

Association of *PCSK7* SNP rs508487 Genotype With Circulating Lipids and CHD

A novel finding from the current GWAS analysis was the significant association of sdLDL-C with SNP rs508487 (at locus 11q23–q24) located in the *PCSK7* gene. We investigated the effect of rs508487 genotype on circulating lipid levels (Table 6). Each copy of the minor allele at this SNP raised sdLDL-C by ≈4 mg/dL and triglycerides by ≈20 mg/dL. In contrast, each copy of the minor allele lowered lbLDL-C by ≈3 mg/dL; rs508487 genotype had no significant effect on circulating LDL-C levels. Interestingly, 2 copies of the minor allele increased HDL-C by ≈5 mg/dL and total cholesterol by ≈14 mg/dL.

Given that the *PCSK7* variant (rs508487) is located in the chromosome 11 region harboring the *APOA5-APOA4-APOC3-APOA1* gene cluster, we investigated whether

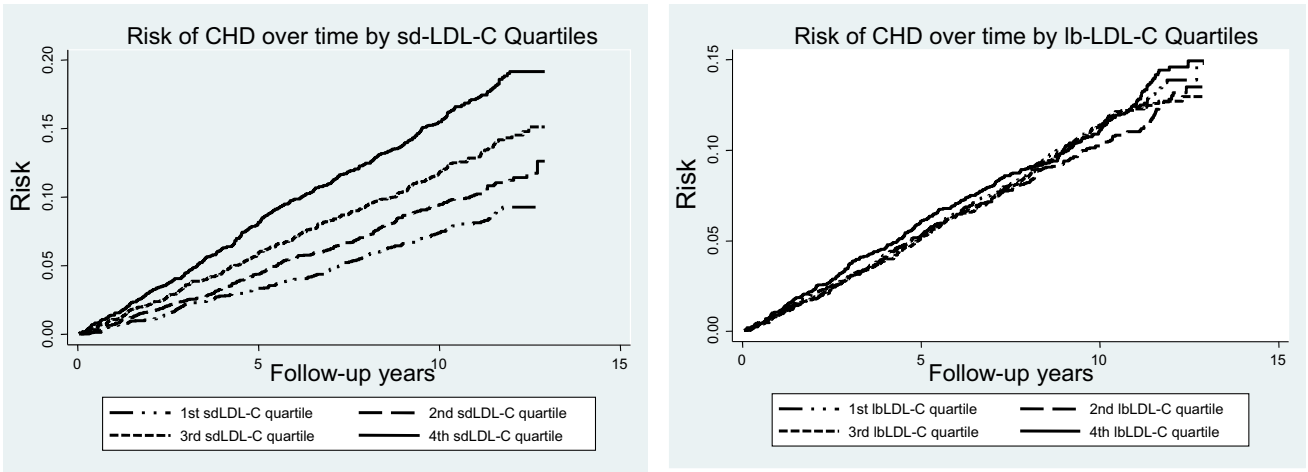

Figure 1. Cumulative incidence curves for risk of coronary heart disease (CHD) by small dense low-density lipoprotein-cholesterol (sdLDL-C) and large buoyant LDL-C (lbLDL-C) quartiles, adjusted for age, race, and sex.

Table 4. Hazard Ratio (95% Confidence Interval) for Incident Coronary Heart Disease by sdLDL-C and LDL-C Quartiles

	2	3	4	*P* Value*
Quartile of sdLDL-C†				
Model 1‡	1.19 (0.99–1.43)	1.44 (1.21–1.72)	2.00 (1.69–2.37)	<0.0001
Model 2§	1.10 (0.90–1.33)	1.21 (0.99–1.48)	1.51 (1.21–1.88)	0.0008
Quartile of LDL-C†				
Model 1‡	1.01 (0.85–1.21)	1.15 (0.97–1.37)	1.56 (1.32–1.83)	<0.0001
Model 2§	1.08 (0.90–1.30)	1.23 (1.03–1.47)	1.68 (1.42–1.99)	<0.0001

sdLDL-C indicates small dense low-density lipoprotein-cholesterol.

*P values ($Pr>\chi^2$) for linear hypothesis testing results of sdLDL-C quartiles.

†Lowest quartile (1) is reference.

‡Adjusted for age, sex, and race.

§Adjusted for model 1 variables+smoking, body mass index, hypertension, high-density lipoprotein-cholesterol, log(triglycerides), lipid-lowering medications, diabetes mellitus, diabetes mellitus medications, and log(high-sensitivity C-reactive protein).

the novel association result with *PCSK7* is because of linkage disequilibrium with previously reported variants in this cluster, especially previously reported functional variants in *APOA5*. One variant, rs662799 (*APOA5* T-1131C), was in significant linkage disequilibrium with rs508487 (R^2=0.53). We repeated the association analysis between *PCSK7* rs508487 and sdLDL-C in 6069 individuals homozygous for the wild-type allele at *APOA5* rs662799, and the results were attenuated a bit but still nominally statistically significant (*P*=0.0027).

We investigated the relationship between *PCSK7* SNP rs508487 genotype and CHD in the ARIC study and did not observe a significant association. However, the power for this particular analysis was limited because of the fact that the number of ARIC CHD cases with 1 or 2 minor alleles at this SNP was low. Therefore, we examined the association of rs508487 with 40 260 CHD cases from the Coronary Artery Disease Genome-Wide Replication And Meta-Analysis (CARDIoGRAM) study and found that rs508487 was significantly associated with CHD (odds ratio, 1.13; 95% CI, 1.06–1.21; *P*=0.00017).

We next analyzed rare variants on the Illumina exome chip designated as nonsynonymous, splicing, or stop gain in the *PCSK7* gene for association with sdLDL-C. We found a total of 7 nonsynonymous *PCSK7* variants among white ARIC participants, resulting in amino acid substitutions in the wild-type

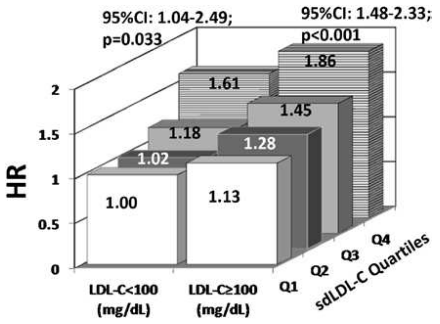

Figure 2. Adjusted hazard ratios (HRs) for incident coronary heart disease by small dense low-density lipoprotein-cholesterol (sdLDL-C) quartiles stratified by LDL-C risk categories, adjusted for age, sex, and race, smoking, body mass index, hypertension, diabetes mellitus, diabetes mellitus medications, and log high-sensitivity C-reactive protein. CI indicates confidence interval.

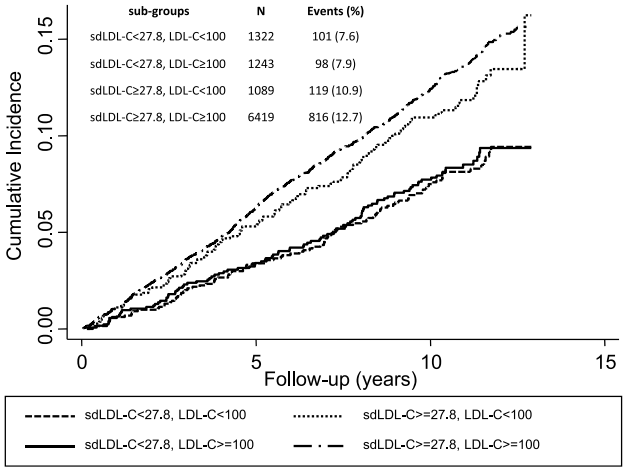

Figure 3. Cumulative incidence of cardiovascular events in subgroups with low-density lipoprotein-cholesterol (LDL-C) <100 mg/dL (<25th percentile) and small dense LDL-C (sdLDL-C) <27.8 mg/dL (<25th percentile), from proportional hazards models adjusted for age, sex, and race.

PCSK7 protein. Because these variants had minor allele frequencies <1%, we analyzed them collectively for their effect on sdLDL-C. Individuals who were carriers of any of the rare *PCSK7* variants had a significant increase in circulating levels of sdLDL-C (≈7.5 mg/dL; *P*=0.012) and triglycerides (ln(TG) ≈0.145; *P*=0.043) when compared with noncarriers (Table 7).

Discussion

In the current study, we investigated the relationship between plasma levels of sdLDL-C and risk of incident CHD in the predominantly biracial ARIC study cohort using a newly developed automated homogeneous sdLDL-C assay. Elevated plasma sdLDL-C levels were associated with increased risk of incident CHD in a multivariable model (HR, 1.51; 95%CI, 1.21–1.88) and even in individuals considered to be at low cardiovascular risk based on their LDL-C levels, sdLDL-C predicted risk for incident CHD (HR, 1.61; 95% CI, 1.04–2.49). Using GWA analyses, we discovered 1 novel locus, PCSK7, for which genetic variation was significantly associated with sdLDL-C levels and other lipid traits. Subsequent examination in the CARDIoGRAM study showed a significant association of the *PCSK7* SNP rs508487 with CHD.

sdLDL-C and Risk for Incident CHD

Among ARIC participants, the mean baseline plasma sdLDL-C level was 43.5 mg/dL, which represented ≈35% of the total LDL-C concentration. sdLDL-C and sdLDL-C/LDL-C ratio were higher in whites than in blacks and higher in men than in women.

Our findings related to circulating sdLDL-C levels seem in general agreement with a report from the Framingham Offspring Study, which showed that men had higher sdLDL-C levels and a higher percentage of LDL-C as sdLDL-C when compared with women.[25] Although we found higher mean sdLDL-C and sdLDL-C/LDL-C ratio overall than those reported in the Framingham Offspring Study, these differences may be, in part, because of differences in study populations (eg, the ARIC cohort has a higher prevalence of obesity

Table 5. Association of the Top SNPs With sdLDL-C

			sdLDL-C							
SNP	Location	Chromosome	No. of Sign SNPs	Coded Allele	Allele Frequency	n	β	SEβ	*P* Value	Gene
rs964184	11q23.3	11	12	C	0.8588	6979	−5.70008	0.511384	7.46×10^{-29}	*APOA5/A4/C3/A1*
rs4420638	19q13.32	19	6	A	0.8264	6979	−5.45295	0.490271	9.77×10^{-29}	*APOE/C1/C4/C2*
rs2075650	19q13.32	19	3	A	0.8611	6979	−7.15912	0.748784	1.17×10^{-21}	*TOMM40*
rs660240	1p13.3	1	10	C	0.7873	6979	3.45306	0.432418	1.40×10^{-15}	*PSRC1/CELSR2/SORT1*
rs6589564	11q23.3	11	18	C	0.0723	6979	5.53037	0.700672	2.95×10^{-15}	*BUD13*
rs2075290	11q23.3	11	5	C	0.0716	6979	5.48955	0.701319	4.98×10^{-15}	*ZNF259*
rs2980853	8q24.13	8	38	A	0.5315	6979	2.63962	0.353669	8.42×10^{-14}	*TRIB1*
rs1260326	2p23.3	2	3	C	0.5856	6979	−2.60009	0.366214	1.25×10^{-12}	*GCKR*
rs7254892	19q13.32	19	1	A	0.0534	6979	−10.9757	1.78507	7.82×10^{-10}	*PVRL2*
rs562338	2p23–2p24	2	24	A	0.1811	6979	−2.65678	0.464554	1.07×10^{-08}	*APOB*
rs508487	11q23–q24	11	1	C	0.9402	6979	−4.95643	0.868883	1.17×10^{-08}	*PCSK7*
rs4803760	19p13.2	19	1	C	0.7692	6979	2.73614	0.49648	3.57×10^{-08}	*BCAM*
rs1178977	7q11.23	7	3	A	0.8000	6979	2.55226	0.466442	4.46×10^{-08}	*MLXIPL*
rs6976930	7q11.23	7	2	A	0.2001	6979	−2.54050	0.464764	4.60×10^{-08}	*BAZ1B*

SdLDL-C indicates small dense low-density lipoprotein-cholesterol; and SNP, single-nucleotide polymorphism.

and metabolic syndrome than the Framingham Offspring Study) and sdLDL-C assay methodologies.

Plasma levels of sdLDL-C were adversely associated with cardiovascular lipid risk factors, a finding consistent with previous reports showing a correlation of sdLDL with an atherogenic lipid profile. We also found significant correlations between sdLDL-C and nonlipid risk factors, such as fasting glucose and lactate levels. Even though sdLDL-C was associated with diabetes mellitus and metabolic syndrome in previous studies, we report a remarkable increase in prevalence of metabolic syndrome among individuals with sdLDL-C levels in the highest quartile (73%) when compared with those in the lowest quartile (23%). sdLDL-C was also correlated with inflammatory markers, such as lipoprotein-associated phospholipase A2 activity and hs-CRP.

During the 11-year follow-up period of this study, 1158 (11.3%) participants developed CHD. The cumulative incidence curves clearly illustrate the direct relation between sdLDL-C levels and CHD risk, whereas we did not find a similar relation between lbLDL-C and CHD. These results suggest that the sdLDL subfraction is a major contributor to the risk for incident CHD that is associated with LDL-C. Circulating levels of sdLDL-C were significantly associated with increased risk for CHD in a model adjusted for age, sex, and race and in a more fully adjusted model that also included smoking, body mass index, hypertension, HDL-C, triglycerides, lipid-lowering medications, diabetes mellitus, diabetes mellitus medications, and hs-CRP. However, sdLDL-C was not an independent predictor of incident CHD when we further adjusted for other lipid risk factors, such as LDL-C, apo B, and total cholesterol, which is not surprising given the strong correlations of sdLDL-C with these other lipid risk factors, and our results are in agreement with previous studies reporting that sdLDL was not an independent predictor of cardiovascular disease.[19–21] Interestingly, sdLDL-C showed predictive power for CHD risk even in individuals with optimal LDL-C levels as defined in the current guidelines (<100 mg/dL).[23] Several investigators have emphasized that the number of particles as measured by nuclear magnetic resonance is more important for assessment of cardiovascular risk than LDL subclass, LDL particle size, or LDL-C concentration.[26,27] Because sdLDL particles contain less cholesterol than lbLDL particles, there are more sdLDL particles than lbLDL particles at a given LDL-C concentration. Whether the total number of particles or the cholesterol payload per particle is more important to cardiovascular risk remains a topic of discussion. However, it is important to note that the particle number theory does not take into account the accumulating evidence pointing to different atherogenic properties of LDL subclasses. A limitation of this study is the fact that we did not have particle number information available and thus we were not able to address this question specifically.

Table 6. Association of *PCSK7* Single-Nucleotide Polymorphism rs508487 Genotype With Circulating Lipids

Lipid, mg/dL (mean±SE)	Genotype			*P* Value
	CC	CT	TT	
sdLDL-C	44.3±20.6	48.5±21.4	51.5±23.4	<0.0001
lbLDL-C	78.7±27.5	75.5±29.7	72.7±23.6	0.02
sdLDL-C/LDL-C	0.36±0.15	0.40±0.16	0.41±0.13	<0.0001
LDL-C	123.0±32.8	124.0±32.6	124.2±33.8	0.75
HDL-C	50.2±16.4	48.6±16.1	54.9±13.4	0.03
Total cholesterol	201.5±35.6	205.0±35.0	215.3±40.4	0.02
Triglycerides	141.5±67.5	162.1±77.5	181.4±88.5	<0.0001

HDL-C indicates high-density lipoprotein-cholesterol; lbLDL-C, large buoyant low-density lipoprotein-cholesterol; and sdLDL-C, small dense low-density lipoprotein-cholesterol.

Table 7. Change in Circulating Lipids Among White Carriers of Rare Nonsynonymous *PCSK7* Variants

Lipid	Change (β)	SE	*P* Value
sdLDL-C, mg/dL	7.53	3.00	0.012
Ln (triglycerides)	0.144	0.071	0.043
HDL-C, mg/dL	−3.58	2.12	0.091

HDL-C indicates high-density lipoprotein-cholesterol; and sdLDL-C, small dense low-density lipoprotein-cholesterol.

Genetics of sdLDL-C

GWAS analysis identified a large number of SNPs clustered at 8 different loci on chromosomes 1, 2, 7, 8, 11, and 19 that were significantly associated with sdLDL-C. With the exception of *PCSK7*, genetic variants located in all the genes associated with sdLDL-C levels have been reported previously in GWAS of blood lipid levels.[28] Our GWAS findings are in general agreement with an earlier report by Chasman et al[29] who used a comprehensive GWAS analysis to identify largely similar loci that affect the nuclear magnetic resonance–based measures of concentration and size of LDL, HDL, and very LDL in women. Although the study by Chasman et al[29] did not find a significant association with LDL particle size or concentration at the *PCSK7* locus, this apparent discrepancy may be because of notable differences between the 2 studies, such as differences in methodologies to measure lipoprotein fractions, genotyping methods, and study populations. Indeed, a recent report from the Multi-Ethnic Study of Atherosclerosis (MESA) compared the identical automated assay of sdLDL-C as was used in our study to nuclear magnetic resonance–derived small LDL concentrations with regards to risk prediction for incident CHD.[30] The authors showed that the new automated assay of sdLDL-C identified the risk of CHD, whereas the nuclear magnetic resonance–derived small LDL concentrations did not convey a significant risk of CHD in the MESA cohort. Therefore, if these 2 different methodologies show different associations with cardiovascular risk, it is plausible that they may also lead to different GWAS findings.

Genetic variants within a number of the genes associated with sdLDL-C in our study have previously been found to be associated with increased risk for cardiovascular disease in meta-analyses of GWA studies.[31,32] SNP rs4420638, which is located in the *APOE-APOC1-APOC4-APOC2* gene cluster, was also associated with lipoprotein-associated phospholipase A2 activity and CHD in a meta-analysis of GWA studies from 5 community-based studies.[33] In addition, we have previously shown an association of the SNP rs780094 in *GCKR* with metabolic syndrome prevalence and incident diabetes mellitus in the ARIC study.[34] rs780094 was also significantly associated with sdLDL-C ($P=4.08\times10^{-12}$) in our current study.

Our findings have important implications in light of recent observations from Mendelian randomization studies investigating genetic determinants of HDL-C levels and risk for incident CHD. Unlike data from human Mendelian diseases, which support a causal role for LDL-C in risk for CHD,[35,36] evidence for a causal role of HDL-C from Mendelian randomization studies is inconsistent and complicated by the fact that most SNPs associated with HDL-C levels affect multiple lipid traits. Voight et al[37] recently showed that a genetic risk score consisting of 14 SNPs exclusively associated with HDL-C was not associated with risk for myocardial infarction, in contrast to a genetic risk score for LDL-C. These investigators had previously shown that a number of SNPs associated with HDL-C were also associated with other lipid traits, such as triglycerides and LDL-C.[38] The SNPs associated with HDL-C that were most strongly associated with increased risk for myocardial infarction and that influenced other lipid traits were located in or near the *APOA5-APOA4-APOC3-APOA1*, *TRIB1*, and *LPL* genes or gene clusters. In the current study, we showed that these genes are also associated with sdLDL-C or sdLDL-C/LDL-C ratio (Table II in the online-only Data Supplement).

Association of *PCSK7* SNP rs508487 Genotype With Circulating Lipids

A novel finding from the current GWAS analysis is the significant association of sdLDL-C with SNP rs508487 (at locus 11q23–q24), located in the *PCSK7* gene. Investigation of the effect of rs508487 genotype on circulating lipid levels showed that each copy of the minor allele at this SNP raised sdLDL-C by ≈4 mg/dL. It is important to note that genetic variation at the *PCSK7* locus was not associated with LDL-C levels in our study. Because LDL-C is a more commonly used lipid measurement, it is plausible that genetic variations in the *PCSK7* gene have not been associated with circulating lipids in previous GWAS studies.

PCSK7 encodes subtilisin-like/kexin proprotein convertase type 7 (PCSK7), a calcium-dependent serine endoprotease. PCSK7 has previously been implicated as a mediator of adipogenesis[39] and in the processing of vascular endothelial growth factor (VEGF)-D, a critical step for binding of the angiogenic receptor VEGFR-2.[40] Furthermore, recent data show that internalization of PCSK7 from the plasma membrane is mediated by clathrin-coated vesicles,[41] which are also implicated in the internalization of other cellular receptors, such as the LDL receptor and various scavenger receptors. Although the physiological role of PCSK7 is not clearly understood, it is plausible that PCSK7 could be involved in the processing of LDL and scavenger receptors, thereby modulating circulating lipid levels. Alternatively, recent studies suggest a potential role of protein convertases, including PCSK7, in lipid metabolism through proteolytic activation of angiopoietins and proteolytic inactivation of lipases.[42,43] In contrast to PCSK9, another member of the proprotein convertase family, to our knowledge PCSK7 has not been previously associated with cardiovascular lipid risk factors in other GWA studies. However, we should be cautious in the interpretation of our findings about the association of *PCSK7* genotype with circulating lipids. A limitation of our study is that we did not measure protein or mRNA levels of PCSK7. Furthermore, the *PCSK7* SNP rs508487 is in close proximity to the *APOA5-APOA4-APOC3-APOA1* gene cluster, which has also been shown to affect circulating triglyceride levels. However, our exome chip data show that rare variants in the *PCSK7* gene, which lead to amino acid substitutions in the PCSK7 protein, are associated with sdLDL-C and other lipid traits. Additional in vitro or animal studies using transgenic or PCSK7-knockout mouse models are needed to investigate the potential role of PCSK7 in lipid metabolism. Our findings also highlight the important

issue of pleiotropy because PCSK7 was associated with circulating levels of sdLDL-C, triglycerides, and HDL-C.

Conclusions

In summary, our results showed that sdLDL-C was highly correlated with an atherogenic lipid profile and, in contrast to lbLDL-C, predicted future CHD events in ARIC participants. Furthermore, sdLDL-C predicted risk for incident CHD even in individuals who would be considered at low cardiovascular risk based on their LDL-C level. This new homogenous sdLDL-C assay could be readily implemented in most routine clinical chemistry laboratories, provided that its clinical value can be confirmed in future studies. GWAS analysis identified significant associations of sdLDL-C with genetic variants in 14 different genes, all but 1 of which have been previously linked to cardiovascular disease risk. Our GWAS findings, together with findings from previous studies showing genetic variants in the same genes associated with other lipid traits, highlight the importance of pleiotropy in the development of cardiovascular disease. The novel finding of a significant association of sdLDL-C with genetic variants in *PCSK7*, a member of the subtilisin-like/kexin proprotein convertase family, provides new insights into the role of this gene in lipid metabolism.

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Disclosures

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Significance

Low-density lipoprotein-cholesterol (LDL-C) is considered one of the most important risk factors for cardiovascular disease and remains the primary target for current cardiovascular risk reduction strategies. LDL particles are a heterogeneous population, and it has long been hypothesized that a subfraction of LDL, small dense LDL, possesses atherogenic potential. In the current study, we investigated the relationship between plasma levels of small dense LDL-C and risk of incident coronary heart disease in the biracial Atherosclerosis Risk in Communities (ARIC) study cohort. Elevated plasma small dense LDL-C levels were associated with increased risk of incident coronary heart disease, even in individuals considered to be at low cardiovascular risk based on their LDL-C levels. Using genome-wide association analyses, we discovered 1 novel locus, *PCSK7*, for which genetic variation was significantly associated with small dense LDL-C levels and other lipid traits. Together these findings provide new insights into the role of the *PCSK7* gene in lipid metabolism and risk of cardiovascular disease.

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Small Dense Low-Density Lipoprotein Cholesterol and the Risk of Coronary Heart Disease in a Japanese Community

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Aims: This study aims to investigate the association between serum small dense low-density lipoprotein (sdLDL) cholesterol level and the development of coronary heart disease (CHD) in a Japanese community.

Methods: A total of 3,080 participants without prior cardiovascular disease, aged 40 years or older, were followed up for 8 years. The participants were divided into the quartiles of serum sdLDL cholesterol levels. The risk estimates were computed using a Cox proportional hazards model.

Results: During the follow-up period, 79 subjects developed CHD. Subjects in the highest quartile had a 5.41-fold (95% confidence interval, 2.12–13.82) higher risk of CHD than those in the lowest quartile after controlling for confounders. In the analysis classifying the participants into four groups according to the levels of serum sdLDL cholesterol and serum low-density lipoprotein (LDL) cholesterol levels, the risk of CHD almost doubled in subjects with sdLDL cholesterol of ≥ 32.9 mg/dL (median), regardless of serum LDL cholesterol levels, as compared with subjects with serum sdLDL cholesterol of <32.9 mg/dL and serum LDL cholesterol of <120.1 mg/dL (median). When serum sdLDL cholesterol levels were incorporated into a model with known cardiovascular risk factors, c-statistics was significantly increased (from 0.77 to 0.79; $p=0.02$), and the net reclassification improvement was also significant (0.40; $p<0.001$).

Conclusions: The present findings suggest that the serum sdLDL cholesterol level is a relevant biomarker for the future development of CHD that offers benefit beyond the serum LDL cholesterol level and a possible therapeutic target to reduce the burden of CHD in a Japanese community.

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Key words: Small dense LDL cholesterol, Coronary heart disease, Risk assessment, Prospective study

Abbreviations: BMI, body mass index; CHD, coronary heart disease; CI, confidence interval; ECG, electrocardiogram; eGFR, estimated glomerular filtration rate; HbA1c, hemoglobin A1c; HDL, high density lipoprotein; HOMA-IR, homeostasis model assessment of insulin resistance; HR, hazard ratio; hs-CRP, high-sensitivity C-reactive protein; IDI, integrated discrimination improvement; LDL, low-density lipoprotein; SD, standard deviation; NRI, net reclassification improvement; sdLDL, small dense low-density lipoprotein

Introduction

Coronary heart disease (CHD) is the leading cause of death worldwide and places a major economic and resource burden on private and public healthcare systems[1]. Globally, an estimated 7.4 mil-

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lion people died from CHD in 2015 [2]. CHD is mainly caused by atherosclerosis and the concomitant low-grade inflammation of the coronary arteries, which develop due to the deposition of atherogenic lipoprotein in the vessel walls[3]. Growing evidence from observational studies and clinical trials has demonstrated that elevated serum low-density lipoprotein (LDL) cholesterol is a significant risk factor for the development of CHD, and lowering the serum LDL cholesterol level with medications, such as statins, has a beneficial effect on the reduction of coronary risk through the prevention of atherothrombosis and plaque rupture in coronary arteries[4-9]. Nevertheless, it has been acknowledged that a relatively high proportion of individuals with serum LDL cholesterol in the normal range still develop CHD[10].

LDL particles are heterogeneous with respect to size, density, and composition[11]. Recently, the difference in the atherogenic effect across LDL particles has attracted attention[12]. Small dense LDL (sdLDL), which is small and highly dense among LDL particles, has high atherogenic potential due to its increased susceptibility to oxidation, high endothelial permeability, and decreased hepatic LDL receptor affinity[13, 14]. Until recently, however, there have been few prospective studies with a large sample size investigating the influence of sdLDL on coronary risk independent of other cardiovascular risk factors probably due to the limited methods available for the measurement of sdLDL (principally ultracentrifugation[15] or gradient gel electrophoresis)[16]. These methods were not suitable for routine analysis because of their long assay times and expensive equipment. Over the last decade, a homogeneous method that allows the easy measurement of serum sdLDL cholesterol levels has been developed[17, 18]. Using this method, several prospective cohort studies have revealed a significant association between higher serum sdLDL cholesterol and the development of CHD[19-21]. However, few studies have addressed the predictive ability of serum sdLDL cholesterol level for future CHD risks.

Aim

The aim of the present study was to evaluate whether serum sdLDL cholesterol levels are a clinically relevant biomarker for CHD that offers benefit beyond serum LDL cholesterol levels and other known cardiovascular risk factors using the prospective longitudinal data from a general Japanese population.

Methods

Study Design and Participants

The Hisayama study, a population-based prospective cohort study of cardiovascular diseases, has been underway since 1961 in the town of Hisayama, which is located in a suburb of Fukuoka City on Kyushu Island in Japan. According to the national census and nutrition surveys, the age and occupational distributions of the Hisayama population have been similar to those in Japan as a whole since the 1960s[22]. The full community surveys of the health status of residents ≥ 40 years of age have been repeated annually since 1961[22]. A screening survey for the present study was performed in 2007 and 2008, and a detailed description has been published previously[23]. Briefly, a total of 3,384 residents aged ≥ 40 years (78.2% of the total population of this age group) underwent the examination. After excluding eight subjects who did not consent to participate in the study, 223 subjects who had past history of stroke or CHD, and 73 subjects with no measurement of serum sdLDL cholesterol, the remaining 3,080 subjects (1,290 men and 1,790 women) were enrolled in the present study.

The study was approved by the Kyushu University Institutional Review Board for Clinical Research, and written informed consent was obtained from all the participants.

Follow-Up Survey

The subjects were followed up prospectively until November 2015 or their death (median, 8.3 years) by annual health examinations or by mail or telephone for any subject who did not undergo the examination or who moved out of the town. The development of CHD was also checked by a daily monitoring system organized by the study team, local physicians, and members of the Health and Welfare Office of the town. Subjects with suspected CHD events were evaluated for their detailed clinical information by study team physicians. When a subject died, an autopsy was performed at the Department of Pathology of Kyushu University, if consent for autopsy was obtained. During the follow-up period, autopsy examination was performed for 192 (57.8%) of 332 deceased subjects. In addition to the deceased cases, four subjects were lost to follow-up, all of whom were subjects who moved out of the town.

Measurement of the Serum sdLDL Cholesterol Level

At the screening examination, the portions of the plasma specimens were stored at −80℃ until serum sdLDL cholesterol concentrations were measured in

2014. Serum sdLDL cholesterol concentrations were directly measured on a Hitachi 7180 automated chemistry analyzer using a homogeneous assay (sdLDL-EX "SEIKEN"; Denka Seiken, Tokyo)[17, 18], which received Food and Drug Administration clearance on August 18, 2017. The subjects were divided into four groups according to the quartiles of serum sdLDL cholesterol levels: Q1, ≤ 24.4 mg/dL [≤ 0.63 mmol/L]; Q2, 24.5–32.8 mg/dL [0.63–0.84 mmol/L]; Q3, 32.9–43.6 mg/dL [0.85–1.12 mmol/L]; and Q4, ≥ 43.7 mg/dL [≥ 1.13 mmol/L].

Outcomes

The primary outcome of the present analysis was CHD. The criteria for the diagnosis of CHD included first-ever fatal and nonfatal myocardial infarction, silent myocardial infarction, sudden cardiac death within 1 h after the onset of acute illness, coronary angioplasty, and bypass grafting. We counted the incident cases of CHD during the follow-up period as events, regardless of the presence or absence of stroke before the onset of CHD. The diagnosis of myocardial infarction was based on detailed clinical information and at least two of the following findings: typical clinical symptoms, electrocardiogram evidence of myocardial infarction, elevated cardiac enzymes, or morphologic findings including echocardiographic, scintigraphic, or angiographic abnormalities compatible with myocardial injury or myocardial necrosis or scars more than 1 cm in diameter at autopsy[24, 25].

Other Risk Factor Measurements

Each subject completed a self-administered questionnaire covering medical history, medication for hypertension, diabetes, and dyslipidemia, smoking habits, alcohol intake, and physical activity. Smoking and drinking habits were categorized as either current use or not. Current smoking was defined as smoking at least one cigarette per day. Current drinking was defined as drinking at least one alcoholic beverage per month. The subjects engaging in sports or other forms of exertion ≥ 3 times a week during their leisure time made up a regular exercise group. The body height and weight were measured in light clothing without shoes, and the body mass index (BMI) was calculated (kg/m^2). The blood pressure was measured three times in a sitting position using an automated sphygmomanometer (BP-203 RVIIIB; Omron Healthcare), and the mean of three measurements was used for the analysis. Hypertension was defined as blood pressure ≥ 140/90 mmHg and/or current use of antihypertensive agents. Electrocardiogram abnormalities were defined as left ventricular hypertrophy (Minnesota code, 3-1), ST depression (4-1, 2, 3), or atrial fibrillation/flutter (8-3).

The blood samples were collected from an antecubital vein. Plasma glucose levels were measured by the hexokinase method, and serum insulin levels were determined by using an electro-chemiluminescence immunoassay. Diabetes mellitus was defined as fasting plasma glucose levels ≥ 126 mg/dL, 2-h post load or casual glucose levels of at least 200 mg/dL, or the current use of glucose-lowering agents (i.e., oral glucose-lowering agents or insulin). Hemoglobin A1c (HbA1c) was measured by latex aggregation immunoassay using determiner HbA1c (Kyowa Medix, Tokyo). The value for HbA1c was estimated as a National Glycohemoglobin Standardization Program equivalent value calculated with the following formula[26]: HbA1c (%) = 1.02 × HbA1c (Japan Diabetes Society) (%) + 0.25%. Insulin resistance was estimated by the homeostasis model assessment of insulin resistance (HOMA-IR) values, which were calculated as follows[27]: HOMA-IR = fasting plasma glucose (mg/dL) × fasting serum insulin (IU/mL)/405. Serum LDL cholesterol and high-density lipoprotein (HDL) cholesterol levels and serum creatinine were measured enzymatically. The estimated glomerular filtration rate (eGFR) was calculated using the Chronic Kidney Disease Epidemiology Collaboration equation with a Japanese coefficient of 0.813 [28]. Serum high-sensitivity C-reactive protein (hs-CRP) concentrations were measured using the frozen serum portion thawed in 2004 by a modified version of the Behring Latex-Enhanced CRP assay on a Behring Nephelometer BN-100 (Behring Diagnostics, Westwood, MA).

Statistical Analysis

The trends in the means (standard deviations [SDs]) and the frequencies of risk factors across the quartiles of sdLDL cholesterol were tested by a linear and a logistic regression analysis, respectively, in which serum sdLDL cholesterol levels were assigned ordinal values of 1, 2, 3, and 4 for the first (Q1), second (Q2), third (Q3), and fourth (Q4) quartiles, respectively. Serum HOMA-IR, triglycerides, and hs-CRP levels were shown as medians and interquartile ranges in the baseline characteristics of the population and were log-transformed in the analyses because their distributions were skewed. The incidence rate of CHD was calculated using the person-year method after adjusting for age and sex by means of the direct method. The Cox proportional hazards model was used to estimate the hazard ratio (HR) and its 95% confidence intervals (CIs) for the development of CHD for the quartiles or the cutoff levels of serum sdLDL cholesterol levels or per 1 SD increment in log-transformed serum sdLDL cholesterol levels (as a continuous vari-

able). The trends in the age- and sex-adjusted incidence rates or HRs across the serum sdLDL cholesterol levels were tested by a Cox proportional hazards model including serum sdLDL cholesterol levels assigned ordinal numbers of 1, 2, 3, or 4 as categorical variables and the relevant covariates. In the multivariable analysis, the risk estimates were adjusted for potential confounding factors at baseline, namely, age, sex, systolic blood pressure, use of antihypertensive agents, HbA1c, use of glucose-lowering agents, serum HDL cholesterol, lipid-modifying agents, BMI, eGFR, electrocardiogram abnormalities, current smoking, current drinking, and regular exercise. Additionally, we made an another multivariable-adjusted model that included the covariates selected from these potential confounders using the backward elimination method at $p<0.20$ for the remaining variables. The interactions in the association between the subgroups of sex, hypertension, diabetes, BMI (<25 or ≥ 25 kg/m^2), serum LDL cholesterol (<120.1 or ≥ 120.1 mg/dl), use of lipid-lowering agents, current smoking, and eGFR (<60 or ≥ 60 ml/min/1.73 m^2) were tested by adding multiplicative interaction terms to the relevant Cox model. The proportions of missing values were less than 0.1% for all the variables included in the model. To compare the accuracy of risk assessment for the development of CHD between the models including known cardiovascular disease risk factors with and without serum sdLDL cholesterol level or serum LDL cholesterol level, the increase in the Harrell's c-statistics among models was evaluated and tested using a method described by Newson[29]. Moreover, the increased predictive ability of the serum sdLDL cholesterol level or the serum LDL cholesterol level was further estimated by the net reclassification improvement (NRI) and integrated discrimination improvement (IDI)[30], where the individual probabilities were estimated by using the Cox proportional hazards model. The cutoff value of serum sdLDL cholesterol levels that optimizes the discriminating ability for the risk of incident CHD was determined as the point closest to (0,1) on the receiver-operating characteristic curve, $\min \sqrt{(1-\text{sensitivity})^2+(1-\text{specificity})^2}$, where sensitivity = the number of subjects above the cutoff value of serum sdLDL cholesterol level/total number of subjects among subjects who developed CHD, and specificity = the number of subjects below the cutoff value/total number of subjects among subjects who did not develop CHD[31]. A two-sided value of $p<0.05$ was considered statistically significant in all analyses. Statistical analyses were conducted using Statistical Analysis Software (SAS) version 9.4 (SAS Institute, Cary, NC), and Stata version 14.0 (StataCorp, College Station, TX).

Results

Table 1 exhibits the baseline characteristics of the study population according to the quartiles of serum sdLDL cholesterol. The mean values of systolic blood pressure, HbA1c, serum total cholesterol, serum LDL cholesterol, BMI, and eGFR; the median values of HOMA-IR and serum hs-CRP; and the frequencies of male sex, use of statins, current smoking, and current drinking increased significantly with high serum sdLDL cholesterol levels. Conversely, the mean values of age and serum HDL cholesterol decreased significantly with high serum sdLDL cholesterol levels.

During the follow-up period, a total of 79 subjects had a first-ever CHD event. Among them, 45 subjects experienced myocardial infarction, 33 subjects experienced coronary angioplasty, and one subject experienced bypass grafting. **Fig. 1** shows that the age- and sex-adjusted cumulative incidence of coronary heart disease increased significantly with the elevating serum sdLDL cholesterol levels (p for trend <0.001). **Table 2** demonstrates the age- and sex-adjusted incidence rates and multivariable-adjusted HRs for the development of CHD according to the quartiles of serum sdLDL cholesterol levels. The incidence rates increased linearly with high serum sdLDL cholesterol levels: 1.04, 3.37, 4.67, and 5.58 per 1000 person-years from the first to the fourth quartile groups, respectively ($p<0.001$ for trend). The HRs of the development of CHD increased significantly with high serum sdLDL cholesterol levels after adjusting for age, sex, systolic blood pressure, use of antihypertensive agents, HbA1c, use of glucose-lowering agents, serum HDL cholesterol, use of lipid-modifying agents, BMI, eGFR, electrocardiogram abnormalities, current smoking, current drinking, and regular exercise (model 1, p for trend <0.001): subjects in the highest quartile had a 5.41-fold (95% CI, 2.12–13.82) higher risk of CHD than those in the lowest quartile. These associations remained significant even after additional adjustments for serum LDL cholesterol levels (p for trend $=0.03$), serum hs-CRP levels (p for trend <0.001), or HOMA-IR (p for trend <0.001) in addition to the abovementioned confounding factors. We also performed a multivariable-adjusted analysis including age, sex, HbA1c, serum HDL cholesterol, electrocardiogram abnormalities, and regular exercise as covariates, which were selected with the backward elimination method from the aforementioned covariates (model 2). Consequently, the HRs of the development of CHD also increased significantly with high serum sdLDL cholesterol levels (p for trend <0.001).

Table 1. Baseline characteristics of participants according to quartiles of serum small dense low-density lipoprotein cholesterol levels (the Hisayama Study, 2007–2008)

Variables	Serum sdLDL cholesterol level, mg/dL				*p* for trend
	Quartile 1 ≤ 24.4 (*n* = 763)	Quartile 2 24.5–32.8 (*n* = 775)	Quartile 3 32.9–43.6 (*n* = 768)	Quartile 4 ≥ 43.7 (*n* = 774)	
Age (years)	65 (15)	63 (12)	64 (11)	61 (10)	< 0.001
Men (%)	35.0	38.3	42.1	52.1	< 0.001
Systolic blood pressure (mmHg)	127 (20)	129 (19)	134 (19)	136 (18)	< 0.001
Use of antihypertensive agents (%)	30.9	27.6	31.6	27.9	0.49
HbA1c (%)	5.3 (0.7)	5.5 (0.7)	5.5 (0.8)	5.7 (0.9)	< 0.001
Use of glucose-lowering agents (%)	5.1	6.8	6.9	7.1	0.14
HOMA-IR	1.1 (0.7–1.5)	1.2 (0.8–1.8)	1.4 (0.9–2.1)	1.8 (1.2–2.7)	< 0.001
Serum total cholesterol (mg/dL)	179.7 (26.6)	204.7 (25.5)	216.8 (30.9)	237.2 (34.2)	< 0.001
Serum LDL cholesterol (mg/dL)	92.4 (19.4)	116.2 (20.3)	131.1 (24.5)	148.3 (31.0)	< 0.001
Serum HDL cholesterol (mg/dL)	72.9 (18.2)	71.6 (18.0)	65.7 (17.0)	58.8 (15.0)	< 0.001
Serum triglycerides (mg/dL)	71 (55–92)	89 (68–115)	107 (83–139)	163 (120–232)	< 0.001
Use of lipid-modifying agents (%)	16.1	12.9	14.5	12.1	0.064
Use of statins (%)	15.7	12.9	13.8	10.3	0.005
BMI (kg/m^2)	21.9 (3.5)	22.3 (3.1)	23.5 (3.5)	24.3 (3.3)	< 0.001
eGFR (ml/min/1.73 m^2)	73.5 (14.9)	75.5 (12.4)	75.0 (11.7)	77.1 (11.1)	< 0.001
Electrocardiogram abnormalities (%)	16.4	12.9	15.8	16.3	0.65
Serum hs-CRP (mg/L)	0.3 (0.1–0.7)	0.4 (0.2–0.7)	0.4 (0.2–0.8)	0.6 (0.3–1.0)	< 0.001
Current smoking (%)	15.1	18.5	19.4	26.7	< 0.001
Current drinking (%)	39.7	45.4	47.5	57.8	< 0.001
Regular exercise (%)	11.6	12.5	11.3	12.0	0.98

SI conversion factors: To convert mg/dL values to mmol/L, multiply serum sdLDL cholesterol, serum total cholesterol, serum LDL cholesterol, and HDL cholesterol values by 0.02586, and multiply serum triglycerides values by 0.01129.
Data are presented as the mean values (standard deviation), percentages, or medians (interquartile range).
The trends in the means and the frequencies of risk factors across the quartiles of sdLDL cholesterol were tested by a linear and a logistic regression analysis. Serum HOMA-IR, triglycerides, and hs-CRP levels were log-transformed in the analyses due to skewed distribution.
Abbreviations: BMI, body mass index; eGFR, estimated glomerular filtration rate; HbA1c, hemoglobin A1c; HDL, high-density lipoprotein; HOMA-IR, homeostasis model assessment of insulin resistance; hs-CRP, high-sensitivity C-reactive protein; LDL, low-density lipoprotein; sdLDL, small dense low-density lipoprotein.

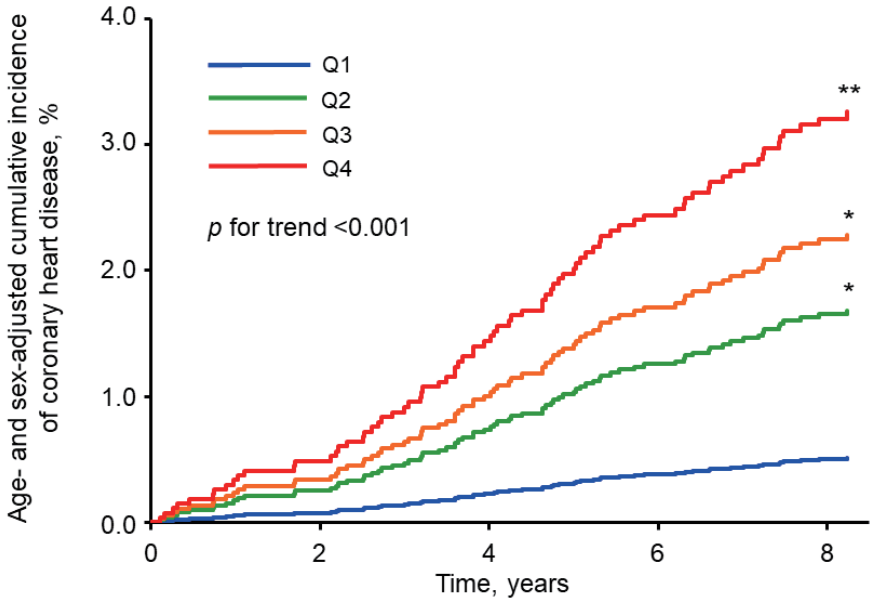

Fig. 1. Age- and sex-adjusted cumulative incidence of coronary heart disease according to quartiles of serum small dense low-density lipoprotein cholesterol

*$p < 0.05$, **$p < 0.001$ versus the lowest quartile of serum small dense low-density lipoprotein cholesterol.

Table 2. Hazard ratios for the development of coronary heart disease according to serum small dense low-density lipoprotein cholesterol levels, 2007–2015

	Serum sdLDL cholesterol level, mg/dL				*p* for trend
	Quartile 1 ≤ 24.4	Quartile 2 24.5–32.8	Quartile 3 32.9–43.6	Quartile 4 ≥ 43.7	
No. of events/subjects	6/763	18/775	24/768	31/774	
Age-and sex-adjusted incidence rate (per 1,000 person-years)	1.04	3.37	4.67	5.58	<0.001
Hazard ratio (95% CI)					
Age-and sex-adjusted	1.00 (Reference)	3.34 (1.32–8.44)	4.55 (1.85–11.20)	6.53 (2.67–15.95)	<0.001
Model 1	1.00 (Reference)	3.18 (1.25–8.09)	4.15 (1.66–10.40)	5.41 (2.12–13.82)	<0.001
Model 1 + serum LDL cholesterol	1.00 (Reference)	2.75 (1.06–7.14)	3.22 (1.20–8.64)	3.76 (1.28–10.99)	0.03
Model 1 + serum triglycerides	1.00 (Reference)	3.06 (1.19–7.84)	3.93 (1.54–10.02)	4.90 (1.78–13.43)	0.003
Model 1 + log hs-CRP	1.00 (Reference)	3.23 (1.27–8.21)	4.21 (1.68–10.55)	5.55 (2.17–14.20)	<0.001
Model 1 + log HOMA-IR	1.00 (Reference)	2.95 (1.16–7.50)	3.93 (1.57–9.85)	4.80 (1.87–12.33)	<0.001
Model 2	1.00 (Reference)	3.04 (1.20–7.68)	3.97 (1.60–9.83)	4.85 (1.94–12.12)	<0.001

SI conversion factors: To convert mg/dL values to mmol/L, multiply serum sdLDL cholesterol values by 0.02586 and multiply serum triglycerides values by 0.01129.
Abbreviations: CI, confidence interval; hs-CRP, high-sensitivity C reactive protein; HOMA-IR, homeostasis model assessment of insulin resistance; LDL, low-density lipoprotein; SD, standard deviation; sdLDL, small dense low-density lipoprotein.
Model 1: Adjusted for age, sex, systolic blood pressure, use of antihypertensive agents, hemoglobin A1c, use of glucose-lowering agents, high density lipoprotein cholesterol, use of lipid-modifying agents, body mass index, estimated glomerular filtration rate, electrocardiogram abnormalities, current smoking, current drinking, and regular exercise.
Model 2: Adjusted for age, sex, hemoglobin A1c, high density lipoprotein cholesterol, electrocardiogram abnormalities, and regular exercise, which were selected with the backward elimination method at $p<0.20$ for the remaining variables.

Moreover, the sensitivity analysis including the use of statins as an adjustment factor instead of the use of lipid-modifying agents in the multivariable-adjusted models or among subjects who were not using lipid-modifying agents were not altered substantially although the association did not reach the statistically significant level in the multivariable-adjusted model including serum LDL cholesterol among subjects who were not using lipid-modifying agents (p for trend = 0.07) (**Supplementary Table 1 and Supplementary Table 2**).

We also investigated the influence of serum sdLDL cholesterol levels on the risk of CHD according to serum LDL cholesterol levels. In this analysis, the subjects were divided into four groups according to sdLDL cholesterol levels (≥ 32.9 mg/dL [median] or <32.9 mg/dL) and LDL cholesterol levels (≥ 120.1 mg/dL [median] or <120.1 mg/dL). The multivariable-adjusted HR of CHD almost doubled in subjects with serum sdLDL cholesterol of ≥ 32.9 mg/dL, regardless of serum LDL cholesterol levels, as compared with subjects with serum sdLDL cholesterol of <32.9 mg/dL and serum LDL cholesterol of <120.1 mg/dL as a reference (**Supplementary Fig. 1**).

In the subgroup analysis of various confounding factors, such as sex, hypertension, diabetes, BMI, serum LDL cholesterol, use of lipid-modifying agents, current smoking status, and eGFR, there was no evidence of heterogeneity in the magnitude of multivariable-adjusted HRs per 1 SD increment in log-transformed serum sdLDL cholesterol levels between the subgroups (all p for heterogeneity >0.3; **Supplementary Table 3**).

In addition, we compared the c-statistics between the basic model including cardiovascular disease risk factors with and without sdLDL cholesterol to evaluate whether sdLDL cholesterol improves the accuracy of CHD risk assessment (**Table 3**). Adding the information on the serum sdLDL cholesterol level to the basic model including the aforementioned cardiovascular risk factors significantly increased the c-statistics (from 0.774 to 0.794; $p=0.02$) and improved the accuracy of the risk assessment for CHD—namely, the continuous NRI was estimated as 0.40 (Z_{NRI} 3.52, $p<0.001$), and the IDI was estimated as 0.008 (Z_{IDI} 2.40, $p=0.02$). Meanwhile, adding the information on the serum LDL cholesterol level tended to increase the c-statistics, but the difference did not reach the level of statistical significance (from 0.774 to 0.793; $p=0.06$) although the changes in continuous NRI and IDI were significant (NRI: 0.49, Z_{NRI} 4.27, $p<0.001$; IDI: 0.007, Z_{IDI} 2.71, $p=0.01$).

Finally, we investigated the cutoff value of sdLDL cholesterol that optimizes the discriminating ability

Table 3. Comparison of the accuracy of risk assessment for the development of coronary heart disease between the models adjusted for potential risk factors with and without small dense low-density lipoprotein cholesterol or low-density lipoprotein cholesterol

	c-statistics (95% CI)	*p* value for difference in c-statistics	cNRI (95% CI) after adding sdLDL cholesterol or LDL cholesterol	*p* value for NRI	IDI (95% CI) after adding sdLDL cholesterol or LDL cholesterol	*p* value for IDI
Basic model	0.774 (0.730–0.817)	reference	-	reference	-	reference
Basic model + log(serum sdLDL cholesterol)	0.794 (0.754–0.835)	0.02	0.40 (0.18–0.62)	<0.001	0.008 (0.001–0.01)	0.02
Basic model + serum LDL cholesterol	0.793 (0.750–0.836)	0.06	0.49 (0.27–0.70)	<0.001	0.007 (0.002–0.01)	0.01

Abbreviations: CI, confidence interval; cNRI, continuous net reclassification improvement; IDI, integrated discrimination improvement; LDL, low-density lipoprotein; sdLDL, small dense low-density lipoprotein.
The basic model was adjusted for age, sex, systolic blood pressure, use of antihypertensive agents, hemoglobin A1c, use of glucose-lowering agents, high-density lipoprotein-cholesterol, lipid-modifying agents, body mass index, estimated glomerular filtration rate, electrocardiogram abnormalities, current smoking, current drinking, and regular exercise.

for the risk of incident CHD. The point closest to (0, 1) on the receiver operating characteristic curve was 35 mg/dL (**Supplementary Fig. 2**). A total of 43.9% of subjects had a serum sdLDL cholesterol level above the cutoff value. Subjects with serum sdLDL cholesterol of ≥ 35 mg/dL were at a 2.09-fold (95% CI, 1.26–3.45) increased risk of the development of CHD after adjusting for the aforementioned confounding factors. In addition, the percentages of subjects with a serum sdLDL cholesterol level above the cutoff value, the sensitivities and specificities to detect subjects who developed CHD in 8 years, and the multivariable-adjusted HRs of the serum sdLDL cholesterol level above the cutoff value compared with that below the cutoff value for the various cutoff levels of serum sdLDL cholesterol are shown in **Supplementary Table 4**.

Discussion

The present study demonstrated that higher serum sdLDL cholesterol levels were significantly associated with the development of CHD after adjustment for serum LDL cholesterol levels in addition to other potential cardiovascular risk factors. In addition, the ability to predict future CHD risk was significantly improved by adding the serum sdLDL cholesterol levels to the known cardiovascular risk factors. It has been well acknowledged that serum LDL cholesterol is an important therapeutic target to reduce the risk of CHD and is a good indicator for predicting CHD events[19-21]. Therefore, lipid-modifying therapy is widely accepted in clinical practice for the treatment of CHD[32, 33]. However, a relatively high percentage of individuals with serum LDL cholesterol in the normal range nonetheless go on to develop CHD[10]. Intriguingly, the present study found that the risk of CHD was significantly higher in subjects with higher serum sdLDL cholesterol level than those with lower serum sdLDL cholesterol level in subjects with serum LDL cholesterol levels below 120 mg/dL. These findings provide evidence that serum sdLDL cholesterol is a clinically valuable biomarker for estimating the future onset of CHD even in subjects with normal range serum LDL cholesterol and that serum sdLDL cholesterol is a possible therapeutic target for the prevention of CHD in general practice.

The recently developed assay for measuring serum sdLDL cholesterol would be readily adaptable to mass screening in general practice[17, 18]. Therefore, several prospective studies have assessed the association between sdLDL cholesterol and the risk of CHD. The Multi-Ethnic Study of Atherosclerosis (MESA) demonstrated a positive association between serum sdLDL cholesterol levels and the risk of CHD but found that higher serum sdLDL cholesterol was a significant risk factor for the development of CHD only in nondiabetic subjects[20]. In the present study, the risk of CHD was significantly increased in the group with high serum sdLDL cholesterol levels regardless of the status of diabetes. The Atherosclerosis Risk in Communities (ARIC) Study and the Suita Study also showed that subjects with higher serum sdLDL cholesterol levels were at a significantly increased risk of CHD compared with those with lower levels, but the significance of the association disappeared after addi-

tional adjustment for other lipid risk factors[19, 21]. In the present study, however, the association of serum sdLDL cholesterol with CHD was robust even after adjustment for known cardiovascular risk factors, including the serum LDL cholesterol level. Although the reasons for these discrepant findings are not entirely clear, they may be partly related to the different environmental and genetic backgrounds of the study populations (i.e., race, medical condition, and absolute coronary risk). The present study also demonstrated that high serum sdLDL cholesterol levels were significantly associated with the development of CHD in subjects with low and those with high LDL cholesterol levels. Similar findings were reported in the two aforementioned observational studies conducted in Western countries[19, 20].

Some cross-sectional studies have shown that the size of serum LDL particles tended to be small in subjects with central obesity (i.e., higher visceral fat area) or insulin resistiance[34, 35]. Moreover, subjects with high serum sdLDL cholesterol levels had high serum hs-CRP levels in the present study. Therefore, the elevation in serum sdLDL cholesterol levels may reflect the insulin resistance and systemic inflammation, which could lead to progression of metabolic disorders and atherosclerosis in the coronary arteries. Meanwhile, the present study showed that the excess risk of CHD in subjects with high serum sdLDL cholesterol levels remained significant even after adjusting for serum hs-CRP levels or HOMA-IR in addition to known cardiovascular risk factors, including metabolic components. These findings raise the possibility that the other mechanisms of CHD development may exist. sdLDL particles have been considered to penetrate into the arterial wall easily because of their small size and to have a high affinity for proteoglycans in the arterial wall, but a low affinity for the hepatic LDL receptor, leading to prolonged residency in plasma[36, 37]. sdLDL particles also lack antioxidant substances, such as vitamin E, and thus are highly susceptible to oxidation[38]. These features of sdLDL particles would be a causal factor promoting atherosclerosis and thus the development of CHD.

In addition, we found that the cutoff value of sdLDL cholesterol that optimizes the ability to discriminate the risk of developing CHD was approximately 35 mg/dL when equal weight was given to sensitivity and specificity and ethical and cost constraints were ignored. On the other hand, approximately 40% of individuals in our community were assigned to the high-risk population for CHD by this cutoff level. The Food and Drug Administration cleared the assay with a serum sdLDL cholesterol cutoff level of 50 mg/dL for detecting subjects with a high risk of CHD based on the results from the MESA and ARIC Study, in which approximately 30% of US community-dwelling individuals had serum sdLDL cholesterol levels of ≥ 50 mg/dL, and the sensitivity and the specificity of this cutoff level to detect subjects who developed CHD were found to be 41% and 71%, respectively[39]. These findings are almost equivalent to those at the cutoff level of 40 mg/dL in the present study (**Supplementary Table 4**). Thus, it may be reasonable to set the cutoff of the high-risk population for CHD in a Japanese community at 35–40 mg/dL. On the other hand, the sensitivity and specificity of this cutoff value to detect subjects with incident CHD were not particularly high. Therefore, a combined risk assessment with sdLDL cholesterol and other cardiovascular risk factors may be necessary rather than the isolated use of sdLDL cholesterol to identify the high-risk population for CHD more effectively. The optimal cutoff level of serum sdLDL cholesterol should be validated in other population-based cohort studies, with due consideration given to the risk assessment method, ethical issues, cost-effectiveness, and racial differences.

The strengths of this study include its longitudinal population-based design, almost perfect follow-up of study subjects, and accurate diagnoses of CHD. However, some potential limitations of this study should be noted. First, the serum sdLDL cholesterol level and other risk factors were based on only one measurement at baseline. In addition, we were unable to obtain information about medical treatment during the follow-up period. Given that serum sdLDL cholesterol levels and other risk factors may have changed during follow-up, this limitation could lead to the misclassification of these variables, which would weaken the association found in the present study, biasing the results toward the null hypothesis. Second, the number of CHD events was insufficient for a more detailed analysis. Third, we measured serum sdLDL cholesterol levels using frozen serum samples collected as part of the survey in 2002 and stored at −80℃ for seven years. However, it has been reported that there is no major difference in sdLDL cholesterol levels between fresh serum samples and stored ones[17]. Finally, the generalizability of our findings, including the cutoff value, may be limited, because these analyses were conducted in only one cohort of Japanese. Therefore, our findings should be validated in other cohorts of various ethnic populations.

Conclusion

The present study demonstrated that elevated sdLDL cholesterol was associated with the development of CHD regardless of LDL cholesterol levels.

Moreover, the incorporation of sdLDL cholesterol values into a model with known risk factors improved the predictive ability of the risk of CHD in a general population. These findings suggest that the measurement of serum sdLDL cholesterol would be useful for assessing future risk of CHD even in patients with LDL cholesterol within a normal range. Therefore, it may be supposed that high serum sdLDL cholesterol levels should be lowered intensively to prevent the onset of CHD. However, further investigations are required to clarify whether sdLDL cholesterol would be a suitable interventional target for reducing the burden of CHD.

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Conflict of Interest

Toshiharu Ninomiya received research funding from DENKA SEIKEN Co., Ltd. The other authors declare that they have no conflicts of interest.

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Supplementary Table 1. Multivariable-adjusted hazard ratios for coronary heart disease according to serum small dense low-density lipoprotein cholesterol levels, 2007-2015

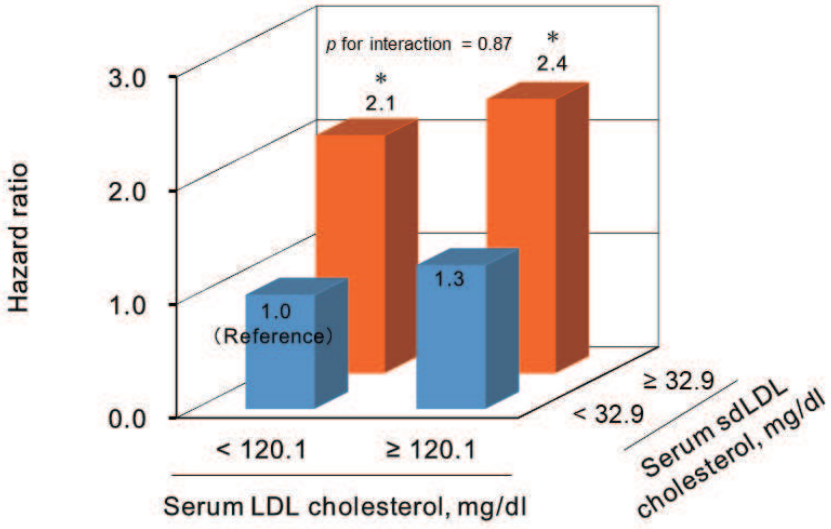
	Serum sdLDL cholesterol level, mg/dL				*p* for trend
	Quartile 1 ≤ 24.4	Quartile 2 24.5-32.8	Quartile 3 32.9-43.6	Quartile 4 ≥ 43.7	
Hazard ratio (95% CI)					
Model 1	1.00 (Reference)	3.18 (1.25-8.06)	4.12 (1.65-10.33)	5.32 (2.08-13.59)	<0.001
Model 1 + serum LDL cholesterol	1.00 (Reference)	2.77 (1.07-7.22)	3.28 (1.22-8.80)	3.84 (1.31-11.24)	0.03
Model 1 + serum triglycerides	1.00 (Reference)	3.04 (1.19-7.77)	3.86 (1.51-9.85)	4.71 (1.72-12.93)	0.003
Model 1 + log hs-CRP	1.00 (Reference)	3.23 (1.27-8.21)	4.19 (1.67-10.49)	5.47 (2.14-14.00)	<0.001
Model 1 + log HOMA-IR	1.00 (Reference)	2.94 (1.15-7.47)	3.88 (1.55-9.73)	4.68 (1.82-12.04)	0.0012

SI conversion factors: To convert mg/dL values to mmol/L, multiply serum sdLDL cholesterol values by 0.02586 and multiply serum triglycerides values by 0.01129.
Abbreviations: CI, confidence interval; hs-CRP, high-sensitivity C reactive protein; HOMA-IR, homeostasis model assessment of insulin resistance; LDL, low-density lipoprotein; SD, standard deviation; sdLDL, small dense low-density lipoprotein.
Model 1: Adjusted for age, sex, systolic blood pressure, use of antihypertensive agents, hemoglobin A1c, use of glucose-lowering agents, high density lipoprotein cholesterol, use of statins, body mass index, estimated glomerular filtration rate, electrocardiogram abnormalities, current smoking, current drinking, and regular exercise

Supplementary Table 2. Multivariable-adjusted hazard ratios for coronary heart disease according to serum small dense low-density lipoprotein cholesterol levels excluding subjects with use of lipid-modifying agents (n=2,651), 2007-2015

	Serum sdLDL cholesterol level, mg/dL				*p* for trend
	Quartile 1 ≤ 24.4	Quartile 2 24.5-32.8	Quartile 3 32.9-43.6	Quartile 4 ≥ 43.7	
No. of events/subjects	5/639	12/675	18/657	29/680	
Age-and sex-adjusted incidence rate (per 1,000 person-years)	1.01	2.38	4.38	5.51	<0.001
Hazard ratio (95% CI)					
Age-and sex-adjusted	1.00 (Reference)	2.46 (0.87-7.01)	3.92 (1.45-10.63)	6.56 (2.49-17.28)	<0.001
Model 1	1.00 (Reference)	2.46 (0.86-7.02)	3.50 (1.27-9.66)	5.25 (1.90-14.48)	<0.001
Model 1 + serum LDL cholesterol	1.00 (Reference)	1.97 (0.67-5.76)	2.41 (0.81-7.11)	3.06 (0.96-9.73)	0.07
Model 1 + serum triglycerides	1.00 (Reference)	2.45 (0.85-7.06)	3.50 (1.24-9.86)	5.27 (1.77-15.75)	0.002
Model 1 + log hs-CRP	1.00 (Reference)	2.52 (0.88-7.21)	3.59 (1.30-9.91)	5.51 (1.99-15.23)	<0.001
Model 1 + log HOMA-IR	1.00 (Reference)	2.29 (0.80-6.54)	3.31 (1.20-9.13)	4.74 (1.71-13.15)	0.001

SI conversion factors: To convert mg/dL values to mmol/L, multiply serum sdLDL cholesterol values by 0.02586 and multiply serum triglycerides values by 0.01129.
Abbreviations: CI, confidence interval; hs-CRP, high-sensitivity C reactive protein; HOMA-IR, homeostasis model assessment of insulin resistance; LDL, low-density lipoprotein; SD, standard deviation; sdLDL, small dense low-density lipoprotein.
Model 1: Adjusted for age, sex, systolic blood pressure, use of antihypertensive agents, hemoglobin A1c, use of glucose-lowering agents, high density lipoprotein cholesterol, body mass index, estimated glomerular filtration rate, electrocardiogram abnormalities, current smoking, current drinking, and regular exercise



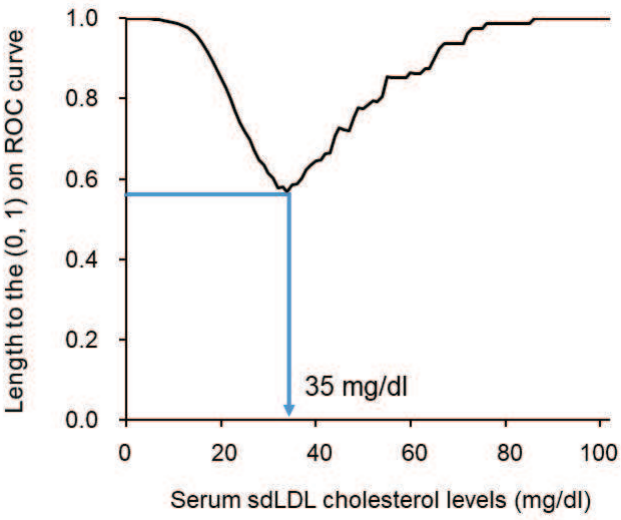
Supplementary Fig. 1. Multivariable-adjusted hazard ratios for the development of coronary heart disease according to serum low-density lipoprotein cholesterol and serum small dense low-density lipoprotein cholesterol

LDL, low-density lipoprotein; sdLDL, smalldense low-density lipoprotein.
*$p<0.05$ vs. reference.
Hazard ratios were adjusted for age, sex, systolic blood pressure, antihypertensive drugs, hemoglobin A1c, antidiabetic medication, high density lipoprotein-cholesterol, lipid-lowering drugs, body mass index, estimated glomerular filtration rate, electrocardiogram abnormalities, current smoking, current drinking, and regular exercise.

Supplementary Table 3. Multivariable-adjusted hazard ratios for coronary heart disease per 1 SD increment in log-transformed small dense low-density lipoprotein-cholesterol levels in various subgroups of the study population, 2007-2015

Variables	Persons at risk	No. of events	HR (95% CI) per 1 SD increment in log (serum sdLDL cholesterol levels)	*p* for heterogeneity
Overall	3,080	79	1.62 (1.23 to 2.16)	-
Sex				
Men	1,290	49	1.48 (1.04 to 2.10)	0.77
Women	1,790	30	1.93 (1.21 to 3.10)	
Hypertension				
No	1,617	22	1.43 (0.86 to 2.39)	0.76
Yes	1,463	57	1.63 (1.17 to 2.28)	
Diabetes				
No	2,619	50	1.66 (1.17 to 2.34)	0.61
Yes	461	29	1.57 (0.96 to 2.59)	
BMI				
<25 kg/m^2	2,294	53	1.67 (1.19 to 2.35)	0.92
≥ 25 kg/m^2	786	26	1.44 (0.89 to 2/34)	
Serum LDL cholesterol				
<120.1 mg/dL	1,538	33	2.01 (1.22 to 3.31)	0.65
≥ 120.1 mg/dL	1,542	46	1.45 (0.92 to 2.28)	
Use of lipid-modifying agents				
No	2,651	64	1.66 (1.21 to 2.27)	0.38
Yes	428	15	1.50 (0.78 to 2.90)	
Current smoking				
No	2,465	58	1.67 (1.20 to 2.32)	0.51
Yes	614	21	1.70 (0.97 to 2.96)	
eGFR				
≥ 60 ml/min/1.73 m^2	2,788	140	1.55 (1.15 to 2.09)	0.70
<60 ml/min/1.73 m^2	292	33	2.64 (1.13 to 6.18)	

SI conversion factors: To convert mg/dL values to mmol/L, multiply the serum LDL cholesterol value by 0.02586.
Abbreviations: CI, confidence interval; eGFR, estimated glomerular filtration rate; HR, hazard ratio; LDL, low-density lipoprotein; SD, standard deviation; sdLDL, small dense low-density lipoprotein.
The model was adjusted for age, sex, systolic blood pressure, use of antihypertensive agents, hemoglobin A1c, use of glucose-lowering agents, serum high-density lipoprotein-cholesterol, use of lipid-modifying agents, body mass index, estimated glomerular filtration rate, electrocardiogram abnormalities, current smoking, current drinking, and regular exercise.
The variables relevant to the subgroup were excluded from the relevant Cox model.



Supplementary Fig. 2. The cut-off value of serum small dense low-density lipoprotein cholesterol that optimizes the ability to discriminate the risk of coronary heart disease: the length to (0, 1) on the receiver operating characteristic curve

Abbreviations: ROC, receiver operating characteristic; sdLDL, small dense low-density lipoprotein

Length to the (0, 1) on ROC curve = $\sqrt{(1 - \text{sensitivity})^2 + (1 - \text{specificity})^2}$

Supplementary Table 4. The sensitivity and specificity of detecting high-risk subjects who developed coronary heart disease during an 8-year follow-up for each cutoff value of serum small dense low-density lipoprotein cholesterol level

Cutoff value of serum sdLDL-C level (mg/dl)	Subjects with a serum sdLDL-C level above the cutoff value among 3,080 subjects		Number of subjects with a serum sdLDL-C level above the cutoff value among 79 subjects who developed CHD during follow-up	Sensitivity (%)	Specificity (%)	HR (95% CI) of a serum sdLDL-C level above the cutoff value vs. that below the cutoff value[a)]
	Number	Frequency (%)				
25	2,275	73.9	71	89.9	26.6	3.10 (1.45-6.63)
30	1,806	58.6	59	74.7	41.8	1.98 (1.15-3.40)
35	1,352	43.9	50	63.3	56.6	2.09 (1.26-3.45)
40	1,018	33.1	36	45.6	67.3	1.52 (0.93-2.46)
45	703	22.8	26	32.9	77.4	1.49 (0.89-2.49)
50	513	16.7	19	24.1	83.5	1.50 (0.86-2.62)
55	336	10.9	16	20.3	89.3	2.08 (1.14-3.78)
60	242	7.9	12	15.2	92.3	2.04 (1.04-3.99)

Abbreviations: CHD, coronary heart disease; sdLDL-C, small dense low-density lipoprotein-cholesterol.

SI conversion factors: To convert mg/dL values to mmol/L, multiply serum sdLDL cholesterol values by 0.02586.

a) Adjusted for age, sex, systolic blood pressure, use of antihypertensive agents, hemoglobin A1c, use of glucose-lowering agents, high density lipoprotein cholesterol, use of lipid-modifying agents, body mass index, estimated glomerular filtration rate, electrocardiogram abnormalities, current smoking, current drinking, and regular exercise.

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Low-Density Lipoprotein Cholesterol 4: The Notable Risk Factor of Coronary Artery Disease Development

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Background: Coronary artery disease (CAD) is the leading cause of death worldwide, which has a long asymptomatic period of atherosclerosis. Thus, it is crucial to develop efficient strategies or biomarkers to assess the risk of CAD in asymptomatic individuals.

Methods: A total of 356 consecutive CAD patients and 164 non-CAD controls diagnosed using coronary angiography were recruited. Blood lipids, other baseline characteristics, and clinical information were investigated in this study. In addition, low-density lipoprotein cholesterol (LDL-C) subfractions were classified and quantified using the Lipoprint system. Based on these data, we performed comprehensive analyses to investigate the risk factors for CAD development and to predict CAD risk.

Results: Triglyceride, LDLC-3, LDLC-4, LDLC-5, LDLC-6, and total small and dense LDL-C were significantly higher in the CAD patients than those in the controls, whereas LDLC-1 and high-density lipoprotein cholesterol (HDL-C) had significantly lower levels in the CAD patients. Logistic regression analysis identified male [odds ratio (OR) = 2.875, $P < 0.001$], older age (OR = 1.018, $P = 0.025$), BMI (OR = 1.157, $P < 0.001$), smoking (OR = 4.554, $P < 0.001$), drinking (OR = 2.128, $P < 0.016$), hypertension (OR = 4.453, $P < 0.001$), and diabetes mellitus (OR = 8.776, $P < 0.001$) as clinical risk factors for CAD development. Among blood lipids, LDLC-3 (OR = 1.565, $P < 0.001$), LDLC-4 (OR = 3.566, $P < 0.001$), and LDLC-5 (OR = 6.866, $P < 0.001$) were identified as risk factors. To predict CAD risk, six machine learning models were constructed. The XGboost model showed the highest AUC score (0.945121), which could distinguish CAD patients from the controls with a high accuracy. LDLC-4 played the most important role in model construction.

Conclusions: The established models showed good performance for CAD risk prediction, which can help screen high-risk CAD patients in asymptomatic population, so that further examination and prevention treatment might be taken before any sudden or serious event.

Keywords: coronary artery disease, LDL-C subfractions, machine learning, coronary angiography, risk factors

INTRODUCTION

Ischemic heart disease (IHD) is the leading cause of death worldwide according to World Health Organization statistics, and its incidence has been increasing over decade (1). IHD pathophysiological mechanisms (2) are mainly involved in atherosclerosis, coronary microvascular dysfunction, inflammation, and vasospasm. Coronary artery disease (CAD) caused by atherosclerosis is the main cause of IHD. Atherosclerosis is a slowly progressing disease, which might be revealed by typical symptoms such as angina pectoris, or an acute event, including sudden death, without any preceding symptoms (3). Hence, it is crucial to screen population at high risk of CAD, and further medical tests, lifestyle changes, or preventive treatment should be proposed before potentially fatal events occur. So far, coronary angiography is the gold standard for CAD diagnosis, but it is an invasive method and is unpractical for universal screening.

Dyslipidemia is a well-studied risk factor for atherosclerosis (4). Numerous epidemiologic studies and randomized clinical trials have suggested that elevated low-density lipoprotein cholesterol (LDL-C) is a major cause and the target to be controlled to reduce atherosclerotic cardiovascular disease (ASCVD) risk (5–7). However, in fact, a large proportion of atherosclerosis and CAD patients have normal range of blood LDL-C level. LDL-C particles are heterogeneous and can be classified into seven subfractions according to their density and size (8). Increasing evidence indicates that small dense LDL-C (sdLDL-C) is more atherogenic than large buoyant LDL-C (9). SdLDL-C is composed of LDL-C subfraction 3 (LDLC-3) to LDLC-7, and large buoyant LDL-C (lbLDL-C) is composed of LDLC-1 and LDLC-2. A few scattered studies with small sample size have reported LDL-C subfractions' role on CAD. For

Abbreviations: CAD, coronary artery disease; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; TC, total cholesterol; TG, triglyceride; SCr, serum creatinine; LR, logistic regression; MLP, the multilayer perceptron network; DT, the decision tree model; SVM, a support vector machine; KNN, k-nearest neighbors classifier; OR, odds ratio; AUC, area under curve.

TABLE 1 | Clinical characteristics and blood lipids of controls and CAD patients.

Characters	Controls (n = 164)	Non-obstructive CAD (n = 57)	Significant CAD (n = 299)	P-value
Age, years	57.41 ± 13.752	56.21 ± 10.20	60.86 ± 11.04	0.004
Median (range)	55 (27–86)	57 (29–76)	60 (33–87)	
BMI (mean ± SD), kg/m^2	22.98 ± 4.06	25.81 ± 3.45	25.17 ± 3.58	<0.0001
Gender				<0.0001
Male	75 (45.73%)	29 (50.88%)	223 (74.58%)	
Female	89 (54.27%)	28 (49.12%)	76 (25.42%)	
Hypertension				<0.0001
Yes	39 (23.78%)	30 (52.63%)	177 (59.20%)	
Taking antihypertensive medications	25/39	25/30	160/177	
No	125 (76.22%)	27 (47.37%)	122 (40.80%)	
Diabetes mellitus				<0.0001
Yes	5 (3.05%)	7 (12.28%)	70 (23.41%)	
Taking hypoglycemic drugs	1/5	5/7	38/70	
No	159 (96.95%)	50 (87.72%)	229 (76.59%)	
Smoke				<0.0001
Yes	18 (10.98%)	14 (24.56%)	114 (38.13%)	
No	146 (89.02%)	43 (75.44%)	185 (61.87%)	
Drink				0.028
Yes	14 (8.54%)	12 (21.05%)	47 (15.72%)	
No	150 (91.46%)	45 (78.95%)	252 (84.28%)	
TC (>5.17 mmol/L)	23 (14.02%)	12 (10.53%)	50 (16.72%)	0.449
TG (>1.7 mmol/L)	27 (16.46%)	29 (50.88%)	135 (45.15%)	<0.001
HDL-C (<0.86 mmol/L)	27 (16.46%)	15 (26.32%)	82 (27.42%)	0.027
LDL-C (>3.4 mmol/L)	9 (5.49%)	6 (10.53%)	40 (13.38%)	0.031
LDLC-1 (>57 mg/dl)	0 (0%)	0 (0%)	0 (0%)	/
LDLC-2 (>30 mg/ dl)	6 (3.66%)	7 (12.28%)	33 (7.69%)	0.018
LDLC-3 (>6 mg/dl)	6 (3.66%)	48 (84.21%)	222 (74.25%)	<0.001
LDLC-4 (>0 mg/dl)	2 (1.22%)	48 (84.21%)	248 (82.94%)	<0.001
LDLC-5 (>0 mg/dl)	1 (0.61%)	23 (40.35%)	142 (47.49%)	<0.001
LDLC-6 (>0 mg/dl)	0 (0%)	11 (19.30%)	62 (20.74%)	<0.001
LDLC-7 (>0 mg/dl)	0 (0%)	5 (8.77%)	34 (11.37%)	<0.001

BMI, body weight index; TC, total cholesterol; TG, triglyceride, HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol.

example, Chaudhary et al. reported that lbLDL-C was negatively correlated with CAD severity (10). In addition, elevated LDLC-4 level was associated with severe CAD in their research, which recruited 179 consecutive patients with suspected CAD, and LDLC-4 was considered as an independent predictive factor for severe CAD based on the result of multivariate analysis (10). Yet there is still lack of substantial studies to elaborate the effects of detailed LDL-C subfractions (LDLC-1 to LDLC-7) on CAD development and its severity.

In this study, we described the blood lipid profile, including LDL-C subfractions, of CAD patients and controls. Then, risk factors of CAD development and severity were analyzed, and predicted models were constructed to assess CAD risk by machine learning method.

METHODS AND MATERIALS

Study Population

A total of 356 newly diagnosed CAD patients and 164 non-CAD controls were recruited (**Table 1**) from the Department of Cardiovascular Medicine, the General Hospital of Tisco Affilated to Shanxi Medical University, the First People's Hospital of Pingdingshan, and Mianxian Hospital. The inclusion criterion in this study was suspected patients who underwent coronary angiography. And the exclusion criteria were as follows: (1) having a history of severe cardiovascular diseases such as CAD and stroke, (2) having received lipid-lowering therapy, and (3) inability to understand the research aims of this study. When patients were admitted to the hospitals, essential and clinical information were collected, including age, gender, body mass index (BMI), smoking and drinking history, and disease history such as hypertension, diabetes mellitus, and so on. Hypertension is defined as a systolic blood pressure of 140 mm Hg or more or a diastolic blood pressure of 90 mm Hg or more, or taking antihypertensive medication. Diabetes mellitus was evaluated as follows: a previous diagnosis or typical clinical symptoms of diabetes mellitus with random plasma glucose $\geq$11.1 mmol/L and/or fasting plasma glucose $\geq$7.0 mmol/L and/or 2-h plasma glucose after 75-g oral glucose tolerance test $\geq$11.1 mmol/L.

Definition of CAD Patients and Controls

The CAD patients were diagnosed by coronary angiography, which were divided into two groups including non-obstructive CAD and significant CAD. Significant CAD is defined as coronary artery stenosis $\geq$50% in at least one main vessel or its major branches. Non-obstructive CAD was defined as visible plaque resulting in $<$50% luminal stenosis. The Gensini score method was used to evaluate the severity of CAD (11, 12). Gensini score of CAD patients was calculated according to coronary angiography result (12). The controls were diagnosed by coronary angiography without any luminal stenosis or plaque in main vessels and branches.

Sample Collection and Laboratory Indices Detection

Blood samples were collected after overnight fasting from all participants before taking coronary angiography and concomitant medications. Then, total cholesterol (TC), triglyceride (TG), high-density lipoprotein cholesterol (HDL-C), LDL-C, serum creatinine (SCr), and other routine detection indexes were tested in the Department of Clinical Laboratory. After having been immediately separated, plasma was subjected to 800$\times g$ centrifugation for 10 min at 4°C. LDL-C subfractions were then classified and quantified by a well-established

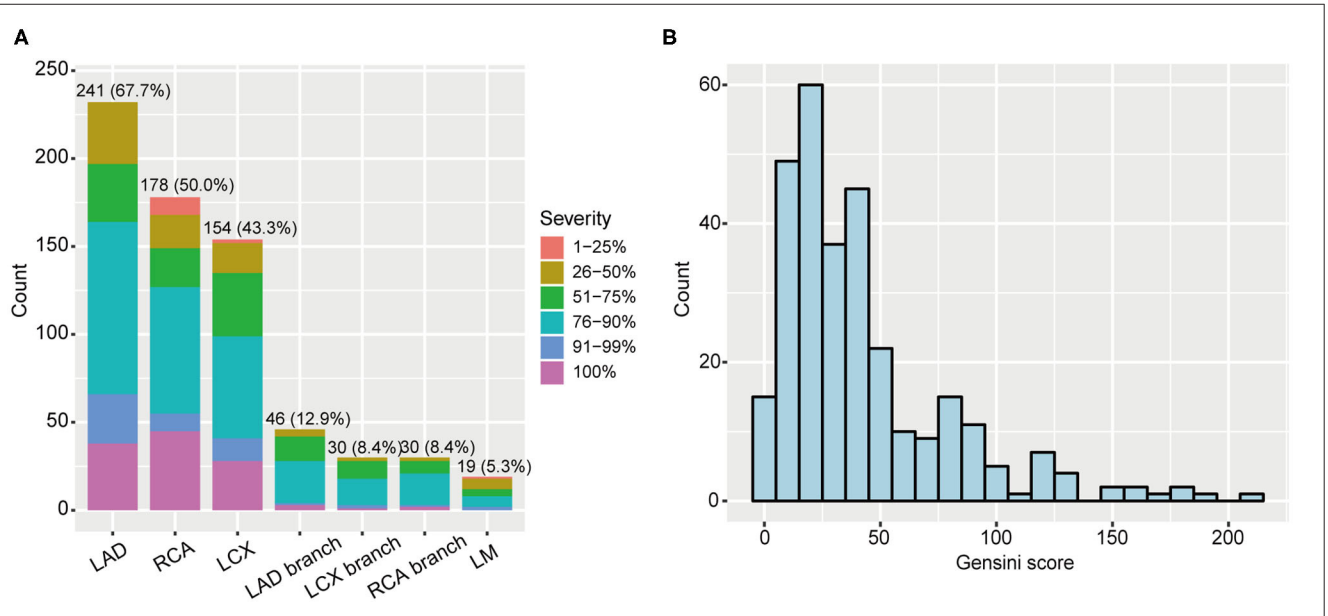

FIGURE 1 | Coronary artery stenosis severity of 356 CAD patients. **(A)** Distribution of stenosis severity and locations. **(B)** Gensini score distribution in CAD patients. CAD, coronary artery disease; LAD, left anterior descending artery; RCA, right coronary artery; LCX, left circumflex artery; LM, left main.

method, namely, Quantimetrix Lipoprint LDL System (8) (Quantimetrix Corporation, Redondo Beach, CA, USA) according to the manufacturer's instructions. Briefly, the plasma mixed with liquid loading gel was added to the top of precast 3% polyacrylamide gel tubes. After 30 min of photopolymerization at room temperature, samples were electrophoresed for 1 h. Then, densitometry was performed at 610 nm.

Statistical Analyses

Statistical analyses were performed using SPSS 19.0 (IBM, NY, USA). Differences of categorical variables in distribution between groups were assessed with χ^2 or the Fisher exact test, as appropriate. Differences of continuous variables among groups were compared by Mann–Whitney *U* or Kruskal–Wallis *H* test. Correlation analysis was conducted by the Pearson correlation method. Graphical plots were generated using GraphPad Prism 6.0 software (La Jolla, CA, USA) and R Project. Logistic regression (LR) analysis was performed to investigate the risk factors of CAD risk by SPSS software. $P < 0.05$ was considered statistically significant. We used Python version 3.7 as the basic language of the whole model and call numpy, panda, sklearn, xgboost, and Matplotlib libraries to process and model the data. After preprocessing the data with numpy and panda, xgboost was used to analyze the importance of features according to the total gain and to select the features with higher importance. StratifiedKFold was used to divide samples into the training set and the test set. The training set was trained by k-nearest neighbors classifier, LR, the support vector machine, the decision tree (DT) model, the multilayer perceptron network, and the XGBoost model. After the training, each model was evaluated

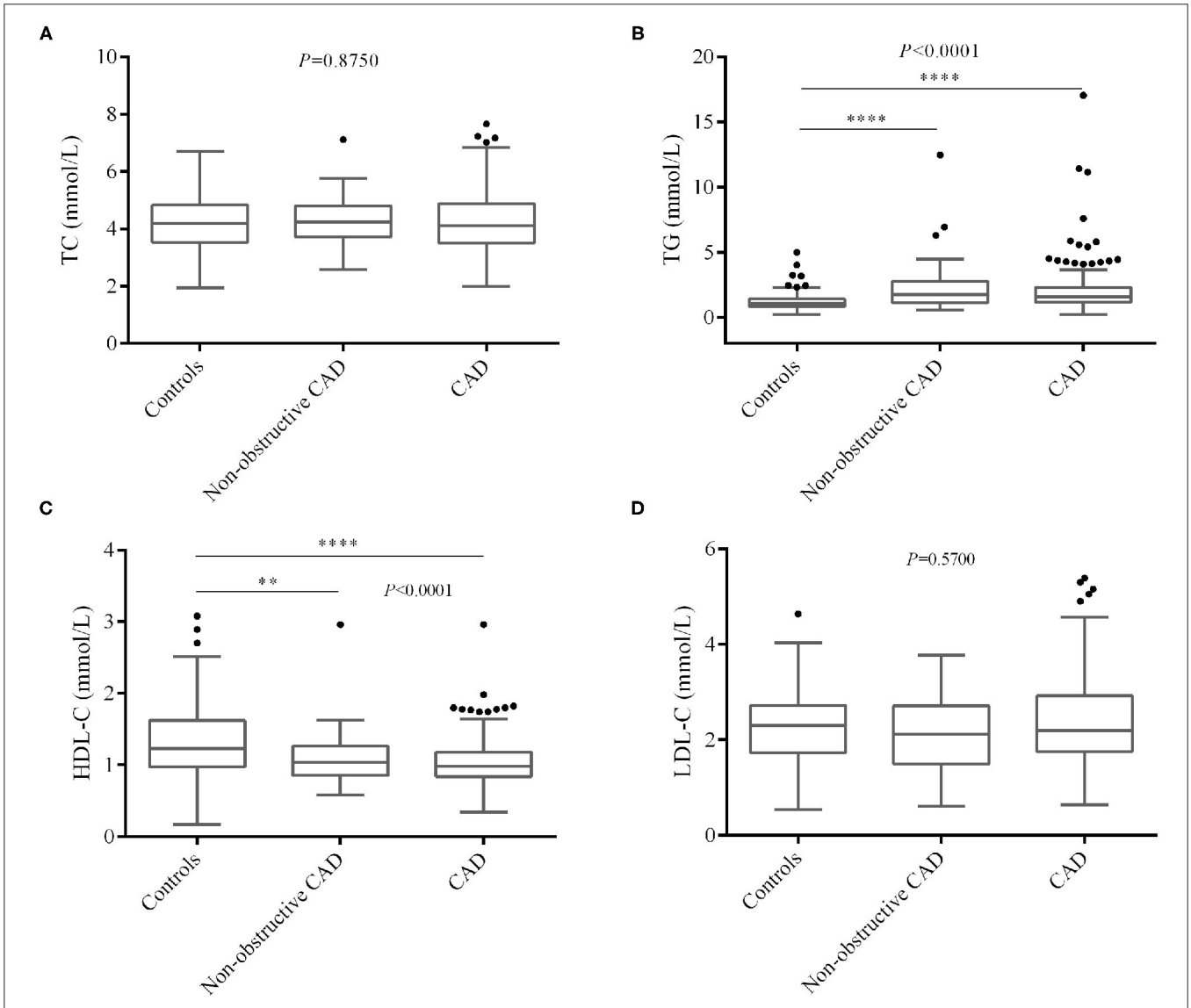

FIGURE 2 | Comparison of blood lipids level among controls and non-obstructive and significant CAD patients. **(A)** TC, **(B)** TG, **(C)** HDL-C, **(D)** LDL-C. CAD, coronary artery disease; TC, total cholesterol; TG, triglyceride, HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

in the test set, and then the accuracy, precision, recall, F1 value, and AUC score of each model were calculated, respectively. The receiver operating characteristic (ROC) curve and precision recall curve (PRC) of each model were drawn to present the judgment ability of the model.

RESULTS

Clinical Characteristics of Participants

Among 356 CAD patients, 57 and 299 patients were divided into non-obstructive and significant CAD patients, respectively (**Table 1**). The distribution of coronary artery stenosis severity and location are shown in **Figure 1A**, from which we can see left anterior descending arteries develop the most frequent stenosis (67.7%) comparing to other vessels. The peak of severity distribution is 76–90%. We also calculated Gensini score of 356 CAD patients, which is shown in **Figure 1B**.

Table 1 shows the clinical information of 164 controls and 356 first-visit CAD patients. We found that controls and non-obstructive CAD patients had comparative gender ratio, but significant CAD patients had significantly higher ratio of male patients (74.58%). Besides, we also found CAD patients had advantageous distribution in hypertension ($P < 0.0001$), diabetes mellitus ($P < 0.0001$), and smoking ($P < 0.0001$) and drinking history ($P = 0.028$) and had elder age ($P = 0.004$) and higher BMI ($P < 0.0001$) than those in controls.

Blood Lipid Profile in Controls and CAD Patients

Table 1 shows the abnormal ratio of blood lipids among controls and non-obstructive and significant CAD patients. We found that the abnormal ratios of total LDL-C were only 10.53 and 13.38% in the two groups of CAD patients, which verified that the blood LDL-C levels in a large proportion of CAD patients were in normal ranges. Besides, after comparing with other LDL-C subfractions, LDLC-3 and LDLC-4 have a higher positive rate in non-obstructive (84.21, 84.21%) and significant CAD (74.25, 82.94%) patients.

We also compared blood lipid levels among controls and non-obstructive and significant CAD patients, and the results are shown in **Figures 2**, **3**. Surprisingly, there is no significant difference of LDL-C (**Figure 2D**) between controls and CAD patients. TG is significantly higher ($P < 0.0001$, **Figure 2B**) and HDL-C ($P < 0.0001$, **Figure 2C**) is lower in CAD patients than in controls. **Figure 3** shows that LDLC-3, LDLC-4, LDLC-5, LDLC-6, and sdLDL-C are significantly higher in CAD patients ($P < 0.0001$), but no significant difference is found between non-obstructive and significant CAD patients. LDLC-1 is higher in controls than those in the two groups of CAD patients, which indicated that LDLC-1 played a protective role in CAD development.

Correlation Analysis Among Clinical Factors and Blood Lipids

Pearson correlation analysis was performed among clinical factors and blood lipids, which is shown in **Figure 4**. Based on our analyses, LDL-C is strongly positively correlated with TC level ($r = 0.82$, $P < 0.001$). HDL-C is negatively correlated with TG ($r = -0.31$, $P < 0.001$), LDLC-3 ($r = -0.18$, $P < 0.001$), LDLC-4 ($r = -0.22$, $P < 0.001$), and sdLDL-C ($r = -0.23$, $P < 0.001$). A large number of studies have demonstrated that sdLDL-C is negatively correlated with HDL-C concentration (13–15).

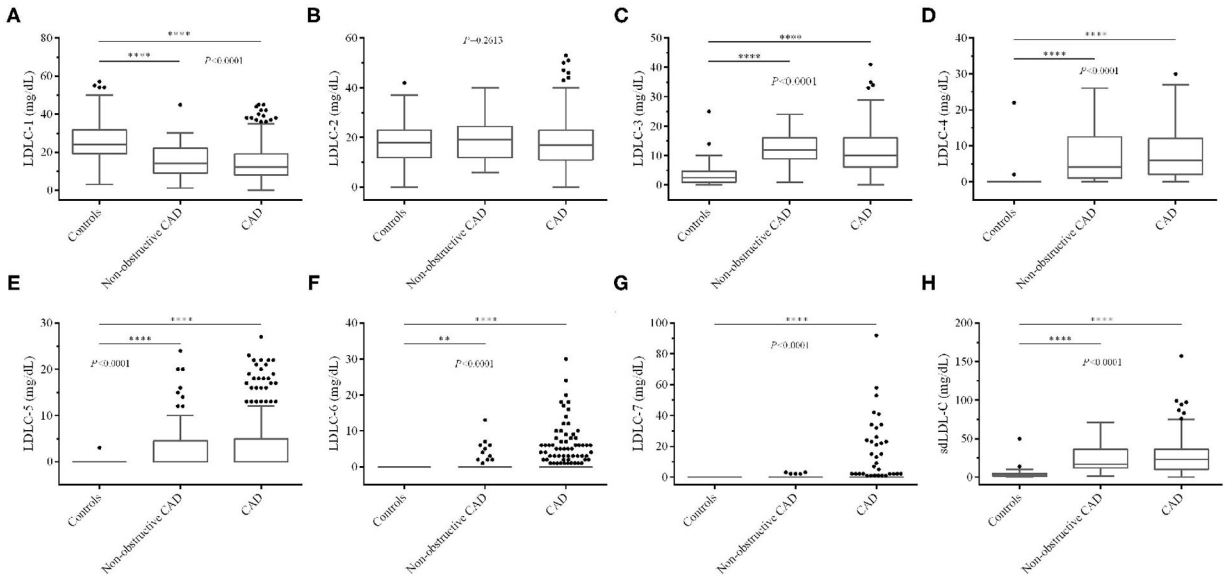

FIGURE 3 | Comparison of LDL-C subfraction levels among controls and non-obstructive and significant CAD patients. **(A)** LDLC-1, **(B)** LDLC-2, **(C)** LDLC-3, **(D)** LDLC-4, **(E)** LDLC-5, **(F)** LDLC-6, **(G)** LDLC-7, **(H)** sdLDL-C. LDL-C, low-density lipoprotein cholesterol; CAD, coronary artery disease. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

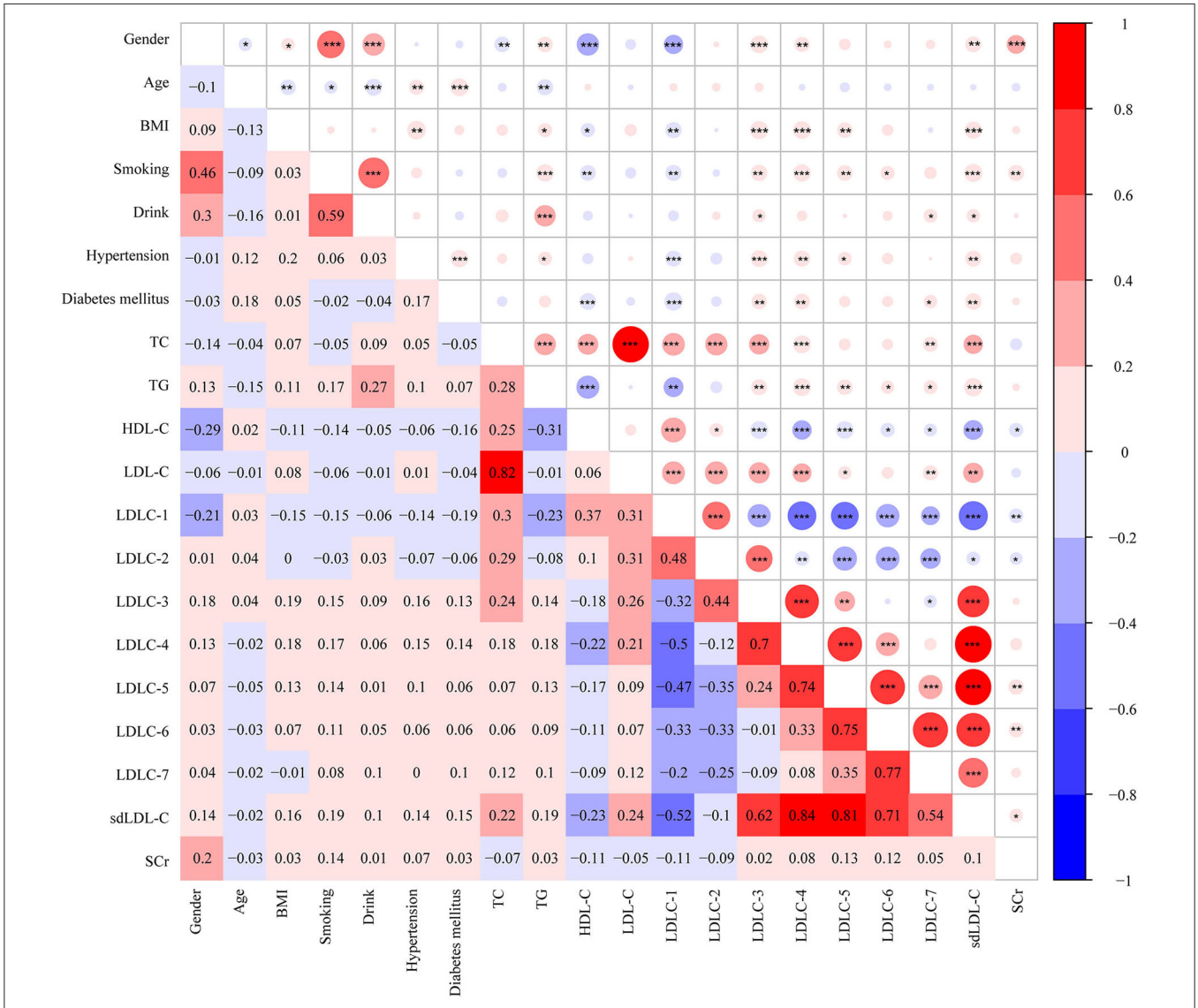

FIGURE 4 | Heatmap showing the correlation among clinical characters and blood markers. The value in grids is Pearson correlation coefficient (Pearson *r*), which is also marked by colors. **P* <0.05, ***P* < 0.01, ****P* < 0.001. BMI, body weight index; TC, total cholesterol; TG, triglyceride, HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; sdLDL-C, small and dense LDL-C; SCr, serum creatinine.

In our study, we found LDLC-1 is negatively correlated with LDLC-3 ($r = -0.32$, $P < 0.001$), LDLC-4 ($r = -0.5$, $P < 0.001$), LDLC-5 ($r = -0.47$, $P < 0.001$), LDLC-6 ($r = -0.33$, $P < 0.001$), LDLC-7 (−0.2, $P < 0.001$), sdLDL-C ($r = -0.52$, $P < 0.001$), and SCr ($r = -0.11$, $P < 0.01$). We also performed linear correlation analysis between Gensini score of CAD patients and aforementioned blood lipids, and HDL-C was negatively correlated with Gensini score ($r = -0.178$, $P = 0.001$). However, no significant correlation was found in other blood lipids.

Risk Factors of CAD Development by Logistic Regression

Table 2 shows the results of LR analysis between the subject's factors and CAD risk. For clinical factors, male [odds ratio (OR) = 2.875, $P < 0.001$], older age (OR = 1.018, $P = 0.025$), BMI (OR = 1.157, $P < 0.001$), smoking (OR = 4.554, $P < 0.001$), drink (OR = 2.128, $P = 0.016$), hypertension (OR = 4.453, $P < 0.001$), and diabetes mellitus (OR = 8.776, $P < 0.001$) are identified as CAD risk factors. For blood lipids, LDLC-3 (OR = 1.565, $P < 0.001$), LDLC-4 (OR = 3.566, $P < 0.001$), and LDLC-5 (OR = 6.866, $P < 0.001$) are identified as risk factors for CAD development.

Prediction of CAD Risk by Model Established

One hundred sixty-four controls and 356 CAD patients were grouped, and six models were established to predict CAD risk by machine learning algorithms. The following features were selected according to their importance including LDLC-4,

TABLE 2 | Logistic regression analysis for independent association between subjects' variables and the risk of CAD.

Variables	Wald	*P*-value	OR	95% CI for Exp(B)	
				Lower	Upper
Gender	29.238	<0.001	2.875	1.961	4.217
Age, y	5.047	0.025	1.018	1.002	1.034
BMI, kg/m^2	20.762	<0.001	1.157	1.086	1.231
Smoking	30.803	<0.001	4.554	2.666	7.778
Drink	5.798	0.016	2.128	1.151	3.936
Hypertension	49.368	<0.001	4.453	2.936	6.754
Diabetes mellitus	21.169	<0.001	8.776	3.479	22.139
TC	0.059	0.809	1.024	0.847	1.237
TG	11.112	<0.001	3.128	2.234	4.379
HDL-C	43.983	<0.001	0.155	0.089	0.269
LDL-C	0.443	0.506	1.079	0.863	1.349
LDLC-1	91.835	<0.001	0.891	0.870	0.912
LDLC-2	0.275	0.600	1.006	0.985	1.027
LDLC-3	107.117	<0.001	1.565	1.438	1.704
LDLC-4	51.558	<0.001	3.566	2.520	5.046
LDLC-5	14.450	<0.001	6.866	2.543	18.539

BMI, body weight index; TC, total cholesterol; TG, triglyceride, HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; OR, odds ratio; CI, confidence interval.

HDL-C, BMI, smoking history, LDLC-3, LDLC-1, hypertension history, age, TG, LDLC-2, SCr, LDL-C, TC, sdLDL-C, diabetes mellitus history, gender, and drinking history, which are shown in **Figure 5A**. Among all features, LDLC-4 played the most important role in model construction. **Table 3** shows the performance of six predictive models. To be specific, LR model is better in accuracy, precision, and F1_score, with the values of 0.880769, 0.920108, and 0.911995, respectively. The DT model gets the highest recall (0.929812). **Figure 5B** shows the ROC curves of six models, and XGboost has the highest AUC score (0.945121).

To further investigate whether CAD severity could be distinguished, we applied similar strategies to establish models between non-obstructive CAD and significant CAD patients. However, the AUC score of the most optimal model in the test set was lower than 0.75, which is not practicable for CAD severity predicted.

DISCUSSION

In this study, we recruited 356 consecutive CAD patients and a group of controls, which were diagnosed with coronary angiogram. First, we analyzed the correlation between blood lipid profile and CAD risk and found LDLC-3 to LDLC-6, as well as TG, were significantly higher in CAD patients. Total LDL-C showed no significant difference between controls and CAD patients. Several studies reported that LDL-C concentration is not always high in patients with acute coronary syndrome (16–18). LDL-C consists of heterogeneous mixture of particles with different density and size, and sdLDL-C is more atherogenic than lbLDL-C (19, 20). The mechanisms behind this phenomenon may include easier transfer from the vessel lumen into the subintimal space (21), higher affinity to proteoglycans (22), decreased binding ability to LDL-C receptors (23), and increased plasma residence time (24), compared with lbLDL-C.

Belonging to lbLDL-C, LDLC-1 level was relatively lower in CAD patients compared with that in controls, which strengthen the hypothesis that lbLDL-C is a protective factor against CAD development. Chaudhary et al. reported that lbLDL-C was negatively correlated with CAD severity (10). In addition, some other studies also focused on the effects of lbLDL-C on metabolic diseases. For example, Srisawasdi and colleagues' study proposed the ratio of sdLDL-C to lbLDL-C as an excellent biomarker for evaluating lipid metabolic status in patients with metabolic syndrome (25). Chen et al. reported that sdLDL-C/lbLDL-C ratio was associated with glucose metabolic status in pregnancy (26). Interestingly, we also found LDLC-1 was negatively correlated with LDLC-3 to LDLC-7. LDL-C is routinely considered as a major cause of ASCVD risk. However, in the present study, no significant difference was observed in LDL-C levels between controls and CAD groups, and LDL-C was not identified as a significant risk factor for CAD development by LR analysis. A possible explanation could be that the effects of total LDL-C are neutralized by sdLDL-C and lbLDL-C. The possible factors impacting on lipoprotein cholesterol size are genetic background (27, 28) and dietary habits (29–31), but the current evidences were weak. It has great clinical significance on lipid-lowering therapy to explore the mechanisms affecting the size of LDL-C particles.

Silent ischemia occurs in about 20–40% of patients with unstable and stable coronary syndromes (32), and the first clinical manifestation in some cases is sudden events. Thus, we investigated the feasibility of predicting CAD risk by machine learning method. Among six models, XGboost (AUC score = 0.945121) and LR (AUC score = 0.944622) models presented excellent performance for predicting CAD development.

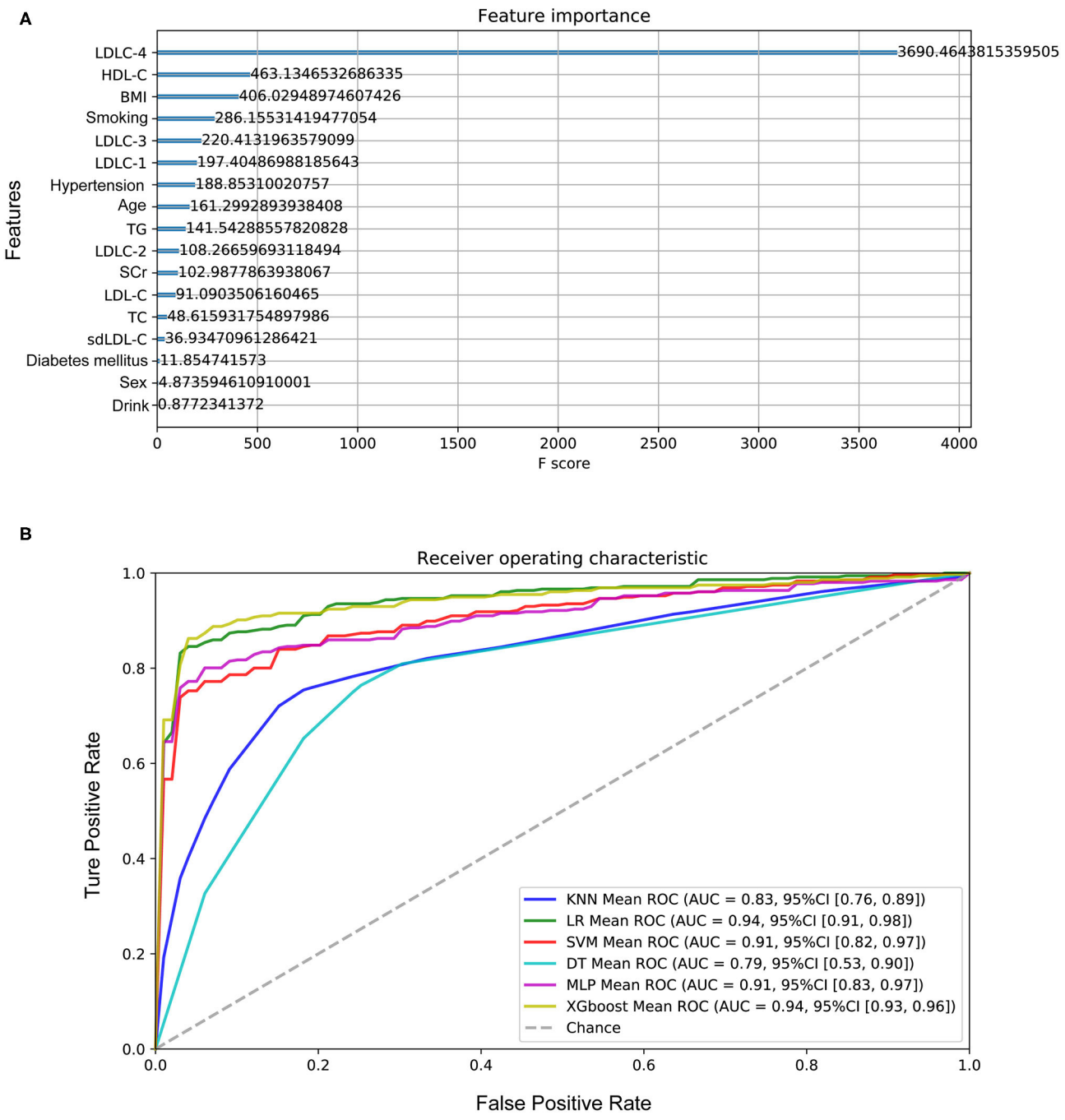

FIGURE 5 | Performance of the models to predict CAD development. **(A)** The importance of features in model construction, including clinical features and laboratory indexes. **(B)** The ROC curves of six models. CAD, coronary artery disease; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; BMI, body weight index; TG, triglyceride; SCr, serum creatinine; TC, total cholesterol; sdLDL-C, small and dense LDL-C.

Previous researchers (3, 33) had made a lot of efforts to assess cardiovascular risk based on known multiple risk factors. The dominant strategy was calculating the total risk score of a person by summing the risk imparted by each of the major risk factors, for example, Framingham risk score and its improved version (34, 35). By comparison, our study has some advantages. First, we employed a machine learning method, which has superiority on algorithms compared with accumulated risk scores. It could analyze diverse data types (e.g., demographic data, clinical data, laboratory data) and incorporate these features according to their weightiness of information gain into predicted models for disease risk, diagnosis, prognosis, and appropriate treatment

TABLE 3 | Performance summary of six machine learning models to predict CAD development.

Models	Accuracy	Precision	Recall	F1_score	AUC score
KNN	0.771154	0.833628	0.851056	0.835283	0.827936
LR	0.880769	0.920108	0.907473	0.911995	0.944622
SVM	0.805769	0.877361	0.848318	0.855256	0.907529
DT	0.846154	0.868333	0.929812	0.895352	0.805235
MLP	0.830769	0.889812	0.862285	0.874413	0.907694
XGboost	0.846154	0.878950	0.915806	0.891967	0.945121

KNN, k-nearest neighbors classifier; LR, logistic regression; SVM, a support vector machine; DT, the decision tree model; MLP, the multilayer perceptron network; AUC, area under curve.

(36). In addition, we not only included classical risk factors, but also explored and selected novel marker LDL-C subfractions. Despite that the recent study published by Sánchez-Cabo et al. (37) also used machine learning to cardiovascular risk definition, sdLDL-C or LDL-C subfractions were not considered. Our results showed that LDLC-4 played the most important role on models' establishment, which made a great contribution for better performance of our models than other studies.

Despite the novelties in the present study, there are some limitations worthy of statement. First, numerous patients were excluded, such as patients who received daily lipid-lowering drugs before recruitment, which might limit the generalizability of our finding to wider population. Clinically, many patients with dyslipidemia take cholesterol-reducing medications to prevent atherosclerosis, but some of them still develop CAD. Thus, the efficacy of various lipid-lowering drugs on LDL-C subfractions, especially LDLC-3 and LDLC-4, is worthy of study. Second, in this study, no significant difference was found in blood lipid profile between non-obstructive and significant CAD patients, which is not consistent with the previous study (10). As our study is a cross-sectional study, the blood lipid profile was tested only when participants were recruited. However, CAD development is a long-period progress; both lipid levels and the duration time of dyslipidemia are important factors for atherosclerosis. Thus, this inconsistency might be caused by the lack of continuous monitoring of blood lipid profile changes. Finally, it needs to be mentioned that not only our study but also previous studies came to the conclusion based on a small size of samples. It is necessary to recruit a large cohort of samples to validate these results and perform a longitudinal study with long-time follow-up to give more robust evidence.

CONCLUSIONS

In this study, we proved that LDL-C was insufficient to be a risk factor for CAD development. LDL-C subfractions have shown their importance to be included in the clinical test for screening population with dyslipidemia or high-risk CAD. The established models presented good performance for CAD risk prediction, and LDLC-4 played the most important role in these models, which can help screen high-risk CAD patients in asymptomatic population so that further examination and prevention treatment can be taken before sudden or serious events.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethical Committee (the General Hospital of Tisco Affilated to Shanxi Medical University, the First People's Hospital of Pingdingshan and Mianxian Hospital). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

DW funded this study. DW, QY, and BL designed project. BS, JH, HM, and HW collected samples and clinical data. WY and JZ performed experiments and analyzed data. DW and JZ wrote the manuscript. BL, HW, JG, FD, and YX revised the manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcvm.2021.619386/full#supplementary-material

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Conflict of Interest: WY, JG, FD, YX, and JZ were employed by the company Shanghai Biotecan Pharmaceuticals Co., Ltd.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Atherogenic Lipoprotein Phenotype

A Proposed Genetic Marker for Coronary Heart Disease Risk

Melissa A. Austin, PhD, Mary-Claire King, PhD,
Karen M. Vranizan, MA, and Ronald M. Krauss, MD

In a community-based study of 301 subjects from 61 nuclear families, two distinct phenotypes (denoted A and B) were identified by nondenaturing gradient gel electrophoretic analysis of low density lipoprotein (LDL) subclasses. Phenotype A was characterized by predominance of large, buoyant LDL particles, and phenotype B consisted of a major peak of small, dense LDL particles. Previous analysis of the family data by complex segregation analysis demonstrated that these phenotypes appear to be inherited as a single-gene trait. In the present study, the phenotypes were found to be closely associated with variations in plasma levels of other lipid, lipoprotein, and apolipoprotein measurements. Specifically, phenotype B was associated with increases in plasma levels of triglyceride and apolipoprotein B, with mass of very low and intermediate density lipoproteins, and with decreases in high density lipoprotein (HDL) cholesterol, HDL_2 mass, and plasma levels of apolipoprotein A-I. Thus, the proposed genetic locus responsible for LDL subclass phenotypes also results in an atherogenic lipoprotein phenotype. (*Circulation* 1990;82:495–506)

Epidemiological studies have established several lipoprotein-related risk factors for coronary heart disease (CHD). Elevated plasma levels of low density lipoprotein (LDL) cholesterol are believed to increase risk,[1–3] whereas high density lipoprotein (HDL) cholesterol levels are inversely related to risk.[4–6] Plasma concentrations of apolipoprotein (apo) B and apo A-I, the major protein components of LDL and HDL, respectively, have also been associated with atherosclerosis.[7,8] The relation of plasma triglyceride levels and triglyceride-rich lipoproteins such as very low density lipoproteins (VLDL) to heart disease risk is less well understood.[9,10] In addition, there is evidence that other classes of lipoproteins, such as intermediate density lipoproteins (IDL) and Lp(a), are involved in the development of atherosclerosis.[11,12]

Genetic influences on lipoproteins have also been demonstrated. Based on population studies, lipid levels have been shown to cluster in families[13–15]; studies of twins indicate significant genetic influences as well.[16,17] Familial forms of hypercholesterolemia may result from deficient or defective LDL receptors[18] or from mutations in apo B leading to defective receptor binding.[19] More common polymorphisms of apo A-I, apo B, and apo E have been associated with variations in lipid and lipoprotein levels.[20–22] Recently, Lp(a) levels and isoforms have also been shown to be under genetic control.[23]

In our laboratory, we have identified distinct lipoprotein phenotypes based on analysis of LDL subclasses.[24] Specifically, two phenotypes, A and B, are characterized by a predominance of large, buoyant LDL particles and small, dense LDL particles, respectively. We have recently demonstrated that phenotype B is associated with increased risk of myocardial infarction,[24] consistent with two previous studies.[25,26] Based on complex segregation analysis of 61 nuclear families, we have also shown that phenotype B appears to be inherited as a single-gene trait with a dominant mode of inheritance.[27] In the present report, we show that in these families, LDL subclass phenotypes are closely associated with other lipoprotein and apolipoprotein profiles that are known to influence risk of atherosclerosis. Thus, we have designated the proposed genetic locus responsible for LDL subclass phenotypes as an atherogenic lipoprotein (ALP) phenotype locus.

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Methods

Subjects

The recruitment of families took place primarily among the Mormon community in the San Francisco

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Bay area between 1984 and 1987, although nonlocal relatives were also screened.[27] Families were not selected for lipid disorders or family history of cardiovascular disease, but sequential sampling of informative kindreds was used.[28] With the exception of one kindred of Portuguese descent ($n=28$) and one small Asian nuclear family ($n=4$), all families were non-Hispanic Caucasian. Three hundred one family members of 29 kindreds participated in the study; included were 61 nuclear families. Of the 301 participants, 100 had married into the kindreds and were not related to each other. Subjects ranged in age from 6 to 95 years, with approximately equal proportions of men and women (49% men and 51% women). Only 22 eligible subjects declined participation, a response rate of 93%. Each subject gave written informed consent.

All participants provided blood samples after an overnight fast and completed a medical history questionnaire. For local family members, heights and weights were measured in the clinic, whereas among nonlocal subjects, reported heights and weights were used. Mormon families were selected for this study because they usually do not smoke tobacco and do not drink beverages containing alcohol or caffeine. Abstinence from these factors among the majority of family members was confirmed by questionnaire and reduced possible confounding in the genetic analysis because these factors have been associated with variations in lipid and lipoprotein levels.[29-33] In addition, because Mormon families are generally large and genealogical records are carefully maintained, they are especially informative for genetic analysis. The segregation results in the non-Caucasian kindreds were not different from the remaining kindreds; therefore, all families were analyzed as a single group.

Lipid, Apolipoprotein, and LDL Subclass Analyses

Plasma lipid and apolipoprotein determinations and LDL subclass analyses were performed on fresh plasma samples after immediate centrifugation of whole blood. Total cholesterol and triglyceride levels were measured by enzymatic techniques with the Gilford 3500 autoanalyzer. HDL cholesterol was measured after precipitation with heparin-$MnCl_2$,[34,35] and LDL cholesterol was calculated from the formula of Friedewald et al.[36] Plasma apo A-I levels and apo B levels were measured by maximal radial immunodiffusion using reagents from Tago, Inc.[37,38] Lipoprotein mass measurements were determined as a function of Svedberg flotation rate using analytic ultracentrifugation in an unselected subset of 211 subjects.[39] The remaining 90 subjects did not have these measurements made due to funding and staff limitations. Measurements of VLDL mass of flotation rate (S_f^o) 20–400, IDL of S_f^o 12–20, large LDL of S_f^o 7–12, small LDL of S_f^o 0–7, HDL_2 of flotation rate ($F^o_{1.20}$) 3.5–9, and HDL_3 of $F^o_{1.20}$ 0–3.5 are reported here.

LDL subclass patterns were determined based on nondenaturing polyacrylamide gradient gel electrophoresis of whole plasma and of the density<1.063 g/ml plasma fraction, using Pharmacia PPA 2–16% gradient gels as described previously.[40,41] Stained gels were scanned with a Transidyne RFT Scanning Densitometer, and particle diameters were calculated from calibration curves using standards of known size.[40] The coefficient of variation of the calculated particle diameters has been estimated to be 3% by this procedure.[40] Based on the resulting scans, two distinct LDL subclass patterns were identified and are denoted here as ALP phenotype A and phenotype B.[24] Examples of these phenotypes among four sibs from a large kindred are shown in Figure 1. Phenotype A is characterized by a major peak of large, buoyant LDL particles and a minor peak of smaller, denser LDL subspecies, as shown in Figures 1a and 1b (peak particle diameters of 268 and 264 Å, respectively). The peak particle diameter for ALP phenotype A scans is generally more than 255 Å. In contrast, the major peak for ALP phenotype B is usually 255 Å or less, as shown in Figures 1c and 1d (peak particle diameters of 252 and 247 Å, respectively). The major peak in this phenotype consists of small, dense LDL particles, with a skewing of the curve toward the larger particle diameters.

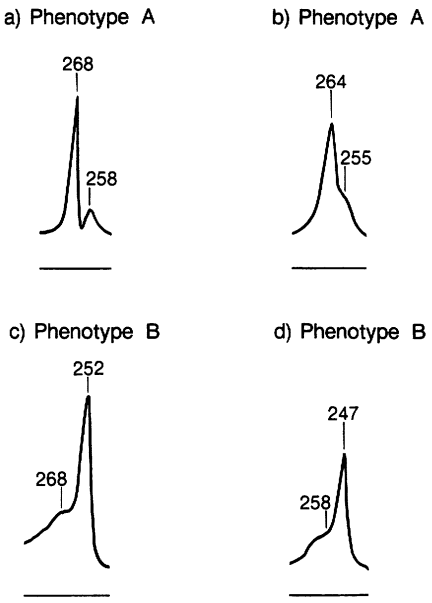


FIGURE 1. *Atherogenic lipoprotein phenotypes for four sibs: 35-year-old man* (a), *44-year-old woman* (b), *38-year-old man* (c), *and 32-year-old man* (d). *Examples of atherogenic lipoprotein phenotype A are seen in panels a and b with peak particle diameters of 268 and 264 Å, respectively. Scans in panels c and d represent atherogenic lipoprotein phenotype B with peak particle diameters of 252 and 247 Å, respectively.*

Among the 301 family members, 87% could be classified into one of these two phenotypes. The remaining 13% of subjects had patterns of an intermediate phenotype. That is, either the peak particle diameter value was close to the 255 Å cutoff point and no skewing of curve was seen, or two distinct major peaks were seen. For the present analysis,

TABLE 1. Observed and Expected Segregation Ratios of Atherogenic Lipoprotein Phenotypes in 49 Nuclear Families

Parental mating type	Matings (*n*)		ALP phenotype A (*n*) (%)	ALP phenotype B (*n*) (%)	Total (*n*)
A×A	14	Observed	41 (100)	0 (0)	41
		Expected	41.0	0.0	
A×B	27	Observed	66 (70)	28 (30)	94
		Expected	64.3	29.7	
B×B	8	Observed	13 (65)	7 (35)	20
		Expected	9.7	10.3	

ALP, atherogenic lipoprotein phenotype.
Based on single-locus dominant model, with allele frequency of 0.25 for phenotype B and reduced penetrance among males less than 20 years old and premenopausal females, as determined by complex segregation analysis.[27] Only families with both parents sampled are included in table.

these subjects have been classified as ALP phenotype B.[27] By this definition, 31% of the study subjects had ALP phenotype B, although the prevalence varied by age, gender, and menopausal status in women. Specifically, among the males, phenotype B had a frequency of 17% in those less than 20 years old and 44% in those 20 years old or older; among females, phenotype B had a frequency of 13% before menopause and 49% after menopause.[27] Mean peak particle diameters were 266.2±5.8 (±SD) and 252.7±7.1 Å for subjects with phenotypes A and B, respectively.

Segregation Analysis

As we have recently reported, the inheritance of ALP phenotypes was investigated using complex segregation analysis based on the mixed model with pointers.[27,42–45] The model that best explained the data was a single-locus model with a dominant mode of inheritance. Based on this model, the frequency of the allele leading to phenotype B was 0.25.[27] Full penetrance (the probability of expressing phenotype B given genotype AB or BB) was observed among men 20 years old or older and among postmenopausal women.[27] Penetrance was 0.4 for younger males and 0.3 for premenopausal females. Thus, complex segregation analysis suggested that ALP phenotype B is a common genetic trait with a dominant mode of inheritance and is fully expressed in adult men and postmenopausal women.

Statistical Procedures

Individual lipid, apolipoprotein levels, and lipoprotein mass measurements were adjusted by analysis of covariance[46,47] for age, gender, and Quetelet index [measured as (wt [kg])/(ht [m])^2]. Main effects for these covariates were included in each model. For HDL cholesterol, apo A-I, HDL_2 mass, and HDL_3 mass, a significant gender by age interaction term was included in the model. For apo B, a significant Quetelet index by gender interaction term was included. Triglyceride, VLDL cholesterol, and VLDL mass were transformed logarithmically due to skewing of the distributions. Means and SDs of these variables are reported in antilog units, however, for ease of interpretation. All mean values were adjusted to expected values for 50-year-old men. Significance levels for comparison of subjects with ALP phenotypes A and B were also based on analysis of covariance models. Skewness of frequency histogram distributions was calculated as the third central moment.[48] Thus, skewness values of more than 0 indicate a long tail to the right, and values less than 0 indicate a long tail to the left.

Interrelations among lipid, lipoprotein, and apolipoprotein variables were assessed by Pearson's product moment correlations.[47] To evaluate the simultaneous associations of lipid and apolipoprotein levels with the ALP phenotypes, multivariate analyses were performed with unconditional logistic regression.[49,50] That is, ALP phenotype was the dependent variable and a series of lipid and apolipoprotein variables were the independent variables in the models. A prior probability of 0.30, based on the overall prevalence in the study sample, was used for phenotype B, and χ^2 goodness-of-fit statistics were determined for each model. All computations were performed with the Statistical Analysis System.[51,52]

Results

Genetic Analysis

Based on the single-locus dominant model and incorporating both the estimated allele frequency and penetrance values,[27] expected segregation ratios were calculated. Table 1 compares these expected frequencies with the observed values based on 49 nuclear families in which both parents were sampled. Among the 14 families in which both parents had phenotype A, all of the 41 offspring had phenotype A, identical to the expected frequency. Among the 27 A×B families, 28 of the 94 total offspring (30%) had phenotype B, corresponding very closely to the expected frequency of 29.7 based on the model. Of the 20 offspring from B×B matings, seven had phenotype B, compared an expected 10.3. Although this comparison is not as close as the other mating types, the sample size in this category of families was small, and sampling variation was likely to be higher. A statistical comparison of the observed and expected was not significant (χ^2=1.021, df=5, p=0.961). Thus, the results of the segregation

TABLE 2. Mean Values of Lipids for Study Subjects Compared With Lipid Research Clinics Program Prevalence Study Results

	Present study subjects (n=301)*	Lipid Research Clinics subjects (n=340)†
Total cholesterol	183±39	213±35
Triglyceride	100±63	153±101
VLDL cholesterol	20±13	27±20
LDL cholesterol	119±35	142±31
HDL cholesterol	43±10	44±11

VLDL, very low density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein.

Values are given as mean±SD mg/dl.

*Mean values adjusted to 50-year-old men with analysis of covariance and adjusting for age, gender, and Quetelet index.

†Based on Lipid Research Clinics Program Prevalence Study data, visit 2 random sample, men 50–54 years old.[53]

analysis appear to explain the observed familial clustering extremely well.

Lipid Levels

Mean lipid values of the 301 study subjects adjusted to values for 50-year-old men are given in Table 2 and are compared with results for men of a similar age from the Lipid Research Clinics Program Prevalence Study random sample.[53] Total cholesterol and triglyceride mean values were 30 and 50 mg/dl less, respectively, in the study subjects than in the Lipid Research Clinics populations. Differences were also seen for VLDL cholesterol and LDL cholesterol. However, the mean values for HDL cholesterol are similar in the two samples.

Lipid, Apolipoprotein, and Lipoprotein Associations

In the present study, ALP phenotypes were found to be closely associated with variation in other lipid, apolipoprotein, and lipoprotein mass measurements. As shown in Table 3, mean values of both total cholesterol and triglyceride were significantly higher among subjects with phenotype B ($p<0.001$). These mean values are within normal ranges, however, because these data are based on a sample of primarily healthy families. The difference in total cholesterol is due to relative increases of LDL cholesterol and VLDL cholesterol among phenotype B subjects, although the difference in mean values for LDL cholesterol was only 10 mg/dl. HDL cholesterol was significantly lower among subjects with phenotype B ($p<0.001$). Differences were also seen for plasma apolipoprotein levels; apo B levels were significantly higher among subjects with phenotype B ($p<0.001$), and apo A-I levels were lower ($p<0.05$).

Based on analytic ultracentrifugal analyses in a subsample of all subjects, VLDL mass was significantly higher among subjects with phenotype B ($p<0.001$), consistent with results for triglyceride and estimated VLDL cholesterol. The differences in large and small LDL reflect primarily the definitions of ALP phenotypes based on LDL subspecies. Mean IDL mass was also higher among phenotype B subjects. HDL_2 mass was significantly lower among subjects with phenotype B, but no difference was seen in HDL_3 mass.

Triglyceride and HDL Cholesterol Distributions

Both triglyceride and HDL cholesterol have been related to risk of CHD in numerous studies.[4–6,9,10,54]

TABLE 3. Adjusted Plasma Lipid, Apolipoprotein, and Lipoprotein Mass Levels by Atherogenic Lipoprotein Phenotype

	ALP phenotype A		ALP phenotype B	
	n	Mean±SD	n	Mean±SD
Total cholesterol*	208	177±37	93	197±40
Triglyceride†*	208	69±26	93	141±79
VLDL cholesterol†*	208	14±5	93	28±16
LDL cholesterol‡	208	116±35	92	126±36
HDL cholesterol*	208	46±15	92	37±14
Apo A-I‡	206	131±29	92	122±31
Apo B*	206	76±34	93	98±36
VLDL mass†*	151	18±31	60	111±68
LDL mass				
Large*	151	119±38	60	87±34
Small*	151	164±55	60	221±64
IDL mass*	151	20±14	60	38±17
HDL_2 mass*	151	55±44	60	13±26
HDL_3 mass	151	189±47	60	180±59

ALP, atherogenic lipoprotein phenotype; VLDL, very low density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein; IDL, intermediate density lipoprotein.

Values are given as mean±SD mg/dl.

Mean values are adjusted to 50-year-old men with analysis of covariance and adjusting for age, gender, and relative weight.

*$p<0.001$, ‡$p<0.05$, for difference in means between phenotype A and phenotype B subjects based on analysis of covariance.

†Log_{10} transformation used in calculations; reported values based on antilogs.

Because of their close association with ALP phenotypes as well, the distributions of these variables, adjusted for age, gender, and relative weight, were examined further.

The percent frequency distribution of triglyceride levels is shown in Figure 2A. The overall distribution has a long tail to the right, with skewness estimated to be 2.35. This has been observed in many other investigations, and log transformations are often used in statistical analyses.[9,54,55] The distribution of triglyceride by ALP phenotype is also shown. As expected from the mean values given in Table 3, the triglyceride distribution for phenotype B subjects is shifted upward in comparison to that for phenotype A subjects. Of particular interest is that the phenotype B subjects appear to be responsible for the skewing of the overall distribution; that is, skewing of the triglyceride distribution is markedly reduced when considered by phenotype; the skewness value was 0.65 for phenotype A subjects, and 1.49 for phenotype B subjects.

In Figure 2B, the cumulative triglyceride distributions for all subjects and for phenotype A and B subjects are shown. The differences in the distributions for phenotypes A and B are clearly demonstrated. The 50th percentile values differ by more than 60 mg/dl (71 versus 133 mg/dl, respectively), and the 90th percentile values are even more divergent (104 versus 278 mg/dl, respectively).

In Figure 2C, the cumulative distribution for phenotype A subjects is reversed to compare the degree of overlap of the distributions for the two phenotypes. The distributions for subjects with phenotypes A and B cross at a triglyceride level of approximately 95 mg/dl. Of subjects with phenotype A, only 17% have triglyceride values of more than 95 mg/dl, whereas 17% of subjects with phenotype B have triglyceride values of less than 95 mg/dl. Because there is very little overlap in these distributions, triglyceride levels of more than and less than 95 mg/dl discriminate ALP phenotype in approximately 83% of the study subjects.

A similar analysis for HDL cholesterol is shown in Figure 3. In Figure 3A, the percent frequency distribution of HDL cholesterol values is shown. In contrast to the triglyceride distribution, little skewness is seen in the overall distribution. As was seen in Table 2, the mean value of this overall distribution is very similar to the Lipid Research Clinics results (43 versus 44 mg/dl, respectively). However, the distribution for phenotype B subjects is shifted downward in comparison to that for phenotype A subjects. This difference is seen even more clearly in the cumulative distributions shown in Figure 3B. For pattern A subjects, the 50th and 10th percentiles were approximately 44 and 34 mg/dl, respectively. The comparable values for phenotype B subjects were considerably lower at 36 and 27 mg/dl, respectively. In Figure 3C, the cumulative distribution for phenotype B subjects is reversed. The distribution curves cross at approximately 39 mg/dl, with 28% of phenotype A subjects having HDL cholesterol values of less than 39 mg/dl and an equal percent of phenotype B subjects having values of more than 39 mg/dl. The HDL cholesterol distributions overlap more than the triglyceride distributions, but the 39 mg/dl threshold discriminates phenotype A and phenotype B subjects relatively well.

A similar cumulative distribution analysis for apo A-I and apo B is shown in Figures 4A and 4B. The apo B distributions cross at 81 mg/dl and 37% of phenotype B subjects have values below this level, whereas an equal percent of phenotype A subjects have values above this level. The apo A-I distribution curves cross at 124 mg/dl, with a similar percent overlap. Thus, apo A-I and apo B levels also discriminate subjects with the two phenotypes but not as well as triglyceride and HDL cholesterol.

Correlations

The interrelations of selected lipid, lipoprotein, and apolipoprotein variables associated with ALP phenotypes, based on correlation coefficients, are shown in Table 4. As expected from the structure of lipoprotein particles, triglyceride, LDL cholesterol, apo B, VLDL mass, and IDL were highly intercorrelated, as were HDL cholesterol, HDL_2 mass, and apo A-I. In addition, triglyceride-related variables were generally inversely correlated with HDL-related variables. For example, the correlation for plasma triglyceride and HDL cholesterol and for VLDL mass and HDL_2 mass was -0.24 ($p<0.001$) and -0.47 ($p<0.001$), respectively.

Multivariate Analysis

Because of these interrelations, the simultaneous associations of lipids and apolipoproteins with ALP phenotypes were investigated by performing logistic regression analysis. That is, the associations of lipid and apolipoprotein variables with ALP phenotypes were investigated by using phenotype as the dependent variable and including various combinations of lipid and apolipoprotein measures as independent variables. The results in Table 5 summarize three models that include age, gender, and Quetelet index as covariates and show χ^2 goodness-of-fit statistics for each model.

In model 1, both HDL cholesterol and plasma triglyceride were strongly and independently associated with ALP phenotypes. In addition to these variables, LDL cholesterol, apo A-I, and apo B were also considered independent variables; however, they did not make a significant contribution to the fit of the model and are not reported in the table. Model 2 shows that apo A-I and apo B, without other lipid variables, were both significantly associated with ALP phenotypes. The model χ^2 values show that model 1 provides a better fit to the data than model 2, however. In model 3, VLDL mass and HDL_2 mass were considered and found to be independently associated with ALP phenotypes in the subset of 211 study subjects with analytic ultracentrifuge data.

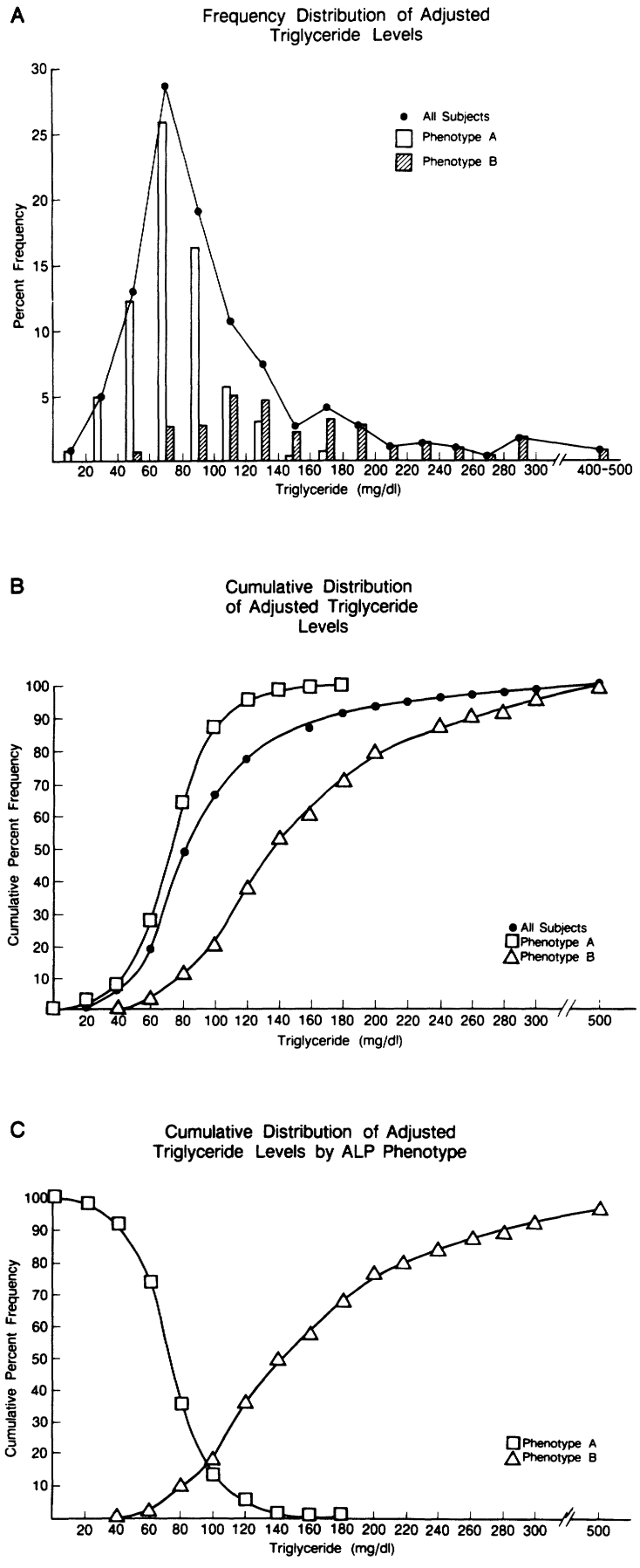
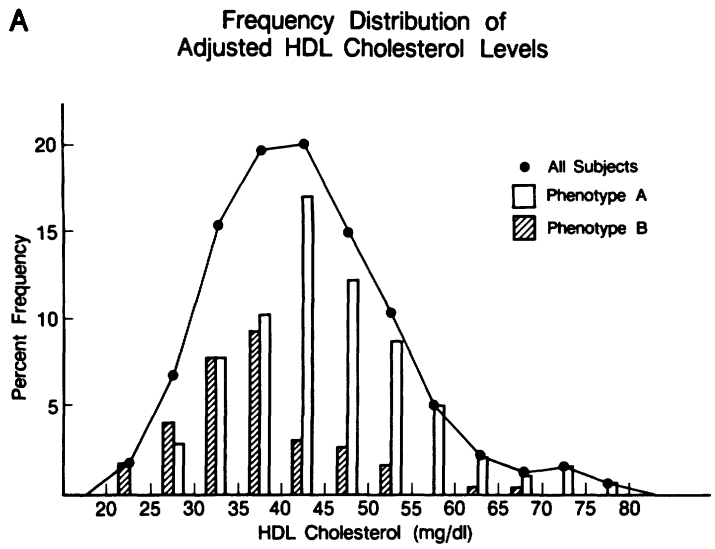
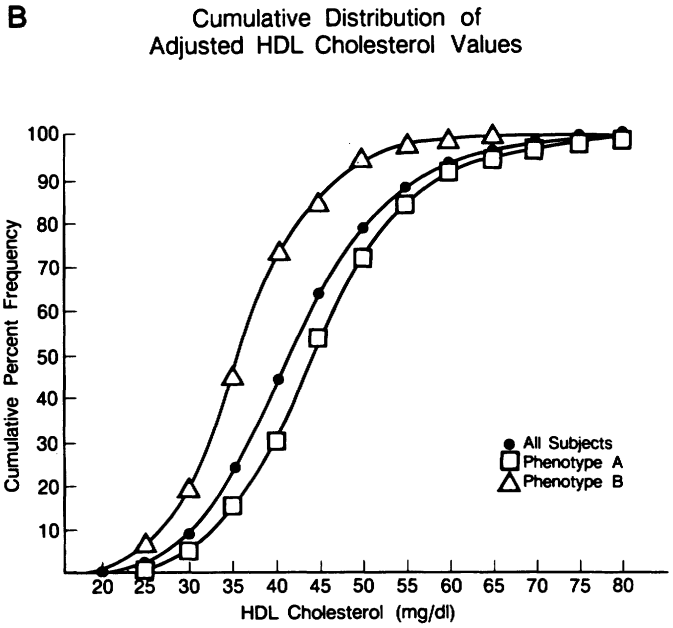


FIGURE 2. *Plots of percent frequency distribution of adjusted triglyceride values for all study subjects and for atherogenic lipoprotein (ALP) phenotype A and phenotype B subjects* (A), *cumulative distributions of adjusted triglyceride values for all study subjects and for phenotype A and phenotype B subjects* (B), *and cumulative triglyceride distributions by ALP phenotype* (C). *Distribution for phenotype A subjects is reversed to compare overlap of two distributions. Triglyceride values are adjusted to mean level for 50-year-old men based on analysis of covariance.*





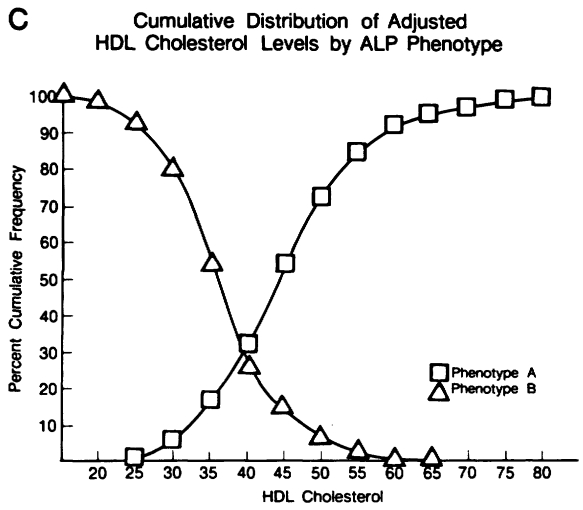


FIGURE 3. *Plots of percent frequency of adjusted high density lipoprotein (HDL) cholesterol values for all subjects and for subjects with atherogenic lipoprotein (ALP) phenotype A and phenotype B* (A), *cumulative distributions of adjusted HDL cholesterol values for all study subjects and for phenotype A and phenotype B subjects* (B), *and cumulative distributions of HDL cholesterol by ALP phenotype* (C). *Distribution for phenotype B is reversed to compare overlap of distributions. HDL cholesterol values are adjusted to mean level for 50-year-old men by analysis of covariance.*

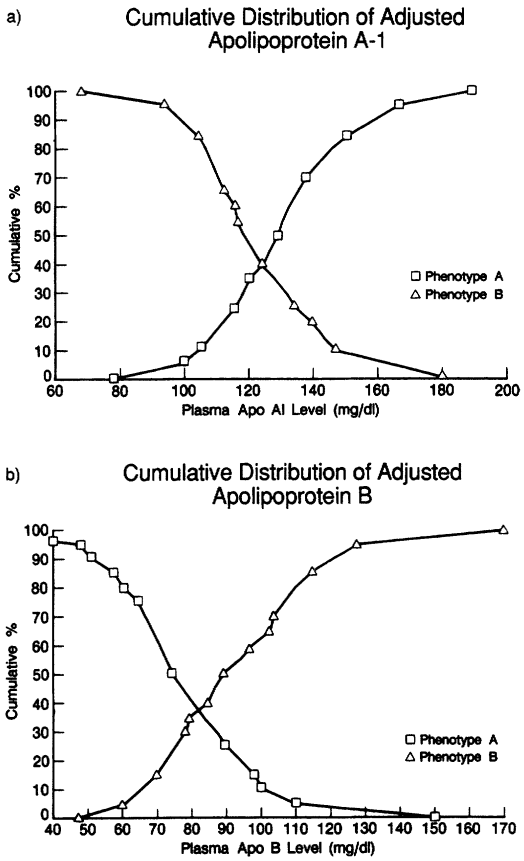


FIGURE 4. *Cumulative distributions of apo A-I levels by ALP phenotype* (a). *Distribution for phenotype B subjects is reversed. Plots of cumulative distributions of adjusted apo B values by atherogenic lipoprotein (ALP) phenotype* (b). *Distribution for phenotype A subjects is reversed. All apolipoprotein values are adjusted for 50-year-old men by analysis of covariance.*

When IDL mass was added to this model, it did not significantly increase the fit of the model to the data.

Taken together, these results demonstrate that a predominance of small, dense LDL particles is strongly associated with an apparently high-risk lipoprotein profile characterized by relative increases in plasma triglyceride, VLDL, and apo B levels and by decreases in HDL cholesterol, HDL_2 mass, and apo A-I levels.

Discussion

We have previously demonstrated that the phenotype characterized by a predominance of small, dense LDL particles (ALP phenotype B) segregates in families consistent with the presence of a single major genetic locus.[27] Based on estimates from the complex segregation analysis, approximately 44% of study subjects would be expected to carry at least one copy of the proposed phenotype B allele. In the same sample of primarily healthy families, we also demonstrate that phenotype B is associated with increased levels of plasma triglyceride, VLDL, IDL, and apo B and with decreased levels of HDL cholesterol, HDL_2 mass, and apo A-I. These results are consistent based on comparisons of mean values, cumulative distributions, and multivariate analysis. Thus, ALP phenotype B may be a common genetic marker for increased susceptibility to CHD.

Although the genetic results reported above are based on a community-based sample of families, we have recently identified a similar mode of inheritance for phenotype B in members of families with familial combined hyperlipidemia.[56] A similar allele frequency was found, and penetrance estimates by age and gender showed comparable trends. However, both studies used complex segregation analysis, a technique that has many advantages as well as limitations. It allows comparisons of a variety of genetic and environmental models, including multifactorial inheritance (polygenic or cultural), single major gene models, and horizontal (environmental) transmission only, using likelihood statistics.[45] However, this is a statistical modeling technique, and the use of this "mixed model" can give spurious results if ascertainment bias is present. Thus, although the two family studies conducted so far provide strong evidence for the presence of a major gene, this can be proven only by using linkage studies to identify the chromosomal location.

In a recent case-control study, we demonstrated that phenotype B was associated with both increased risk of myocardial infarction (odds ratio, 3.0) and a high-risk lipoprotein profile.[24] Specifically, subjects in that study with phenotype B had significantly increased levels of triglyceride, VLDL mass, IDL

TABLE 4. Correlations of Lipid, Apolipoprotein, and Lipoprotein Mass Levels

	LDL cholesterol	HDL cholesterol	Apo B	Apo A-I	VLDL mass*	IDL mass*	HDL_2 mass*
Triglyceride	0.31†	−0.24†	0.54†	−0.004	0.93†	0.61†	−0.43†
LDL cholesterol		−0.06	0.87†	0.10	0.29†	0.67†	−0.24†
HDL cholesterol			−0.09	0.73†	−0.38†	−0.25†	0.81†
Apo B				0.11	0.52†	0.74†	−0.34†
Apo A-I					0.09	0.07	0.60†
VLDL mass						0.63†	−0.47†
IDL mass							−0.35†

LDL, low density lipoprotein; HDL, high density lipoprotein; Apo, apolipoprotein; VLDL, very low density lipoprotein; IDL, intermediate density lipoprotein.

*Sample size was 211 study subjects for correlations including these variables.

†$p<0.001$.

TABLE 5. Logistic Regression Equations Associating Atherogenic Lipoprotein Phenotypes With Lipid, Lipoprotein, and Apolipoproteins

	Regression coefficients		
	Model 1 (n=300)	Model 2 (n=298)	Model 3 (n=211)
Age (yr)	0.018	0.016	0.028*
Gender (0, female; 1, male)	0.133	0.459	0.212
Quetelet index (wt [kg])/(ht [m])^2	−0.118*	0.030	−0.127*
Triglyceride (mg/dl)	0.049†	...‡	...
HDL cholesterol (mg/dl)	−0.070§	...	...
VLDL mass (mg/dl)	...	...	0.041†
HDL_2 mass (mg/dl)	...	...	−0.036§
Apo B (mg/dl)	...	0.032†	...
Apo A-I (mg/dl)	...	−0.014*	...
Model χ^2	141.54	60.06	112.28
df	5	5	5
p	<0.001	<0.001	<0.001

*p<0.05, †p<0.001, §p<0.01.
‡This variable was not included in the model.

mass, and apo B and decreased levels of HDL cholesterol, HDL_2 mass, and apo A-I (Reference 24 and personal observations), each of which has been associated with increased risk of CHD.[4-11,57-59] The variations in lipid and apolipoprotein levels seen in the present study of primarily healthy relatives confirm these associations, although mean levels are within normal ranges. In addition, mean total cholesterol and LDL cholesterol levels are slightly, but significantly, higher among phenotype B subjects in this study.

Another recent study has shown similar associations among healthy blood donors.[60] Although the underlying mechanism for phenotype B has not been identified, it is tempting to speculate that this constellation of lipid and apolipoprotein variations is the result of pleiotropic effects of a single gene. That is, the proposed ALP gene may simultaneously influence both LDL particle size and these other lipoprotein-related variables through a common metabolic mechanism. In addition, a borderline association between increased Quetelet index and phenotype B was observed (p=0.054) after adjustment for age and gender.

The particularly strong association between ALP phenotype B and plasma triglyceride level reported in the present study could indicate that phenotype B is a marker for a defect having a primary action on triglyceride metabolism. As shown in Figure 2C, there is very little overlap in the cumulative triglyceride distributions of phenotype A and phenotype B subjects. In addition, the skewing of the triglyceride distribution appears to be largely explained by ALP phenotype B (Figure 2A). However, the triglyceride cutoff point that best distinguishes the two phenotypes (95 mg/dl) may be low because of the healthy study sample (Table 2). Many studies have demonstrated relations between LDL particle size and triglyceride metabolism.[61-64] In particular, a recent kinetic study of relatives of probands with primary hypertriglyceridemia provided evidence for genetic control of triglyceride removal.[62] Changes in both the core and surface of LDL particles have been shown to occur when plasma triglyceride levels are increased, possibly due to exchange of core lipids between lipoproteins.[64,65] Studies of postprandrial lipemia in normal individuals have also suggested that HDL cholesterol is influenced by triglyceride metabolism through the action of lipolytic enzymes.[66]

The association of phenotype B with variations in lipid and apolipoprotein levels among these family members also suggests that phenotype B may be involved in other reported familial lipid disorders. A predominance of small, dense LDL has been shown to be common in families with familial combined hyperlipidemia.[67] This disorder is characterized by elevations of plasma total cholesterol and/or triglyceride levels in family members, and affected relatives have variable lipid phenotypes and increased risk of myocardial infarction.[68,69] As mentioned above, we have recently demonstrated that ALP phenotype B appears to be inherited as a single-gene trait in a sample of families with this disorder, and phenotype B was closely associated with the hypertriglyceridemia found in family members.[56] In addition, both ALP phenotype B and familial combined hyperlipidemia are characterized by relative increases in plasma apo B levels. Two recent studies, also using complex segregation analysis, have provided data to indicate that apo B levels are controlled by a single, major locus.[70,71] Finally, a condition termed hyperapobetalipoproteinemia, in which a subset of coronary artery disease patients were found to have elevated apo B levels but normal LDL cholesterol levels,[72] could also involve or interact with ALP phenotype B. To date, the interrelations of these proposed genes and lipid disorders have not been investigated.

It should be noted, however, that the study of genetic control of ALP phenotypes is complicated by many factors. Phenotype B, as determined by gradi-

ent gel electrophoresis analysis, is often not expressed in young males and premenopausal women. This finding suggests that hormonal factors might be involved in the apparent full penetrance of phenotype B in adult men and postmenopausal women. Severe elevations of triglyceride due to mechanisms other than the genetic model proposed here may give rise to phenocopies. Behavioral and environmental influences such as diet, exercise, and use of lipid-altering medications may also affect the expression of the trait.[73] Other genes could potentially influence the expression of ALP phenotypes through epistatic effects, and segregation analysis cannot rule out the possibility of genetic heterogeneity of the ALP phenotypes. For example, a syndrome named "familial dyslipidemic hypertension" has recently been described and could involve the proposed ALP locus.[74]

If genetic control of lipoprotein and apolipoprotein levels by the proposed ALP locus is confirmed, there are important clinical implications for reduction of risk of CHD. For example, intervention strategies might be designed specifically for individuals who carry an ALP B allele. These strategies may need to differ from general recommendations to be effective in reducing risk in these individuals.

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KEY WORDS • low density lipoproteins • coronary heart disease • triglycerides • genetics

Association between low density lipoprotein and all cause and cause specific mortality in Denmark: prospective cohort study

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ABSTRACT

OBJECTIVE

To determine the association between levels of low density lipoprotein cholesterol (LDL-C) and all cause mortality, and the concentration of LDL-C associated with the lowest risk of all cause mortality in the general population.

DESIGN

Prospective cohort study.

SETTING

Denmark; the Copenhagen General Population Study recruited in 2003-15 with a median follow-up of 9.4 years.

PARTICIPANTS

Individuals randomly selected from the national Danish Civil Registration System.

MAIN OUTCOME MEASURES

Baseline levels of LDL-C associated with risk of mortality were evaluated on a continuous scale (restricted cubic splines) and by a priori defined centile categories with Cox proportional hazards regression models. Main outcome was all cause mortality. Secondary outcomes were cause specific mortality (cardiovascular, cancer, and other mortality).

RESULTS

Among 108 243 individuals aged 20-100, 11 376 (10.5%) died during the study, at a median age of 81. The association between levels of LDL-C and the risk of all cause mortality was U shaped, with low and high levels associated with an increased risk of all cause mortality. Compared with individuals with concentrations of LDL-C of 3.4-3.9 mmol/L (132-154 mg/dL; 61st-80th centiles), the multivariable adjusted hazard ratio for all cause mortality was 1.25 (95% confidence interval 1.15 to 1.36) for individuals with LDL-C concentrations of less than 1.8 mmol/L (‹70 mg/dL; 1st-5th centiles) and 1.15 (1.05 to 1.27) for LDL-C concentrations of more than 4.8 mmol/L (›189 mg/dL; 96th-100th centiles). The concentration of LDL-C associated with the lowest risk of all cause mortality was 3.6 mmol/L (140 mg/dL) in the overall population and in individuals not receiving lipid lowering treatment, compared with 2.3 mmol/L (89 mg/dL) in individuals receiving lipid lowering treatment. Similar results were seen in men and women, across age groups, and for cancer and other mortality, but not for cardiovascular mortality. Any increase in LDL-C levels was associated with an increased risk of myocardial infarction.

CONCLUSIONS

In the general population, low and high levels of LDL-C were associated with an increased risk of all cause mortality, and the lowest risk of all cause mortality was found at an LDL-C concentration of 3.6 mmol/L (140 mg/dL).

WHAT IS ALREADY KNOWN ON THIS TOPIC

Conflicting results have been reported on the association between levels of LDL-C and all cause mortality

Most previous studies were conducted in individuals aged over 65 in historical populations

WHAT THIS STUDY ADDS

Low and high levels of LDL-C were associated with an increased risk of all cause mortality in the general population

The lowest risk of all cause mortality was found at a concentration of LDL-C of 3.6 mmol/L (140 mg/dL)

Introduction

Low density lipoprotein cholesterol (LDL-C) is a well established causal risk factor for the development of atherosclerosis and cardiovascular disease.[1] High levels of LDL-C consistently predict a risk of future atherosclerotic cardiovascular events in a variety of populations throughout the world. Also, many randomised controlled trials of treatment with lipid lowering agents have clearly shown that lowering LDL-C levels reduces the risk of atherosclerotic cardiovascular events in the future.[1-4]

Because lowering levels of LDL-C reduces cardiovascular disease outcomes, the general perception is that high levels of LDL-C are associated with an increased risk of mortality but low levels are not. Studies on the association between LDL-C levels and the risk of all cause mortality, however, have provided conflicting results, with some studies showing a counterintuitive inverse association (lower mortality with increasing levels of LDL-C) [5-7] and some showing no association.[8-10] Most of these studies were conducted in individuals aged 65 and older, and in historical population based cohorts. Also, a recent study in young Koreans not taking lipid lowering drugs showed a U shaped relation between levels of LDL-C and mortality.[11] Studies on the association between levels of LDL-C and cardiovascular mortality found different results, with some studies showing a positive association only[8 12] and some showing a U shaped association.[11] Thus the association between LDL-C levels and the risk of all cause and cause specific mortality in the general population is unclear. Also, the concentration of LDL-C where the risk of mortality is lowest is not defined.

In this study, we determined the association between levels of LDL-C and the risk of all cause and cause

specific mortality. Also, we identified the LDL-C level associated with the lowest mortality in individuals in the contemporary ongoing Copenhagen General Population Study.

Methods

Study population

The study included individuals of Danish descent from the Copenhagen General Population Study, an ongoing cohort study with the first round of examinations in participants recruited in 2003-15. Invited individuals were aged 20-100 and randomly selected from the national Danish Civil Registration System, reflecting the Danish general population (43% participation rate). All participants completed a self-administered questionnaire, including questions on lifestyle factors and medical treatment, underwent a physical examination, and gave blood samples for biochemical measurements.

Endpoints

The number of deaths from any cause was obtained from the Danish Civil Registration System, a complete register of all residents in Denmark since 1968 without losses to follow-up. The cause of death from January 1977 onwards was retrieved from the national Danish Causes of Death Registry, based on the codes of the International Classification of Diseases, seventh, eighth, and 10th revisions (ICD-7, ICD-8, and ICD-10), and classified as cardiovascular, cancer, or other mortality. If one of the first three ranked causes of death had a cardiovascular diagnosis (ICD-10 codes I00-I90), death was categorised as cardiovascular mortality. The remaining deaths were classified as cancer mortality if one of the first three ranked causes of death had a cancer diagnosis (ICD-10 codes C00-C96), and as other mortality if death was not classified as cardiovascular or cancer mortality.

Information on diagnoses of non-fatal and fatal myocardial infarction (ICD-8 code 410 and ICD-10 codes I21-I22) was obtained from the national Danish Patient Registry, a registry with information on all hospital contacts in Denmark from January 1977 onwards (outpatients and emergency wards from 1995), and the national Danish Causes of Death Registry (ICD-9 was never used in Denmark). Information on diagnoses of non-fatal and fatal cancer (ICD-7 codes 140-205 and ICD-10 codes C00-D09, excluding common skin cancers) was obtained from the national Danish Cancer Registry and the national Danish Causes of Death Registry.

Laboratory analyses

All blood samples were collected in the non-fasting state.[13] Concentrations of LDL-C were calculated with the Friedewald equation as:

Total cholesterol − high density lipoprotein cholesterol − triglycerides/2.2 in mmol/L (total cholesterol − high density lipoprotein cholesterol − triglycerides/5 in mg/dL) when triglyceride concentrations were less than 4 mmol/L (352 mg/dL), and were measured directly (Konelab) when triglyceride concentrations were 4 mmol/L or more (≥352 mg/dL). Concentrations of total cholesterol, high density lipoprotein cholesterol, triglycerides, and direct LDL-C were measured by standard hospital assays (Roche and Konelab).

Covariates

Statistical analyses were adjusted for a priori defined covariates (that is, for well known risk factors for mortality).[14] Sex and age were derived from the Civil Registration Number. Blood pressure was measured at the physical examination. In the questionnaire, participants reported on their smoking status and cumulative number of pack years, lipid lowering treatment, and diabetes. Diagnoses of diabetes, cardiovascular disease, cancer, or chronic obstructive pulmonary disease before entry into the study were obtained from the national Danish Patient Registry. Individuals with diabetes were identified as those having a registered diagnosis in the national Danish Patient Registry, a non-fasting plasma glucose concentration of more than 11 mmol/L (198 mg/dL), treatment with antidiabetic drugs, or self-reported diabetes from the questionnaire.

Statistical analyses

Only participants with an LDL-C measurement at baseline were included in the study; 847 individuals were excluded because of missing LDL-C measurements. Data on potential confounders were more than 99% complete. The remaining missing values were imputed by multivariable chained imputation with fully conditional specification[15]; imputed and reported results were similar.

Associations between levels of LDL-C and the risk of all cause mortality, cause specific mortality, myocardial infarction, and cancer were estimated by Cox proportional hazards regression models with 95% confidence intervals, with age as the underlying time scale (participants enter the risk set at baseline age and exit at censoring/event age=age adjustment) and left truncation (delayed entry at study examination). Follow-up started on the day of examination and ended at the first occurrence of death, myocardial infarction, cancer, emigration, or in December 2018. Individuals with a previous myocardial infarction or cancer were excluded when myocardial infarction or cancer was the endpoint. Multivariable adjusted statistical analyses were adjusted for age (as time scale), sex, current smoking, cumulative number of pack years, systolic blood pressure, lipid lowering treatment, diabetes, cardiovascular disease, cancer, and chronic obstructive pulmonary disease at baseline.

The associations between levels of LDL-C and all endpoints were evaluated on a continuous scale with restricted cubic spline curves based on Cox proportional hazards models. To balance best fit and overfitting in the main splines for mortality, myocardial infarction, and cancer, the number of knots, between three and seven, was chosen as the lowest value for

the Akaike information criterion, but if within two of each other for different knots, the lowest number of knots was chosen. The same number of knots from the main splines was also applied in splines for stratified analyses to allow direct comparison of overall and stratified analyses, including test of interaction. Interaction of levels of LDL-C with covariates for stratification on all cause mortality was examined by including two factor interaction terms in the Cox proportional regression model. The concentration of LDL-C associated with the lowest risk of mortality was the concentration with the lowest hazard ratio on the spline curve. The association between levels of LDL-C on a continuous scale and all cause mortality was also evaluated with fractional polynomials based on the Cox proportional hazards models. Furthermore, the associations between seven predefined LDL-C categories and all cause mortality were examined: five equally distributed categories of LDL-C were defined by the 20th, 40th, 60th, and 80th centiles, and to evaluate the highest and lowest levels of LDL-C, two additional categories were defined by the 5th and 95th centiles. The reference category for these analyses was the LDL-C level associated with the lowest risk of all cause mortality.

Hazard ratios and 95% confidence intervals for categories of LDL-C are presented with and without regression dilution bias; restricted cubic splines and the results in the main paper are reported without this correction. Correction for regression dilution bias was done with a non-parametric method to correct for underestimation caused by random measurements and long term fluctuations.[16] With LDL-C measurements from 9604 individuals with no atherosclerotic cardiovascular disease and who were not treated with lipid lowering agents participating in the 2003-15 examination and in the follow-up examination about 10 years later, a regression dilution ratio of 0.64 was determined for LDL-C (this ratio was used for the overall analyses for all individuals, regardless of follow-up time for the individual person—that is, the regression coefficients were multiplied by 1/0.64). Spline curves were not adjusted for regression dilution bias as we are not aware of a method to do this calculation.

In sensitivity analyses, pretreatment levels of LDL-C were estimated in individuals receiving lipid lowering treatment as baseline LDL-C measurements multiplied by 1.43 for individuals with no concurrent diagnoses of ischaemic heart disease or stroke, and by 1.67 for individuals with known ischaemic heart disease or stroke, corresponding to a 30% and 40% reduction, respectively.[17] All statistical analyses were performed in Stata/SE 15.1.

Patient and public involvement

No patients were involved directly in the design of the study, recruitment, or conduct of the study because our cohort consisted of normal individuals from the population at large (not patients) and because our study was planned in the year 2000 when direct patient involvement was not used in Denmark.

Results

The study included 108 243 individuals with 1 002 361 person years of follow-up (median follow-up 9.4 years, range 0-15 years). We found 11 376 (10.5%) deaths during follow-up, with a median age of 81 (range 26-106) at the time of death. Table 1 shows the baseline characteristics by LDL-C centile categories.

LDL-C and all cause mortality

The association between levels of LDL-C on a continuous scale and risk of all cause mortality was U shaped; low and high levels of LDL-C were associated with an increased risk of all cause mortality (fig 1). This association was also found in those not receiving lipid lowering treatment. For individuals receiving lipid lowering treatment, however, the 95% confidence

Table 1 | Baseline characteristics of 108 243 individuals in the Copenhagen General Population Study

	Centile (mmol/L, mg/dL)							
	1st-5th (<1.8, <70)	6th-20th (1.8-2.3, 70-92)	21st-40th (2.4-2.8, 93-112)	41st-60th (2.9-3.3, 113-131)	61st-80th (3.4-3.9, 132-154)	81st-95th (4.0-4.8, 155-189)	96th-100th (>4.8, >189)	All
No of individuals	6412 (6)	15 681 (14)	21 289 (20)	22 207 (21)	21 892 (20)	15 999 (15)	4763 (4)	108 243
Women	3202 (50)	9068 (58)	11 973 (56)	12 385 (56)	11 710 (53)	8537 (53)	2699 (57)	59 574 (55)
Age (years)	62 (47-72)	56 (45-68)	56 (46-67)	58 (48-67)	59 (50-67)	60 (51-67)	60 (52-67)	58 (48-67)
Smoker	961 (15)	2441 (16)	3291 (15)	3674 (17)	3872 (18)	3203 (20)	1107 (23)	18 549 (17)
Pack years, ever smokers	20 (8-38)	15 (5-30)	14 (5-29)	15 (6-30)	16 (7-30)	18 (7-31)	19 (8-32)	16 (6-30)
Systolic blood pressure (mm Hg)	137 (124-151)	136 (122-151)	137 (124-152)	140 (126-154)	141 (128-156)	144 (130-159)	145 (132-160)	140 (126-155)
Lipid lowering treatment	3030 (47)	4166 (27)	2891 (14)	1584 (7)	849 (4)	373 (2)	132 (3)	13 025 (12)
Diabetes	1249 (19)	1218 (8)	822 (4)	563 (3)	401 (2)	269 (2)	83 (2)	4605 (4)
Atherosclerotic cardiovascular disease	1806 (28)	2262 (14)	1756 (8)	1413 (6)	1200 (5)	817 (5)	223 (5)	9477 (9)
Cancer	557 (9)	1093 (7)	1393 (7)	1474 (7)	1508 (7)	1081 (7)	327 (7)	7433 (7)
Chronic obstructive pulmonary disease	1218 (19)	2517 (16)	3224 (15)	3324 (15)	3246 (15)	2331 (15)	666 (14)	16 526 (15)
LDL-C (mmol/L)	1.6 (1.4-1.7)	2.2 (2.0-2.3)	2.7 (2.6-2.8)	3.2 (3.1-3.3)	3.7 (3.6-3.9)	4.4 (4.2-4.6)	5.3 (5.1-5.7)	3.2 (2.6-3.8)
LDL-C (mg/dL)	62 (54-66)	85 (77-89)	104 (101-108)	124 (119-128)	143 (139-150)	170 (162-178)	205 (197-219)	124 (101-147)

Values are median (interquartile range) or number (%).
LDL-C=low density lipoprotein cholesterol.

interval included the hazard ratio of 1.0 for any level of LDL-C (P value for interaction between LDL-C levels and lipid lowering treatment on all cause mortality was <0.001) (fig 1, eFig 1). Compared with individuals with concentrations of LDL-C of 3.4-3.9 mmol/L (132-154 mg/dL; 61st-80th centiles), the multivariable adjusted hazard ratio for all cause mortality was 1.25 (95% confidence interval 1.15 to 1.36) for individuals with concentrations of LDL-C less than 1.8 mmol/L (<70 mg/dL; 1st-5th centiles) and 1.15 (1.05 to 1.27) for individuals with concentrations of LDL-C greater than 4.8 mmol/L (>189 mg/dL; 96th-100th centiles) (fig 2).

An increased risk of all cause mortality at low levels of LDL-C were seen in men and women (eFigs 2-3). Also, the association was most pronounced in individuals aged 65 or younger (eFig 4). For categories of age, the P value for interaction between low levels of LDL-C and age on all cause mortality was <0.001 (eFig 5).

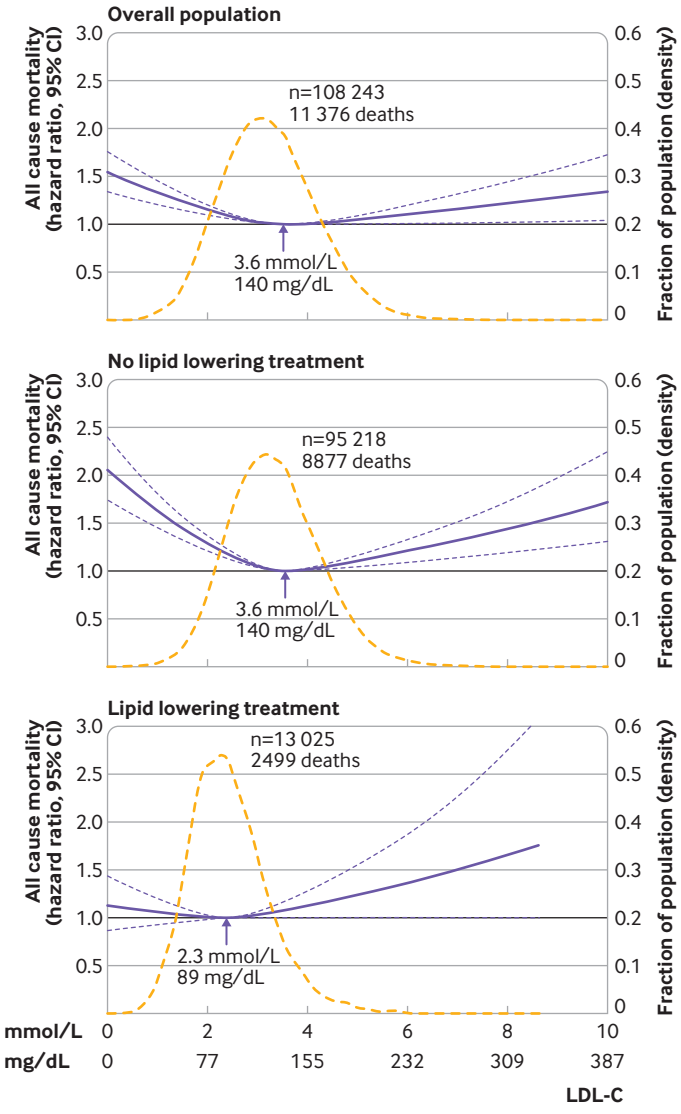

Fig 1 | Multivariable adjusted hazard ratios for all cause mortality according to levels of low density lipoprotein cholesterol (LDL-C) on a continuous scale. Solid blue lines are multivariable adjusted hazard ratios, with dashed blue lines showing 95% confidence intervals derived from restricted cubic spline regressions with three knots. Reference lines for no association are indicated by the solid bold lines at a hazard ratio of 1.0. Dashed yellow curves show the fraction of the population with different levels of LDL-C. Arrows indicate the concentration of LDL-C with the lowest risk of all cause mortality. Analyses were adjusted for age, sex, current smoking, cumulative number of pack years, systolic blood pressure, lipid lowering treatment, diabetes, cardiovascular disease, cancer, and chronic obstructive pulmonary disease at baseline. Based on individuals from the Copenhagen General Population Study followed for a mean 9.4 years

LDL-C level with the lowest risk of all cause mortality

The concentration of LDL-C associated with the lowest risk of all cause mortality in multivariable adjusted analyses was 3.6 mmol/L (140 mg/dL) in the overall population and in individuals not receiving lipid lowering treatment, compared with 2.3 mmol/L (89 mg/dL) in individuals receiving lipid lowering treatment (fig 1). Similar levels were seen in men and women and across age groups, except for men and women receiving lipid lowering treatment where the lowest risk of all cause mortality was at a concentrations of LDL-C of 2.7 mmol/L (105 mg/dL) and 1.9 (74 mg/dL), respectively (eFig 2, eFig 4).

LDL-C and cause specific mortality

In the overall population, the 95% confidence interval included the hazard ratio of 1.0 at any concentration of LDL-C for cardiovascular mortality whereas low levels of LDL-C were associated with an increased risk of cancer and other mortality (fig 3, eFig 6). In individuals not receiving lipid lowering treatment, the associations with cardiovascular, cancer, and other mortality were U shaped (eFig 6). In individuals receiving lipid lowering treatment, low levels of LDL-C were associated with increased cancer mortality but otherwise the 95% confidence interval included the hazard ratio of 1.0 at any concentration of LDL-C for cardiovascular, cancer, and other mortality (eFig 6). Also, the P value for interaction between levels of LDL-C and lipid lowering treatment was <0.001 for cardiovascular and other mortality, and 0.04 for cancer mortality.

Analysing cardiovascular mortality by ICD-10 codes showed that 13% of individuals died from myocardial infarction, 13% from heart failure, and 25% from any stroke (eTable 1). For cardiovascular mortality not including fatal myocardial infarction, the results were similar to overall cardiovascular mortality (fig 4). Any increase in LDL-C levels was associated with an increased risk of fatal myocardial infarction, although low levels of LDL-C were associated with an increased risk of fatal heart failure but the 95% confidence interval was wide (fig 4). For any fatal stroke, the 95% confidence interval included a hazard ratio of 1.0 at any concentration of LDL-C (fig 4).

LDL-C and myocardial infarction

Any increase in LDL-C levels was associated with an increased risk of myocardial infarction in the overall cohort and in individuals not receiving lipid lowering

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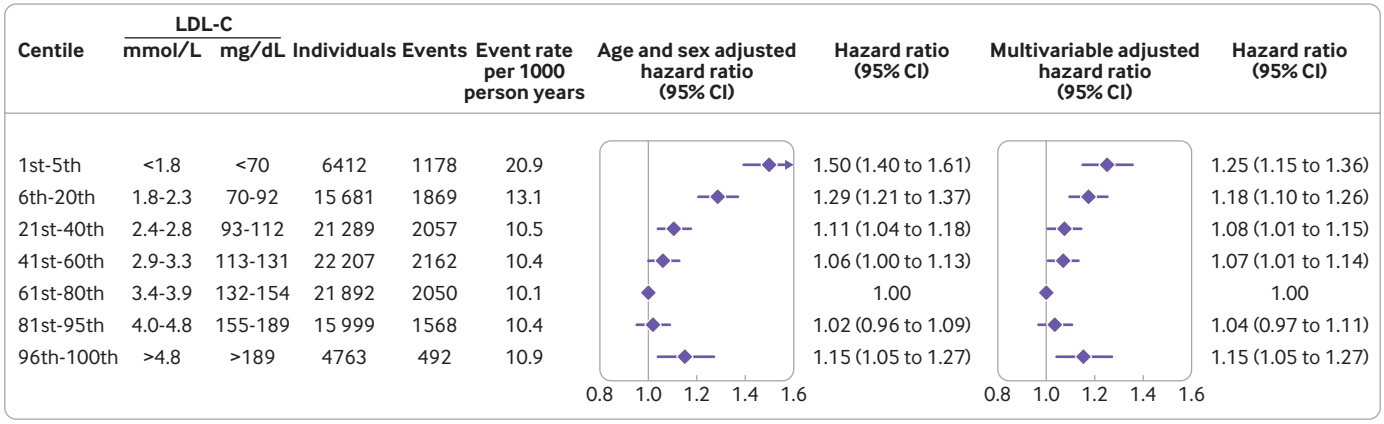

Fig 2 | Hazard ratios for all cause mortality according to categories of levels of low density lipoprotein cholesterol (LDL-C), sex and age adjusted, and multivariable adjusted. Multivariable adjusted analyses were adjusted for age, sex, current smoking, cumulative number of pack years, systolic blood pressure, lipid lowering treatment, diabetes, cardiovascular disease, cancer, and chronic obstructive pulmonary disease at baseline. Based on individuals from the Copenhagen General Population Study followed for a mean 9.4 years

treatment, although the 95% confidence interval included a hazard ratio of 1.0 at any concentration of LDL-C in individuals receiving lipid lowering treatment (P value for interaction between LDL-C levels and lipid lowering treatment on risk of myocardial infarction was 0.04) (fig 5).

LDL-C and cancer

Very low levels of LDL-C were associated with an increased risk of cancer in the overall population and in individuals not receiving lipid lowering treatment, although the 95% confidence interval included a hazard ratio of 1.0 at any concentration of LDL-C in individuals receiving lipid lowering treatment (P value for interaction between LDL-C levels and lipid lowering treatment on risk of cancer was 0.02) (fig 5).

Sensitivity analyses

The U shaped association between LDL-C levels on a continuous scale and all cause mortality was similar when a statistical method other than restricted cubic splines was used: with fractional polynomials, the concentration of LDL-C associated with the lowest risk of all cause mortality was 4.1 mmol/L (159 mg/dL) in the overall population, 4.0 mmol/L (155 mg/dL) in individuals not receiving lipid lowering treatment, and 2.1 mmol/L (82 mg/dL) in individuals receiving lipid lowering treatment (eFig 7 versus fig 1).

To assess whether the positive association between low levels of LDL-C and an increased risk of all cause mortality could be explained by reverse causation as a result of severe disease, we excluded individuals with less than five years of follow-up (start of follow-up began five years after the baseline examination) and individuals with atherosclerotic cardiovascular disease, cancer, and chronic obstructive pulmonary disease at the start of the study. We found that the results were similar to the main analyses although the association was slightly reduced (fig 6, and eFigs 8-10 versus fig 1). Starting follow-up five years after the baseline examination excluded individuals dying within five years of baseline and individuals with less than five years of follow-up. Excluding only those dying within five years of the baseline examination gave similar results. Also, we found similar results when restricting analyses to individuals aged 40-70 and with no chronic diseases at baseline.

Estimated pretreatment levels of LDL-C were examined to evaluate whether low levels of LDL-C caused by lipid lowering treatment, representing a high risk group with higher pretreatment levels, could explain the association between low levels of LDL-C and mortality. The results from these analyses gave similar results for all cause, cancer, and other mortality at low levels of LDL-C whereas the hazard ratios were nominally higher at high levels of LDL-C (eFig 11 versus fig 1, and fig 3). Low and high levels of LDL-C were associated with an increased risk of cardiovascular mortality. To assess the magnitude of effect size underestimation caused by random measurements and long term fluctuations, the hazard ratios and 95% confidence intervals for the association between LDL-C categories and all cause mortality were corrected for regression dilution bias (eFig 12 versus fig 2).

Discussion

In this study of 108 243 individuals from a contemporary ongoing general population cohort, we found a U shaped association between levels of LDL-C and the risk of all cause mortality, with low and high levels associated with an increased risk. The concentration of LDL-C with the lowest risk of all cause mortality was 3.6 mmol/L (140 mg/dL), well above the generally considered optimal concentration. These new results are likely to have implications for the interpretation of levels of LDL-C in clinical practice. As expected, the risk of myocardial infarction increased with any increase in the level of LDL-C.

Possible explanations for our findings

The association between low levels of LDL-C and an increased risk of all cause mortality could be explained

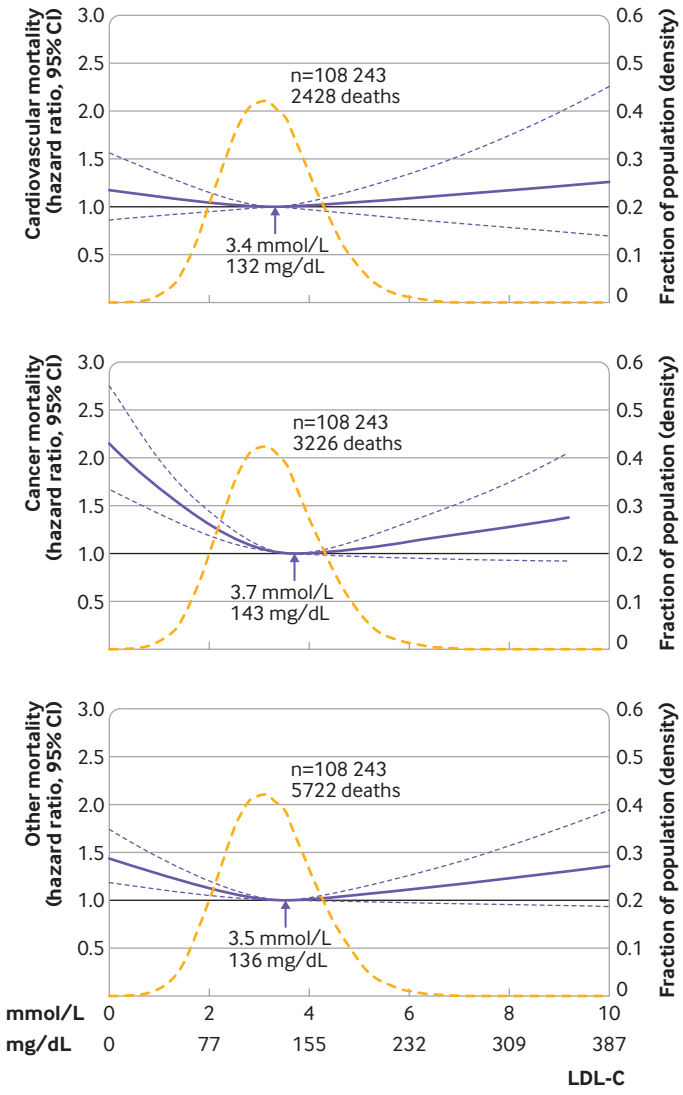

Fig 3 | Multivariable adjusted hazard ratios for cause specific mortality according to levels of low density lipoprotein cholesterol (LDL-C) on a continuous scale in the overall population. Solid blue lines are multivariable adjusted hazard ratios, with dashed blues lines showing 95% confidence intervals derived from restricted cubic spline regressions with three knots. Reference lines for no association are indicated by solid bold lines at a hazard ratio of 1.0. Dashed yellow curves show fraction of population with different levels of LDL-C. Arrows indicate the concentration of LDL-C with the lowest risk of mortality. Analyses were adjusted for age, sex, current smoking, cumulative number of pack years, systolic blood pressure, lipid lowering treatment, diabetes, cardiovascular disease, cancer, and chronic obstructive pulmonary disease at baseline. Based on individuals from the Copenhagen General Population Study followed for a mean 9.4 years

by reverse causation. Debilitation and illness have been hypothesised to cause a decrease in levels of cholesterol[18 19] and, in this study, comorbidities were more frequent in individuals with the lowest levels of LDL-C. Also, consistent with the theory that low levels of LDL-C are an indirect marker of severe disease, the association between low levels of LDL-C and the risk of all cause mortality was strongest in the age and sex adjusted model, and substantially reduced when adjusting for baseline comorbidities. An association remained after this adjustment, however, and after excluding individuals with less than five years of follow-up and known cardiovascular disease, cancer, and chronic obstructive pulmonary disease at baseline. Whether the remaining association, despite extensive comorbidity adjustment, can be attributed to residual confounding in terms of alternative mechanisms is unclear. The more pronounced association in individuals aged 65 or younger could point to an epiphenomenon where a pathophysiological abnormality, possibly genetic, causes an increased risk of mortality and decreased levels of LDL-C in parallel.

The U shaped association between levels of LDL-C and mortality might be similar to the obesity paradox, which is largely explained by methodological issues, including reverse causation.[20] In contrast with the obesity paradox, however, the U shaped association between levels of LDL-C and mortality in our study remained similar when analyses were restricted to healthy individuals aged 40-70 with no chronic diseases. This finding indicates that the obesity paradox and the U shaped association between levels of LDL-C and mortality are caused by different mechanisms.

Previous studies

Most studies investigating the relation between levels of LDL-C and the risk of all cause mortality have found no association [8-10] or an inverse association.[5-7] Our study showed that the inverse association can be explained by the increased risk of all cause mortality associated with low levels of LDL-C rather than representing an actual decreased risk at high levels of LDL-C. Also, a recent study in young Koreans not taking lipid lowering drugs showed an association between low levels of LDL-C and an increased risk of all cause mortality, cardiovascular mortality, and cancer mortality,[11] similar to our results in the group of individuals not receiving lipid lowering treatment.

No previous study has examined the concentration of LDL-C associated with the lowest risk of all cause mortality in a general population cohort. One study in people aged 65 and over reported the lowest all cause risk of mortality at a concentration of LDL-C of 4.9 mmol/L (190 mg/dL) for women and 3.8 mmol/L (147 mg/dL) for men.[21] In our study, we consistently found the lowest risk of all cause mortality at concentrations of LDL-C of 3.6-3.7 mmol/L (140-143 mg/dL) for men and women and across the age groups (≤65 or >65).

Previous studies on the association between total cholesterol and risk of mortality showed a reversed J shaped or U shaped association, with the highest risk of all cause, cancer, and other mortality found at the lowest levels of total cholesterol, although positive, inverse, and no association with cardiovascular mortality have been reported.[18 22 23] Also, we have recently found a similar U shaped association between levels of high density lipoprotein cholesterol and risk of all cause mortality.[24]

Lipid lowering treatment

The relatively low number of individuals receiving lipid lowering treatment in Denmark has been confirmed

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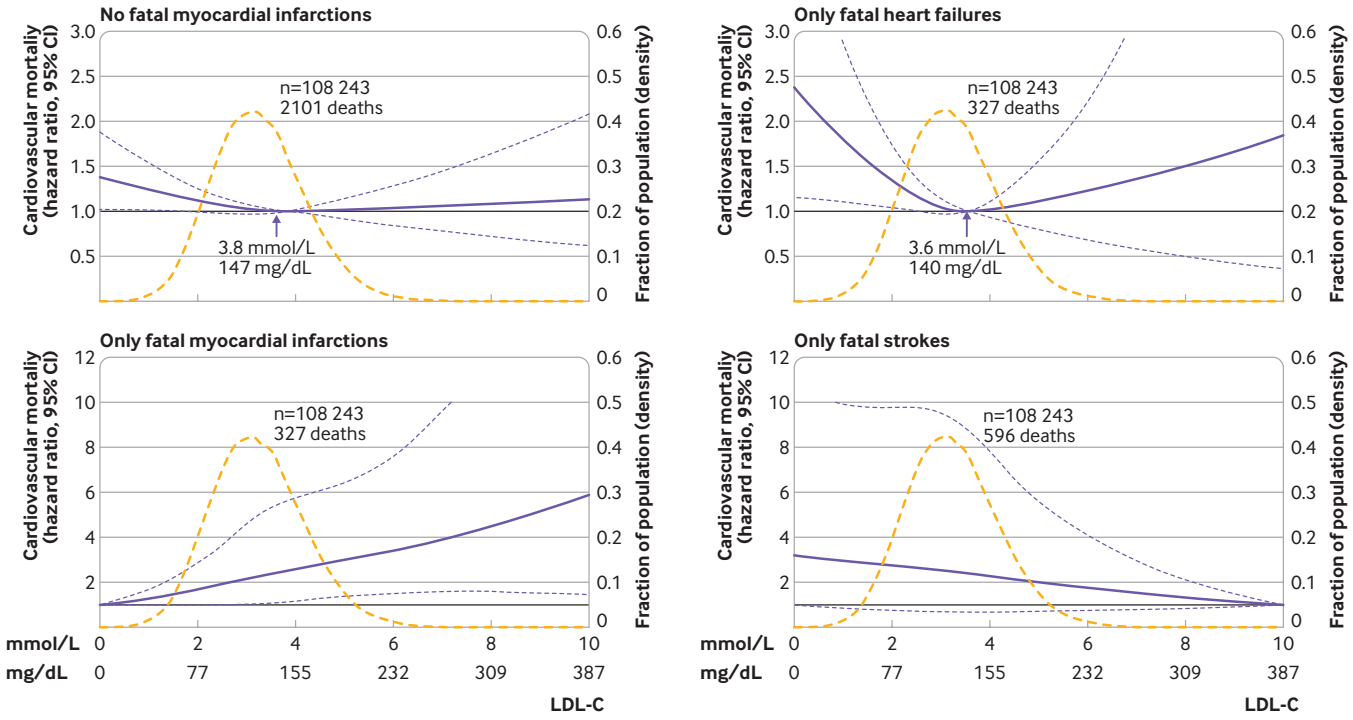

Fig 4 | Multivariable adjusted hazard ratios for different cardiovascular mortality endpoints according to levels of low density lipoprotein cholesterol (LDL-C) on a continuous scale. Solid blue lines are multivariable adjusted hazard ratios, with dashed blue lines showing 95% confidence intervals derived from restricted cubic spline regressions with three knots. Reference lines for no association are indicated by solid bold lines at a hazard ratio of 1.0. Dashed yellow curves show fraction of population with different levels of LDL-C. Arrows indicate the concentration of LDL-C with the lowest risk of mortality. Analyses were adjusted for age, sex, current smoking, cumulative number of pack years, systolic blood pressure, lipid lowering treatment, diabetes, cardiovascular disease, cancer, and chronic obstructive pulmonary disease at baseline. Based on individuals from the Copenhagen General Population Study followed for a mean 9.4 years

in previous studies.[25 26] In our study, in individuals receiving lipid lowering treatment, the association between low levels of LDL-C and an increased risk of all cause, cancer, and other mortality was weaker than for individuals not receiving lipid lowering treatment. Any increase in levels of LDL-C, however, was associated with an increased risk of cardiovascular mortality but the 95% confidence intervals were wider and included a hazard ratio of 1.0 for all cause, cardiovascular, and other mortality at any concentration of LDL-C. This finding indirectly indicates a non-causal association and suggests that the reduction in levels of LDL-C caused by lipid lowering treatment does not explain the increased risk of mortality at low levels of LDL-C but rather low LDL-C levels is a predictor for mortality. Hence it would be incorrect to use our data as an argument against the use of lipid lowering treatment in the prevention of atherosclerotic cardiovascular disease and mortality. A recent meta-analysis of studies in individuals at high risk of atherosclerotic cardiovascular disease showed that more intensive lowering of levels of LDL-C was associated with a greater reduction in the risk of all cause and cardiovascular mortality.[4] The remaining association between low levels of LDL-C and cancer mortality together with the association between very low levels of LDL-C and an increased risk of cancer (fatal and non-fatal) supports the hypothesis of a decrease in LDL-C levels because of debilitation and illness.

Clinical importance

Our results could be important for understanding what is a "normal and healthy" level of LDL-C in the general population (that is, when the focus is not limited to myocardial infarction and atherosclerotic cardiovascular disease). The finding of the lowest risk of all cause mortality at a concentration of LDL-C of 3.6 mmol/L (140 mg/dL) implies that in individuals with an otherwise low risk of atherosclerotic cardiovascular disease, an LDL-C level of around this value is not necessarily hazardous in itself. Any increase in LDL-C, however, was associated with an increased risk of myocardial infarction and death from myocardial infarction. Together, these results indicate the importance of assessing the absolute risk of atherosclerotic cardiovascular disease in deciding when to use lipid lowering treatment,[27 28] rather than starting treatment based solely on a moderate increase in levels of LDL-C.

Strengths and limitations

The strengths of our study include, firstly, the size of the cohort in terms of the large number of individuals recruited, with no individuals lost to follow-up. Secondly, information on cause of death for every individual was obtained from Danish registries. Thirdly, we adjusted for several confounders with an effect on mortality risk.[14] Fourthly, the strong positive association between any increase in levels of LDL-C

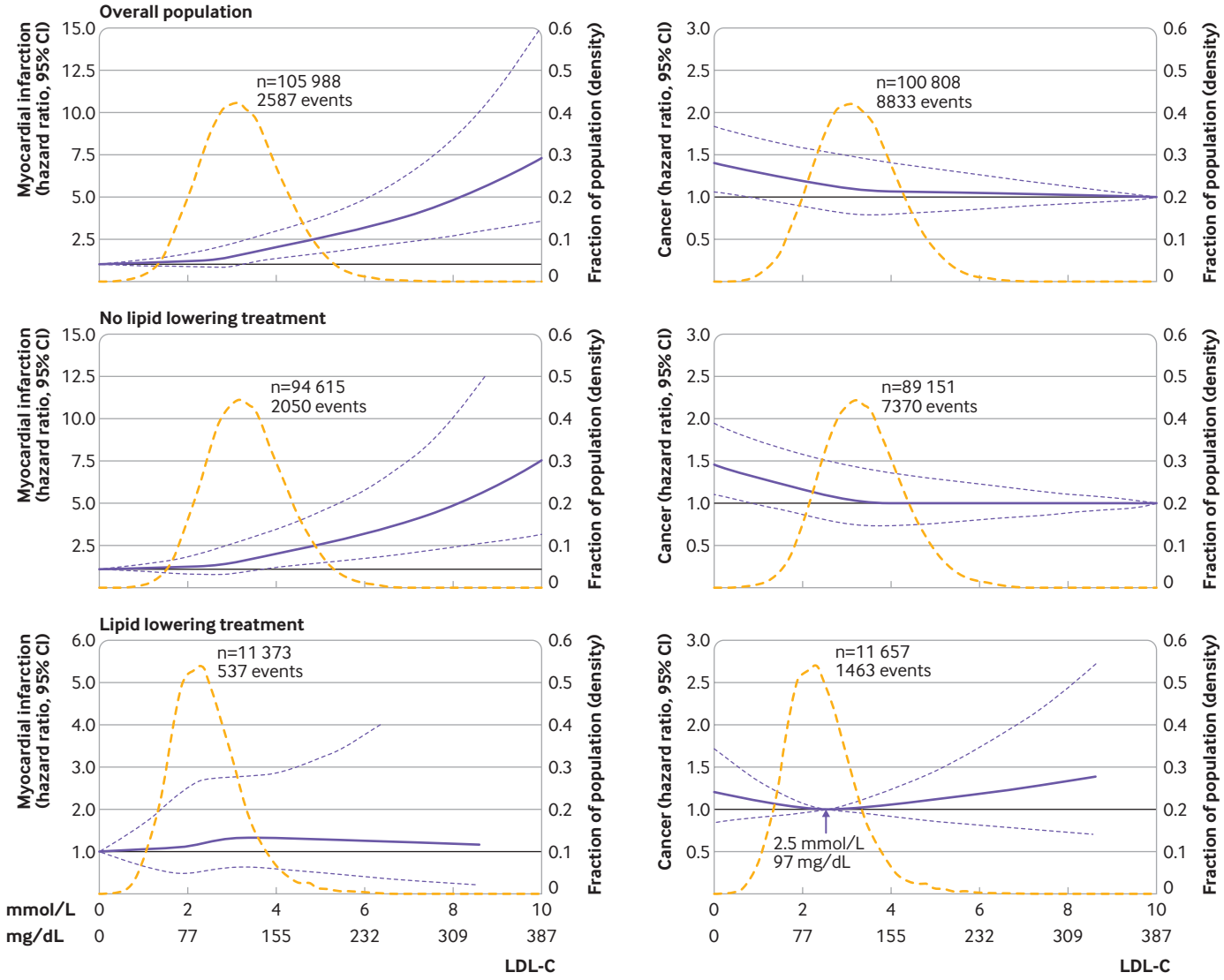

Fig 5 | Multivariable adjusted hazard ratios for myocardial infarction and cancer according to levels of low density lipoprotein cholesterol (LDL-C) on a continuous scale. Solid blue lines are multivariable adjusted hazard ratios, with dashed blue lines showing 95% confidence intervals derived from restricted cubic spline regressions, with four knots for myocardial infarction and three knots for cancer. Reference lines for no association are indicated by solid bold lines at a hazard ratio of 1.0. Dashed yellow curves show fraction of population with different levels of LDL-C. Arrow indicates the concentration of LDL-C with the lowest risk of cancer. Analyses were adjusted for age, sex, current smoking, cumulative number of pack years, systolic blood pressure, lipid lowering treatment, diabetes, cardiovascular disease, cancer, and chronic obstructive pulmonary disease at baseline. Based on individuals from the Copenhagen General Population Study followed for a mean 9.4 years

and an increased risk of myocardial infarction supports the validity of this study.

A limitation of our study is that it included only white individuals living in a Western country, which could limit the applicability of our results to other ethnicities; however, we are not aware of data to suggest that our results are not applicable to other ethnicities living in countries with a similar standard of living and healthcare system to Denmark. A recent study in young Koreans of supposedly comparable affluence to people in Denmark showed similar results to our study.[11] In less affluent and less developed countries, levels of LDL-C associated with the lowest mortality could differ from our results. We only had information on lipid lowering treatment at baseline and cannot rule out that the results might have been influenced by individuals starting or stopping treatment with lipid lowering agents during follow-up. We could not adjust for weight loss, which has been associated with decreases in LDL-C levels, as this information was not available in our cohort. Some results were corrected for regression dilution bias to visualise the possible underestimation of the effect estimates; however, the main figures show unadjusted results and the true values are likely to lie somewhere between the corrected and uncorrected values. Finally, we could not deal with the question of causality because the design of the study was observational. This question could theoretically be looked at in mendelian randomisation analyses, modelling non-linear and

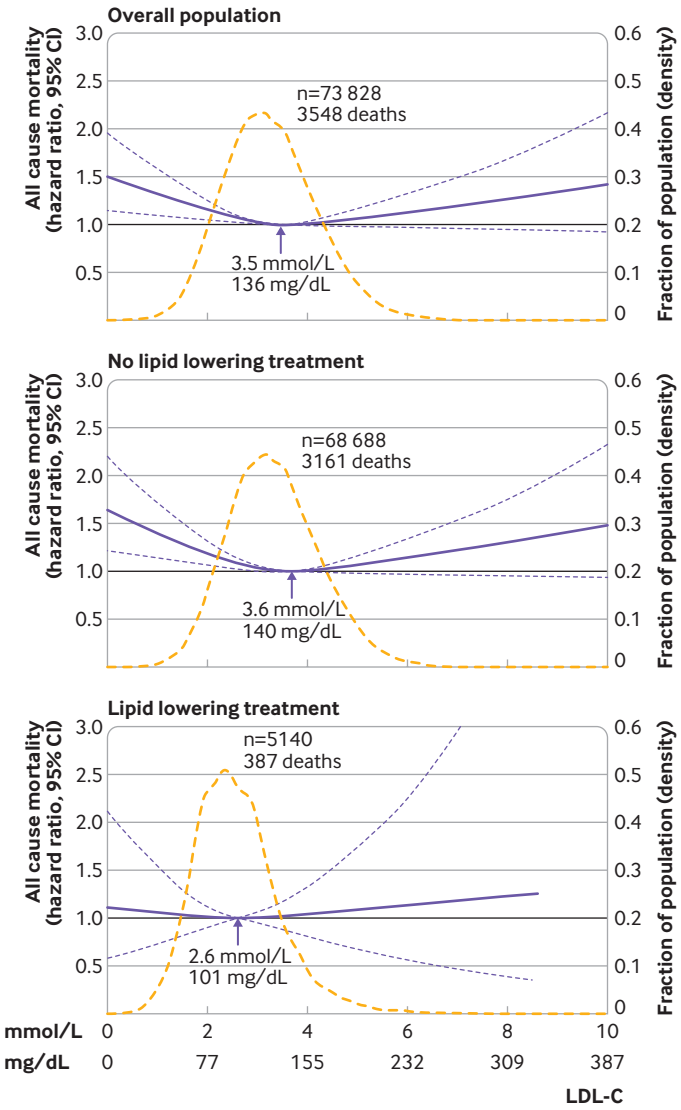

Fig 6 | Multivariable adjusted hazard ratios for all cause mortality according to levels of low density lipoprotein cholesterol (LDL-C) on a continuous scale with the start of follow-up at year 5 and after exclusion of individuals with known atherosclerotic cardiovascular disease, cancer, and chronic obstructive pulmonary disease at baseline. Solid blue lines are multivariable adjusted hazard ratios, with dashed blue lines showing 95% confidence intervals derived from restricted cubic spline regressions with three knots. Reference lines for no association are indicated by solid bold lines at a hazard ratio of 1.0. Dashed yellow curves show fraction of population with different levels of LDL-C. Arrows indicate the concentration of LDL-C with the lowest risk of all cause mortality. Analyses were adjusted for age, sex, current smoking, cumulative number of pack years, systolic blood pressure, lipid lowering treatment, diabetes, cardiovascular disease, cancer, and chronic obstructive pulmonary disease at baseline. Based on individuals from the Copenhagen General Population Study followed for a mean 9.4 years

U shaped relations.[29-31] Modelling of U shaped associations in mendelian randomisation analyses, however, requires high statistical power and numerous genetic instruments explaining a large fraction of the variation in plasma concentrations of LDL-C. Such genetic data with sufficient statistical power were not available in our cohort. Nevertheless, future studies with more statistical power than our study could provide further insight into the potential causal nature of the association of levels of LDL-C with mortality with non-linear mendelian randomisation analyses.

Conclusions

Low and high levels of LDL-C were associated with an increased risk of all cause mortality in individuals in the general population. Similar results were seen for cancer and other mortality whereas no association was found for cardiovascular mortality overall. Also, individuals in the general population with a concentration of LDL-C of 3.6 mmol/L (140 mg/dL) live the longest. This finding, if confirmed in more studies, will have important clinical and public health implications.

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Competing interests: All authors have completed the ICMJE uniform disclosure form at www.icmje.org/coi_disclosure.pdf and declare: support from Herlev and Gentofte Hospital's Research Fund and the Department of Clinical Biochemistry, Herlev and Gentofte Hospital, Copenhagen University Hospital, Denmark for the submitted work; no financial relationships with any organisations that might have an interest in the submitted work in the previous three years; no other relationships or activities that could appear to have influenced the submitted work.

Ethical approval: The study was approved by Herlev and Gentofte Hospital, the ethics committee of the Capital Region of Denmark (H-KF-01-144/01), and the Danish Data Protection Agency. Written informed consent was given by each participant.

Data sharing: Additional data are available from the corresponding author on reasonable request.

The lead author affirms that the manuscript is an honest, accurate, and transparent account of the study being reported; that no important aspects of the study have been omitted; and that any discrepancies from the study as originally planned (and, if relevant, registered) have been explained.

Dissemination to participants and related patient and public communities: Results will, after scientific publication, be disseminated to the public in general.

Provenance and peer review: Not commissioned; externally peer reviewed.

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Web appendix: Supplementary material

Red meat, dairy, and insulin sensitivity: a randomized crossover intervention study[1–3]

Kirsty M Turner, Jennifer B Keogh, and Peter M Clifton

ABSTRACT

Background: Epidemiologic studies have linked high consumption of red and processed meat with risk of developing type 2 diabetes, whereas high dairy consumption has been associated with decreased risk, but interventions have been limited.

Objective: We compared the effects on insulin sensitivity of consuming a diet high in lean red meat with minimal dairy, a diet high in primarily low-fat dairy (from milk, yogurt, or custard) with no red meat, and a control diet that contained neither red meat nor dairy.

Design: A randomized crossover study was undertaken with 47 overweight and obese men and women divided into 2 groups as follows: those with normal glucose tolerance and those with impaired fasting glucose or impaired glucose tolerance. Participants followed the 3 weight-stable dietary interventions for 4 wk with glucose, insulin, and C-peptide measured by using oral-glucose-tolerance tests at the end of each diet.

Results: Fasting insulin was significantly higher after the dairy diet than after the red meat diet ($P < 0.01$) with no change in fasting glucose resulting in a decrease in insulin sensitivity after the high-dairy diet ($P < 0.05$) as assessed by homeostasis model assessment of insulin resistance (HOMA-IR). A significant interaction between diet and sex was observed such that, in women alone, HOMA-IR was significantly lower after the red meat diet than after the dairy diet (1.33 ± 0.8 compared with 1.71 ± 0.8, respectively; $P < 0.01$). Insulin sensitivity calculated by using the Matsuda method was 14.7% lower in women after the dairy diet than after the red meat diet ($P < 0.01$) with no difference between diets in men. C-peptide was not different between diets.

Conclusion: In contrast to some epidemiologic findings, these results suggest that high consumption of dairy reduces insulin sensitivity compared with a diet high in lean red meat in overweight and obese subjects, some of whom had glucose intolerance. This trial was registered at the Australian New Zealand Clinical Trials Registry as ACTRN12613000441718. *Am J Clin Nutr* 2015;101:1173–9.

Keywords: dairy, dietary proteins, insulin sensitivity, red meat, type 2 diabetes

INTRODUCTION

Insulin resistance increases the likelihood of impaired glucose tolerance (IGT)[4] and development of type 2 diabetes (T2D) (1). Improved diet quality, energy restriction, and weight loss and increased physical activity lowers risk of T2D, but the role of specific dietary components is still debated (2).

Red meat is a good source of protein as well as vitamins and minerals (3); however, high consumption of red meat has been linked to risk of developing T2D. A meta-analysis of 12 cohort studies showed a 20% increase in risk of diabetes per 120-g/d increase in red meat intake and, for processed red meat, a 57% increase in risk per 50-g/d increase (4). There have been only a few intervention studies that assessed meat intake and insulin sensitivity without a weight-loss component, and these studies showed mixed results. A crossover study in healthy young women compared an 8-wk diet high in oily fish to one high in red meat. Fasting glucose concentrations decreased after both diets, and the effect of diet was NS. Fasting insulin concentrations increased after the red meat diet and decreased significantly after the oily fish diet with an almost 20% difference between the 2 diets, which resulted in increased insulin sensitivity after the oily fish diet (5). Fasting glucose concentrations did not change after a comparison of lean lamb and chicken in a crossover intervention of two 5-wk diets, but insulin was not measured (6).

Dairy foods such as milk, yogurt, and cheese, which are sources of high-quality protein, calcium, and other vitamins and minerals (7), have been associated with protection from developing T2D. A recent meta-analysis examined 14 cohorts and showed a significant inverse linear association between consumption of total dairy products, low-fat dairy, cheese, and yogurt and risk of T2D (8). A United Kingdom study showed 24% lower risk with 4.5 (125-g) servings of low-fat fermented dairy (primarily yogurt but including low-fat cheese), whereas total dairy, high-fat dairy, milk, cheese, and high-fat fermented dairy showed no association (9). In contrast, high-fat dairy had the strongest inverse association with T2D in a recent Swedish study, with no association shown for low-fat dairy intake, which indicated a possible protective effect of dairy fat (10). The most-recent meta-analysis (11) showed no effect of total or low fat dairy, and

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[4] Abbreviations used: BCAA, branched-chain amino acid; IFG, impaired fasting glucose; IGT, impaired glucose tolerance; NGT, normal glucose tolerance; OGTT, oral-glucose-tolerance test; T2D, type 2 diabetes.

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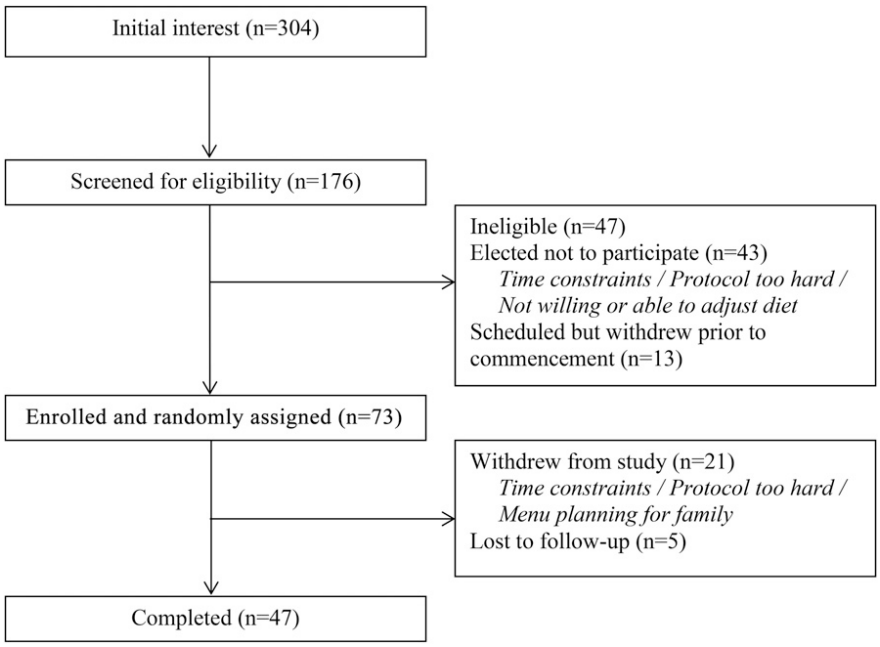

FIGURE 1 Participant recruitment and withdrawal.

only yogurt was protective. There was no suggestion of harm from dairy despite its high saturated fat content.

Few intervention studies have evaluated red meat or dairy for their effect on insulin sensitivity in the absence of weight loss; therefore, this study was designed to maintain weight stability to isolate the effect that lean red meat and dairy have on insulin sensitivity. The primary hypothesis was that a red meat diet would produce greater insulin resistance than would a high-dairy diet with secondary hypotheses that the high-dairy diet would produce greater insulin sensitivity than would the diet without dairy and that these changes would be greater in individuals with impaired fasting glucose (IFG) or IGT than in those with normal glucose tolerance (NGT).

METHODS

Participants

Participants were recruited by public advertisement and screened for eligibility. Inclusion criteria included overweight and obese men and women >20 y old. Exclusion criteria included diagnosed diabetes, medication or supplements that would influence glucose metabolism, pregnancy or breastfeeding, recent weight gain or weight loss, or a history of metabolic illness such as kidney or liver disease. Participants were excluded if they had a known allergy or intolerance to dairy or lactose or were considered unlikely to comply with the study protocol. Participants were separated into 2 groups as follows: those with NGT and those with IGT or IFG as established by a 75-g oral-glucose-tolerance test (OGTT) performed at the baseline visit or from a previous medical diagnosis of IGT. The University of South Australia Human Research Ethics committee approved the study, and all participants provided written informed consent before participating. The trial was registered with the Australian New Zealand Clinical Trials Registry as ACTRN12613000441718. AUD $150 was offered to participants at completion of the study.

Dietary intervention

During the high–red meat diet, participants were instructed to consume ≥200 g red meat/d for 6 d/wk and consume minimal (<1 serving) dairy per day. During the high-dairy diet, participants were instructed to consume 4–6 servings of primarily low-fat dairy (from milk, yogurt, or custard) and cheese per day with chicken and fish as additional sources of protein but no red meat. Serving sizes were defined by using the guidelines of the Australian National Health and Medical Research Council (7) (e.g., 250 g milk, 200 g yogurt, 40 g hard cheese, or 120 g ricotta cheese). The low-dairy, no red meat control diet contained ≥200 g fish or chicken/d with <1 serving of dairy/d. Usual food items were replaced with red meat or dairy for weight to remain stable. Participants attended the clinic on 3 occasions during each diet to monitor weight and ensure dietary compliance. Participants were asked not to consume processed meat for the duration of the study. The diet order was randomized with all participants completing each 4-wk diet with a 2-wk washout period in between. Verbal and written instructions, including explanations of serving sizes, were provided for each diet along with digital kitchen scales (Homemaker Slimline Electronic Scale; KMart Australia).

TABLE 1
Baseline characteristics of the participants[1]

	NGT	IFG/IGT
Sex (M/F), *n*	12/15	6/14
Age, y	44.3 ± 12.9[2,a]	52.5 ± 12.0[b]
BMI, kg/m^2	30.7 ± 4.1	31.6 ± 6.3
Baseline SBP, mm Hg	124.9 ± 16.8	128.9 ± 12.9
Baseline DBP, mm Hg	81.7 ± 10.3	83.6 ± 7.9
Total fat mass,[3] %	39.8 ± 9.3	38.3 ± 9.2
Total lean mass,[3] %	60.6 ± 9.4	61.7 ± 9.2
Total fat mass,[3] kg	35.3 ± 10.5	29.8 ± 7.6
Total lean mass,[3] kg	50.6 ± 10.0	45.7 ± 9.9

[1]Values in a row that do not share a common superscript letter are significantly different, $P < 0.05$. DBP, diastolic blood pressure; IFG, impaired fasting glucose; IGT, impaired glucose tolerance; NGT, normal glucose tolerance; SBP, systolic blood pressure.

[2]Mean ± SD (all such values).

[3]n = 45.

TABLE 2
Effect of diet on glucose, insulin, and insulin-sensitivity indexes[1]

	All (n = 47)			Men (n = 18)			Women (n = 29)		
	Red meat	Dairy	Control	Red meat	Dairy	Control	Red meat	Dairy	Control
Fasting glucose, mmol/L	5.24 ± 0.6	5.23 ± 0.6	5.15 ± 0.6	5.31 ± 0.6	5.32 ± 0.8	5.23 ± 0.6	5.20 ± 0.6	5.18 ± 0.5	5.10 ± 0.6
Fasting insulin, mU/L	5.47 ± 2.4[a]	6.64 ± 4.1[b]	5.78 ± 2.9[a]	5.23 ± 2.1	5.43 ± 2.8	5.13 ± 1.5	5.62 ± 2.6[a]	7.38 ± 4.5[b]	6.16 ± 3.5[a]
HOMA-IR	1.30 ± 0.7[a]	1.55 ± 1.0[b]	1.34 ± 0.7[a,b]	1.24 ± 0.5	1.29 ± 0.7	1.19 ± 0.4	1.33 ± 0.8[a]	1.71 ± 1.1[b]	1.42 ± 0.9[a]
Matsuda index	7.89 ± 4.1[a]	7.28 ± 3.89[b]	7.95 ± 5.64[a,b]	7.49 ± 3.8	8.03 ± 4.3	6.82 ± 3.4	8.14 ± 0.8[a]	6.81 ± 0.6[b]	8.61 ± 1.2[a]
Stumvoll index	0.084 ± 0.027	0.082 ± 0.029	0.082 ± 0.028	0.088 ± 0.020	0.090 ± 0.025	0.088 ± 0.025	0.081 ± 0.006[a]	0.077 ± 0.006[b]	0.078 ± 0.005[a,b]

[1] All values are means ± SDs. A repeated-measures ANOVA was used with the 3 diets. $P < 0.05$ for insulin, HOMA-IR, and the Matsuda index. Red meat compared with dairy for insulin: diet, $P = 0.02$; diet by sex, $P = 0.02$. Red meat compared with dairy for HOMA-IR: diet, $P = 0.04$; diet by sex, $P = 0.03$. Red meat compared with dairy for the Matsuda index: diet, $P = 0.07$; diet by sex, $P = 0.01$. Red meat compared with dairy for the Stumvoll index: diet, $P = 0.39$; diet by sex, $P = 0.17$. Values in a row that do not share a common superscript letter are significantly different, $P < 0.05$ [after 2-diet repeated-measures ANOVAs (red meat compared with dairy; dairy compared with control; red meat compared with control)].

Dietary measurements

A food-frequency questionnaire was completed at the baseline visit to assess usual dietary intake over the previous 12 mo. The food-frequency questionnaire is a validated tool for measuring dietary intake in the Australian population (12) and provides information regarding food choice, portion size, and frequency of consumption. A daily checklist was completed during each dietary period to obtain the amounts of red meat, dairy, or alternate protein sources consumed each day. A 3-d weighed food record was also completed within each 2-wk period. All food and beverages consumed over these 3-d weighed periods were recorded and entered into FoodWorks Professional Edition 7.0 software (Xyris) for dietary analysis.

Clinical measurements

Height was measured on a wall-mounted stadiometer (Seca) at the baseline visit. Body weight was measured at each visit by using electronic digital scales (Tanita Corp.) in light clothing and without shoes. BMI (in kg/m^2) was calculated as weight divided by height squared. Body composition was assessed at baseline by using whole-body dual-energy X-ray absorptiometry (Luna Prodigy; Lunar Radiation Corp.). After an overnight fast, participants came to the Sansom Institute for Health Research Clinical Trial facility at the University of South Australia for OGTTs. These tests were performed at the end of each diet with blood samples taken every 30 min for a total of 5 time points. Blood for serum was collected in tubes with no additives and allowed to clot at room temperature for 30 min. Blood for plasma was collected in tubes containing sodium fluoride and stored on ice until processed. Blood samples were separated by a centrifuge at 1780 g at 4°C for 10 min (Universal 32R; Hettich Zentrifugen). Plasma glucose was measured by using an automated spectrophotometric analyzer (Konelab 20XTi; Thermo Electron), and serum insulin and C-peptide were measured by using commercial ELISA kits (kit 0030N for insulin, kit 0040 for C-peptide; Alpha Diagnostic).

Analysis

Insulin sensitivity was assessed from the OGTT by using the methods of Stumvoll et al. (13), calculated as

$$[0.226 - (0.0032 \times \text{BMI}) - (0.0000645 \times \text{Ins}_{120}\,(\text{pmol/L})] - [0.0037 \times G_{90}(\text{mmol/L})] \quad (1)$$

whereby Ins_{120} denotes insulin at 120 min and G_{90} denotes glucose at 90 min, and Matsuda and DeFronzo (14), calculated as

$$10,000 \div \sqrt{\{[G_{\text{fasting}}\,(\text{mg/dL}) \times \text{Ins}_{\text{fasting}}\,(\mu\text{U/mL})] \times [G_{\text{meanOGTT}} \times \text{Ins}_{\text{meanOGTT}}]\}} \quad (2)$$

whereby Ins denotes insulin, and G denotes glucose. HOMA-IR was also calculated from fasting glucose and insulin as

$$\text{HOMA-IR} = [\text{FPI}(\text{mU/L}) \times \text{FPG}\,(\text{mmol/L})] \div 22.5 \quad (3)$$

whereby FPI denotes fasting plasma insulin, and FPG denotes fasting plasma glucose. Each of these methods previously showed strong correlations with the euglycemic hyperinsulinemic clamp method, which is considered the reference standard for assessing

insulin sensitivity (15). A sample size of 68 was calculated initially from the literature, and thus, the aim was to recruit 80 participants, allowing for withdrawals. This calculation was revised after the first 5 volunteers completed the study, and we showed the SD of insulin and HOMA-IR was lower than expected, and a sample size of 45 would have provided 90% power to see a 20% change in insulin sensitivity as assessed by using the Matsuda index.

The statistical analysis was performed with SPSS V22 software (IBM). The Kolomogorov-Smirnov test, Q-Q plots, and histograms were used to test for the normality of distribution. Variables that were not normally distributed were log transformed. Differences between groups were tested by using a repeated-measures ANOVA and paired samples *t* tests. A mixed-model analysis was used to examine the influence of weight changes during each dietary period. Analyses were conducted with and without outliers >2 SDs from the mean to assess any effect on outcomes. Outliers were included in the final analysis. The incremental AUC was calculated by using the trapezoidal equation. Data are expressed as means ± SDs, and significance was set at $P < 0.05$.

RESULTS

Of 304 people who initially responded to advertising, 176 people were screened, and 86 people satisfied the inclusion criteria. **Figure 1** outlines the recruitment and withdrawal of participants. Forty-seven people (age: 47.8 ± 13.0 y; BMI: 31.1 ± 5.1) completed the study. Twenty-seven subjects had NGT, 6 subjects had IFG, and 14 subjects had IGT. Baseline characteristics of each group are shown in **Table 1**.

Sensitivity indexes were not normally distributed, and thus, data were log transformed before analysis. A repeated-measures analysis of variance showed a significant difference between diets for fasting insulin concentrations (**Table 2**). Fasting insulin was significantly higher in the dairy diet compared with the red meat diet (6.6 ± 4.1 compared with 5.5 ± 2.4 mU/L, respectively; $P < 0.01$). Because there was no difference in fasting glucose concentrations between diets, this resulted in a 16% decrease in insulin sensitivity after the high-dairy diet as assessed by using HOMA-IR ($P < 0.05$). There was no effect of age, BMI, percentage of fat mass, percentage of lean mass, or glucose-tolerance group when added as covariates or factors; however, a post hoc analysis revealed a significant interaction between diet and sex ($P < 0.05$), with insulin and HOMA-IR significant for women between red meat and dairy diets ($P < 0.01$). In women alone, the glucose-tolerance group or percentage of fat mass was NS.

Similarly, when red meat and dairy were compared, the Matsuda index showed a 14.7% reduced sensitivity after the dairy diet in women ($P = 0.01$) with no difference between diets in men (*P*-diet by sex interaction < 0.05). The Stumvoll sensitivity index showed a significant effect for women between red meat and dairy diets ($P < 0.05$); however, the removal of an outlier >2 SDs from the mean attenuated the significance. No interaction between group (NGT or IFG and IGT), BMI, age, or diet order was seen when added as covariates or factors. Fasting insulin concentrations after the dairy diet were also higher than after the control diet ($P < 0.05$), but HOMA-IR and the Matsuda index were only significantly different between the 2 diets in women (*P*-both comparisons < 0.05). The Stumvoll index was not significantly different between dairy and control diets.

The glucose incremental AUC was significantly different between glucose-tolerance subgroups ($P < 0.01$) (**Figure 2**), but there were no differences between diets overall and no effect when age, BMI, sex, or diet order was used as a covariate or factor. Insulin and C-peptide incremental AUCs were not significantly different between diets or groups (**Table 3**).

Energy intake was higher with the dairy diet (**Table 4**) than with both red meat and control diets (*P*-both comparisons $<$ 0.001), and total and saturated fat intakes were also higher during the dairy diet than during either the red meat or control diet (*P*-both comparisons < 0.01). Carbohydrate intake was

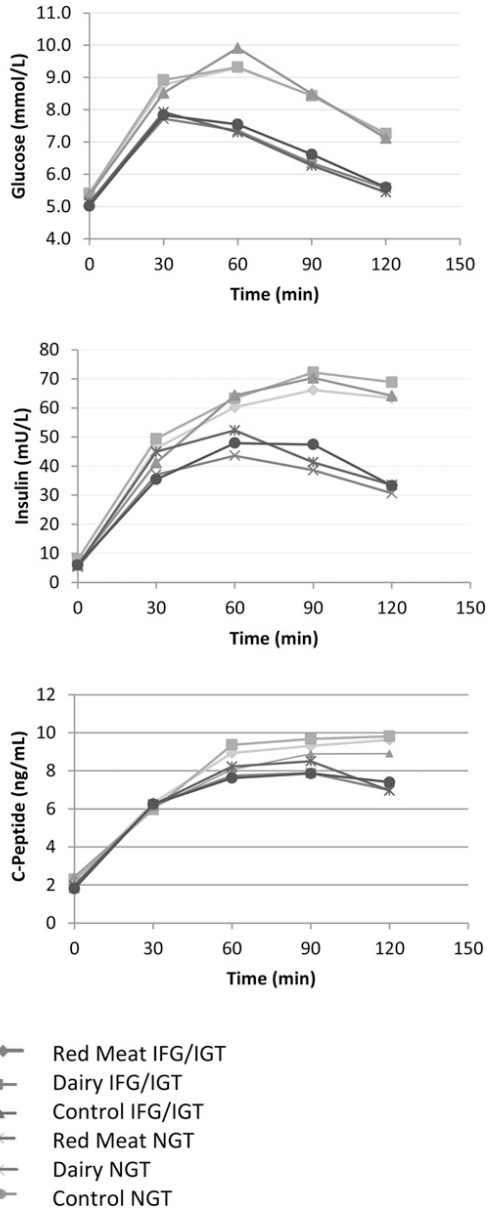

FIGURE 2 Postprandial glucose, insulin, and C-peptide concentrations in response to a 75-g glucose OGTT after three 4-wk diets. NGT: $n = 27$, IFG/IGT: $n = 20$. Between glucose-tolerance group difference, $P < 0.01$ for glucose; NS for insulin or C-peptide (repeated-measures ANOVA by glucose-tolerance group). IFG, impaired fasting glucose; IGT, impaired glucose tolerance; NGT, normal glucose tolerance; OGTT, oral-glucose-tolerance test.

TABLE 3
Incremental AUC for glucose, insulin, and C-peptide by glucose-tolerance group[1]

	Red meat		Dairy		Control	
	NGT	IFG/IGT	NGT	IFG/IGT	NGT	IFG/IGT
Glucose	6.6 ± 4.5	10.3 ± 4.9	6.4 ± 3.5	10.8 ± 5.1	7.4 ± 4.4	10.9 ± 6.9
Insulin	118.2 ± 76.9	174.4 ± 97.6	134.8 ± 103.1	183.7 ± 105.9	128.1 ± 89.0	176.8 ± 104.4
C-peptide	19.03 ± 6.08	21.3 ± 6.7	19.6 ± 6.4	21.5 ± 5.8	19.2 ± 5.5	18.6 ± 8.1

[1]All values are means ± SDs. No effect of diet or diet by group was shown for the incremental AUC (3-diet repeated measures ANOVA by glucose-tolerance group). Between-group difference: glucose, $P < 0.01$. NS for insulin or C-peptide. IFG, impaired fasting glucose; IGT, impaired glucose tolerance; NGT, normal glucose tolerance.

similarly higher with the dairy diet than with either the red meat or control diet (P-both comparisons < 0.001). Adjustment for carbohydrate did not abolish the diet effect. The dairy diet resulted in a small weight gain from the start of the diet (0.1 ± 1.2 kg), whereas red meat and control diets each resulted in a loss of 0.4 ± 1.1 kg. Men had a greater decrease in weight during red meat and control diets than did women (data not shown; P-change < 0.05); however, the weight change between diets did not have a significant effect on any of the sensitivity indexes for the group as a whole or when analyzed by group or sex. Similarly, energy intake and total and saturated fat intakes were unrelated to changes in insulin sensitivity.

DISCUSSION

Fasting insulin increased after the dairy diet with no change in fasting glucose, which resulted in a higher HOMA-IR index. Calculated insulin sensitivity from the OGTT showed reduced sensitivity after the dairy diet in women. The red meat diet had a similar insulin and glucose response to that of the control diet that contained white meat. In contrast to some epidemiologic findings, these results suggest that, in overweight and obese individuals, high consumption of dairy may reduce insulin sensitivity compared with a diet high in lean red meat. The low carbohydrate amount in our diets may have influenced the results. Hoppe et al. (16) showed similar results when 2 groups of healthy 8-y-old boys consumed either 1.5 L skim milk each day for 1 wk or 250 g lean red meat, although weight gain in the dairy group may have played a role in the decreased insulin sensitivity seen.

In contrast to the current study, a decrease in fasting insulin was observed when an adequate dairy diet was compared with a low dairy diet in overweight and obese subjects (17) over 12 wk, but there was a 1.7-kg fall in fat mass in the adequate dairy group. Fasting insulin concentrations were also significantly different between 2 groups of overweight and obese subjects (n = 121) assigned to either increase dairy to 3–5 servings/d or to continue their habitual intake of <2 servings/d over a 6-mo period (18). However, this change was due solely to increased insulin concentrations in the control group in Norway in the absence of weight change. In studies with normal-weight volunteers, high dairy consumption appeared to have no effect (19, 20), whereas for overweight and obese subjects, some studies showed an improvement in homeostasis model assessment with higher dairy intake (17, 21), and other studies reported no difference (22, 23). Our increase in dairy consumption was similar to increases in these studies. Although the amounts consumed in these studies were higher than Australian National Health and Medical Research Council dietary guidelines, which recommend 2.5 servings of dairy each day (24), typical intake in Australia is <2 servings/d (25). Our data were partly consistent with that of Chiu et al. (26) who showed, in a large study, that increasing predominantly dairy fat and dairy protein did not improve insulin sensitivity. Although no impairment of insulin sensitivity was shown, dairy branched-chain amino acids (BCAAs) were related to fasting insulin concentrations and insulin clearance.

Whey proteins in dairy foods were shown to increase serum insulin concentrations more than casein or other animal and plant proteins (27, 28). This finding may have been due to the activation of the incretin hormones glucagon-like peptide-1 and glucose-dependent insulinotropic polypeptide by BCAAs, known to enhance insulin secretion (29). Whey protein consumed before or with a carbohydrate meal was shown to substantially increase the AUC for insulin, glucagon-like peptide-1, and glucose-dependent insulinotropic polypeptide (P-all < 0.05) in people with diet-controlled T2D (30). In contrast with our study, after a chronic 12-wk parallel intervention study, Pal et al. (21) noted a decrease in fasting plasma insulin concentrations after whey supplementation compared with a glucose control. However, within the Framingham cohort, higher fasting concentrations of BCAAs along with tyrosine and phenylalanine were higher in subjects who later developed diabetes than in their matched controls (31). Other metabolomic studies also confirmed a correlation between

TABLE 4
Dietary intake from weighed food records[1]

	Red meat	Dairy	Control
Energy, kJ	8205 ± 1840[a]	9332 ± 1525[b]	7811 ± 1946[a]
Protein, g	118 ± 23 [a]	118 ± 24[a]	103 ± 20[b]
Total fat, g	74 ± 21[a]	85 ± 20[b]	69 ± 19[c]
Saturated fat, g	25 ± 9[a]	39 ± 11[b]	21 ± 9[c]
Carbohydrate, g	182 ± 55[a]	231 ± 56[b]	186 ± 70[a]
Dietary fiber, g	26 ± 9[a]	23 ± 8[b]	25 ± 10 [a,b]
Calcium, mg	485 ± 168[a]	1763 ± 303[b]	533 ± 225[c]
Kilojoules from protein, %	25 ± 4[a]	22 ± 4[b]	23 ± 5[c]
Kilojoules from fat, %	34 ± 6	34 ± 6	33 ± 7
Kilojoules from saturated fat, %	12 ± 3[a]	15 ± 4[b]	10 ± 3[c]
Kilojoules from carbohydrate, %	36 ± 6[a]	40 ± 6[b]	38 ± 8[c]
Kilojoules from fiber, %	3 ± 0.8[a]	2 ± 0.6[b]	3 ± 0.7[a]
Fat as saturated, %	37 ± 6[a]	49 ± 7[b]	34 ± 7[c]

[1]All values are means ± SDs. Values in a row that do not share a common superscript letter are significantly different, $P < 0.05$ (2-diet repeated-measures ANOVA).

raised BCAA and insulin resistance (32, 33). The leucine content is comparable between dairy and red meat (34), and in the current study, protein intake did not differ between dairy and red meat diets, and thus, leucine was unlikely to be an explanation for the differences.

Dairy fat contains ~70% saturated fat (35), and in healthy subjects, insulin sensitivity was significantly impaired with an SFA diet compared with a diet containing MUFAs (36); therefore, it is surprising that dairy is not associated with more T2D rather than less. Higher concentrations of dairy fat were inversely associated with fasting plasma glucose in an observational study, and an OGTT showed higher systemic and hepatic insulin sensitivity in high-dairy consumers (37). A trial in overweight individuals showed no effect when saturated fats were replaced with either MUFAs or carbohydrates (38), and a review in this area (39) similarly did not find an association between fat quality and insulin sensitivity. Only 3 of 12 interventions evaluated showed a negative effect of saturated fat; and thus, it is not clear that dairy saturated fat would be adverse. Food diaries indicated 4% significantly higher saturated fat intake during the dairy diet, but an analysis of serum lipids or fatty acids would be unlikely to detect this difference. Carbohydrate intake was similarly higher in the dairy diet than in either the red meat or control diets. Adjustment for carbohydrate differences in this study did not abolish the effect.

A recent meta-analysis showed that, although higher intake of yogurt was associated with reduced risk of T2D, other dairy products showed no association (11). Participants in this study could choose from a range of dairy products including yogurt and high-fat products, and the latter may have had adverse effects.

Participants were instructed to consume lean red meat and avoid processed meat, which may be why the red meat diet resulted in an insulin response that was not different from that of the control diet that contained white meat.

Glucose concentrations did not change between diets, which was consistent with the effect of increased insulin balancing the effect of increased insulin resistance. The IFG and IGT group was not more sensitive to dairy than the NGT group, which suggested that dietary recommendations should cover all individuals.

The sex difference was not expected, and the reasons were unclear but not related to glucose-tolerance group or the percentage of fat mass. Bedard et al. (40) showed that only men benefited from a Mediterranean diet, and women had an increase in insulin AUC with an OGTT. Similarly, Sumner et al. (41) showed that, relative to their fat-free mass, African American women were more insulin resistant than were men. A 14% reduction in insulin sensitivity was seen in women in this study with dairy. If maintained, this reduction would increase risk of T2D by a similar amount in the population genetically at risk, which may be one-third of the whole population, leading to perhaps a 5% increase in T2D incidence.

One of the strengths of this study was the crossover design with each participant serving as their own control. This was a free-living study and, as far as is possible to determine from the checklists and diaries provided, adherence to the protocol was good. Although the checklists indicated that compliance to the protocol was met, food diaries indicated that overall energy intake was significantly higher in the dairy diet than both red meat and control diets; however, the weight gain was minimal and unrelated to insulin sensitivity. Self-reporting of food intake is a limitation, as underreporting is a common issue across all methods of dietary accounting (42), and it was possible that participants underreported dietary intake in red meat and control diets or, perhaps, may have overestimated dairy consumption to meet the expectations of the protocol. An analysis of biomarkers of dairy consumption may also be useful to assess compliance; however, this analysis was not performed in this study.

In conclusion, in contrast to some epidemiologic findings, these results suggest that high consumption of mixed dairy reduces insulin sensitivity compared with that of a diet high in lean red meat in this population of overweight and obese individuals. Interventions with yogurt only are required.

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The authors' responsibilities were as follows—PMC: designed the research; KMT: conducted the research and had primary responsibility for the final content of the manuscript; KMT and PMC: analyzed data; and all authors: wrote the manuscript and read and approved the final manuscript. None of the authors reported a conflict of interest related to the study.

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Research Article

Dairy Consumption and Insulin Resistance: The Role of Body Fat, Physical Activity, and Energy Intake

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The relationship between dairy consumption and insulin resistance was ascertained in 272 middle-aged, nondiabetic women using a cross-sectional design. Participants kept 7-day, weighed food records to report their diets, including dairy intake. Insulin resistance was assessed using the homeostatic model assessment (HOMA). The Bod Pod was used to measure body fat percentage, and accelerometry for 7 days was used to objectively index physical activity. Regression analysis was used to determine the extent to which mean HOMA levels differed across low, moderate, and high dairy intake categories. Results showed that women in the highest quartile of dairy consumption had significantly greater log-transformed HOMA values (0.41 ± 0.53) than those in the middle-two quartiles (0.22 ± 0.55) or the lowest quartile (0.19 ± 0.58) ($F = 6.90$, $P = 0.0091$). The association remained significant after controlling for each potential confounder individually and all covariates simultaneously. Adjusting for differences in energy intake weakened the relationship most, but the association remained significant. Of the 11 potential confounders, only protein intake differed significantly across the dairy categories, with those consuming high dairy also consuming more total protein than their counterparts. Apparently, high dairy intake is a significant predictor of insulin resistance in middle-aged, nondiabetic women.

1. Introduction

Increasing rates of overweight and obesity worldwide have generated concern about a diabetes epidemic, with associated negative effects on quality of life, life expectancy, and healthcare costs. Recent data from the United States suggests that about 26 million people (8.3% of the population) are affected by diabetes [1], with more than 90% of these suffering from type 2 diabetes mellitus [2]. Danaei et al. reported that the number of individuals with diabetes worldwide has nearly doubled over the past 30 years [3]. The substantial economic and healthcare burdens placed on society by type 2 diabetes mellitus (T2DM) demonstrate a need for improved prevention efforts, particularly given its largely avertable nature.

To better control T2DM, considerable effort has been devoted to research aimed at isolating the determinants of this widespread disorder. To date, many modifiable risk factors of T2DM have been identified [4–7]. Of the various contributors, diet has become a primary focus [8, 9]. Consumption of a healthy diet, commonly characterized by sensible intakes of unsaturated fats and fiber, as well as low intakes of saturated and trans fats and foods with a high glycemic load, has been associated with a decreased risk of developing T2DM [6, 10, 11].

Several studies have also investigated the impact of milk and dairy products on the development of T2DM. Most epidemiological investigations have identified an inverse relationship between dairy consumption, as part of an overall healthy diet, and T2DM [12–14] and the metabolic syndrome [15–17]. However, conflicting results have surfaced [18–22], leaving the relationship inconclusive.

The natural disease progression of T2DM is characterized by the inability of the body to respond to consumption of a glycemic load with the appropriate amount of insulin to mediate glucose uptake [23, 24]. This is known as insulin resistance. Insulin resistance precedes T2DM and is strongly related to obesity and cardiovascular disease [25, 26].

Milk and dairy products have been identified as potent insulin secretagogues, as their consumption stimulates acute hyperinsulinemia [27–31]. The hyperinsulinemia resulting from milk and dairy consumption may be considered

a beneficial and even protective effect for regulating blood glucose levels, particularly in individuals with elevated levels or those with T2DM [32]. However, consumption of milk and dairy products and the resultant hyperinsulinemia may produce less-than-desirable long-term effects in healthy individuals, including insulin resistance. Research in humans [33] and in rats [34] suggests that regular hyperinsulinemia can lead to insulin resistance.

Prevention of T2DM is probably best achieved by avoiding the development of insulin resistance. Several modifiable risk factors of insulin resistance have been identified [35–38], among which diet plays a principal role [37, 38]. Unfortunately, the dairy and insulin resistance relationship has not been extensively investigated [39–42], and results have been contradictory.

Measurement method shortcomings are common in studies that have investigated the role of milk and dairy products on disease outcomes. To date, body weight has largely been self-reported or the body mass index (BMI) has been used to estimate body composition. Both of these strategies result in considerable measurement error and frequent misclassification [43]. In addition, questionnaires have been used almost exclusively to assess physical activity levels. Unfortunately, self-reported physical activity is known to be highly biased and contain significant error [44, 45]. Lastly, the vast majority of investigations designed to study the relationship between diet and insulin resistance or T2DM have measured diet using food frequency questionnaires or the 24-hour recall method. Research shows that subjects struggle to recall precisely what they have consumed in the past, and additional error is introduced when subjects are required to estimate portion sizes [46–48]. Energy intake is commonly underreported using these methods [48].

The present study was designed to overcome these measurement deficiencies. A high quality measurement method, air-displacement plethysmography (Bod Pod), was employed to evaluate body fat, rather than body weight. Moreover, physical activity was measured objectively using accelerometers, rather than relying on self-reported estimates of activity. Further, diet and energy intake were evaluated using 7 days of weighed food records.

In conclusion, studies designed to examine the relationship between dairy intake and insulin resistance are sparse, and few investigations have adjusted adequately for differences in body fat, physical activity, diet, energy intake, and other potential confounding factors measured using high quality methods. Research on the association between dairy intake and insulin resistance, using high quality measurement methods, is clearly warranted.

2. Methods

2.1. Design. The relationship between dairy intake and insulin resistance in a sample of 272 middle-aged women was studied using a cross-sectional design. In addition, determining the extent to which age, weight, body fat percentage, total energy intake, physical activity level, education, grams consumed of carbohydrate, protein, and fat, and insoluble and soluble fiber intake influenced the relationship between dairy consumption and insulin resistance was an ancillary purpose of the investigation.

2.2. Participants. Potential subjects were recruited by word-of-mouth and through newspaper advertisements and e-mails circulated to individuals and companies in approximately 20 cities in the Mountain West, United States. Initial inclusion screening was conducted via telephone and focused on recruiting individuals who were female, premenopausal, not pregnant, nondiabetic, nonsmokers, and apparently healthy. Informed consent was obtained from each subject prior to study commencement and was approved by the University's Institutional Review Board.

2.3. Procedures. Subject information and measurements were gathered at the university. Measurements and training instructions lasted 60 to 90 minutes. Height and weight were measured for each participant during the initial appointment while wearing a one-piece, lab-issued swimsuit. While wearing the same swimsuit and a swim cap, a Bod Pod test (Life Measurements Instruments, Concord, CA) was performed on each subject to estimate body fat percentage. Subjects were taught how to accurately measure food intake using a digital food scale (Ohaus 2000, Florham Park, NJ) and were instructed to keep a seven-day weighed food record. A nine-page diet log, including specific directions for recording dietary intake, a sample page, and blank records for each day of the week, was given to each subject. Each subject was asked to read the instructions. Common recording mistakes were explained to subjects to improve detail and compliance. Next, each subject was given written and verbal instructions regarding the proper way to weigh food with the Ohaus 2000 portable electronic scale using plastic food models.

Each subject was issued an Actigraph accelerometer (Health One Technology, formerly CSA, Pensacola, FL), which they were instructed to wear continuously over the left hip for seven consecutive days, with the exception of bathing or water events. Participants were encouraged to maintain their normal lifestyle and to avoid implementing new dietary or exercise practices. Explanations of proper techniques were provided to all participants so that they understood correct procedures.

During the 7-day period, participants were contacted by study personnel by telephone to ensure that they were accurately recording everything consumed and that they were maintaining a typical diet and physical activity levels and to answer questions. Participants were given a blood requisition form, which they took to a local hospital during the seven days, following a 12-hour fast, to have their blood drawn by lab phlebotomists. At the end of the 7 days, subjects returned the food record, food scale, and accelerometer. Subjects were weighed again wearing a one-piece lab-issued swimsuit. An average of the two body weight measures allowed body weight to be indexed based on the average of two measures taken a week apart, rather than one assessment. Once it was determined that the data obtained was accurate and

complete, subjects were mailed a thank you letter with a $25 gift certificate.

2.4. Instrumentation and Measurements. The criterion variable for this study was insulin resistance, assessed using the homeostatic model assessment (HOMA). The primary predictor variable was servings of dairy foods, which were measured using 7-day weighed food records. Partial correlation was used to determine the extent to which potential confounding variables, namely, age, education, total energy intake, multiple dietary variables, objectively measured physical activity level, and body fat percentage, affected the dairy consumption and insulin resistance relationship.

Insulin Resistance. Lab phlebotomists withdrew a blood sample from the antecubital vein after the subjects had fasted for at least 12 hours. Drinking water during the 12 hours was allowed. The samples were stored at about −20°C after being centrifuged for 15 minutes at 2000 g at 4°C. The Access Ultrasensitive Insulin assay (Beckman Coulter, Brea, CA) was used to determine fasting insulin (μU/L). Dimension Vista System and Flex reagent cartridges (Siemens, Deerfield, IL) were used to measure fasting glucose levels (mg/dL). HOMA [49] was used to assess insulin resistance using the following formula: fasting plasma insulin (μU/mL) × fasting plasma glucose (mg/dL)/405. HOMA has been shown to be comparable to the euglycemic clamp as a means of assessing insulin resistance ($r = 0.82$, $P = 0.0002$) and is considered valid and reliable [49, 50].

Dietary Intake. Seven-day weighed food records were used to measure dairy consumption, total energy intake, carbohydrate, protein, and fat consumption, and insoluble and soluble fiber intake. This method minimizes subject recall bias and effectively represents an individual's normal dietary patterns by covering weekdays and weekends [47, 48]. Weighed food records have frequently been employed as a standard for comparison when assessing the validity of other dietary intake measurements [51, 52], and seven days has been shown to be an appropriate length of time to accurately assess intake [47, 48].

Subjects were issued a digital food scale (Ohaus 2000, Florham Park, NJ) and were instructed how to properly weigh and record foods and beverages using plastic food models and verbal directions. Printed instructions were also given to each participant. Subjects were taught about the importance of maintaining a typical diet over the week of recording. To help combat the tendency to underreport food consumption [48], participants were informed that they would be weighed before and after the week of recording and were asked to not gain or lose weight during the week. If a participant's food record indicated her daily intake was less than 130% of her estimated resting metabolic rate, determined using the Ravussin formula [53], she was required to repeat the weighed food record. Completed food records were returned following the 7-day recording period and were examined for accuracy. A registered dietician input all food records into the ESHA Research software (ESHA Research Inc., Salem, OR, USA) for further analysis.

Dietary analysis categorized dairy intake based on the American Dietetic Association (now Academy of Nutrition and Dietetics) and American Diabetes Association (ADA) Exchange Lists program. In the Exchange Lists program, a fat-free/low-fat serving of dairy is defined as 12 g of carbohydrate, 8 g of protein, and 0–3 g of fat. In the present study, typical fat-free/low-fat dairy foods included fat-free milk, 1/2% milk, 1% milk, low-fat buttermilk, evaporated fat-free milk, low fat soy milk, and fat-free yogurt, including those with artificial sweeteners. A serving of reduced-fat dairy foods included those with 12 g carbohydrate, 8 g of protein, and 5 g of fat. Specific foods included 2% milk, soy milk, sweet acidophilus milk, and plain low-fat yogurt. Similarly, one serving of whole dairy was defined as 12 g of carbohydrate, 8 g protein, and 8 g of fat. In the present study, specific foods included whole milk, evaporated whole milk, goat's milk, kefir, and yogurt made with whole milk. The American Dietetic and American Diabetes Associations (ADA) Exchange Lists do not include cheese in the dairy category because the macronutrient composition of cheese differs significantly from other dairy foods, as defined above. Differences in fat across the dairy categories, fat free/low-fat, reduced fat, and whole dairy, were controlled in the present study, so that each dairy serving had the same energy content, as used in other studies [54, 55]. Partial servings were calculated to within 0.1 servings.

Physical Activity. Physical activity was assessed using Actigraph accelerometers, model 7164 (Health One Technology, Fort Walton Beach, FL, USA). Accelerometers are superior to self-reported physical activity, which is known to be biased and contain significant error [44, 45]. Many investigations have been conducted to validate the Actigraph in adults, showing a close representation between the physical activity levels of free living subjects and doubly labeled water and portable metabolic systems [56–58].

A pilot study testing 15 women from the present investigation evaluated the reliability of the accelerometers as they took part in seventeen different activities, such as walking, jogging, and stair climbing at different speeds and grades. The same assessments were performed one week following the baseline tests. The test-retest intraclass reliability for each activity was greater than 0.90 and was greater than 0.98 for the sum of the seventeen activities.

Physical activity was measured objectively for 7 consecutive days using the accelerometer. Other than during bathing or water activities, the accelerometer was worn constantly throughout the day and night. The accelerometer was worn over the left hip, attached to a nylon belt that was worn around the waist. Following the testing period, participants returned the accelerometers and investigators downloaded their activity data and checked for accuracy. Any participant who failed to wear the accelerometer for at least 12 hours during waking hours was required to re-wear it for the corresponding day(s) of the week as the noncompliant day(s). Final data included 7 days of valid wear time for every subject. Average wear time from 7 A.M. to 10 P.M., a 15-hour period, was 13.9 hours (93% wear-time compliance) across the 7 consecutive days.

In the present study, total physical activity was indexed using the sum of all the activity counts acquired over the seven days of assessment. Concurrent validity for this measure has been shown by several investigations [59–62].

Body Fat Percentage. Air-displacement plethysmography (Bod Pod) was used to estimate body fat percentage (Life Measurements Instruments, Concord, CA). The Bod Pod was also used to assess thoracic lung volume, which was subtracted from body volume. Subjects were instructed to fast and avoid exercise for at least three hours before their appointment, according to standard protocol. They were given a lab-issued swimsuit and a swim cap to complete the test in and were asked to void, if possible, before the assessment. Body composition was measured in the Bod Pod at least twice. If the body fat percentage results differed by more than one percentage point, then another measurement was taken. This process was repeated until two results were within one percentage point, and then the average of these two outcomes was used to index body fat percentage.

The Bod Pod has been shown to be valid and reliable in estimating body fat percentage. Maddalozzo et al. [63] demonstrated concurrent validity for the Bod Pod compared to dual energy X-ray absorptiometry. Concurrent validity of the body fat percentage measure resulting from the Bod Pod and dual energy X-ray absorptiometry was also established with a sample of 100 women from the current study, with an intraclass correlation of 0.97 ($P < 0.0001$) [64]. In addition, test-retest using the Bod Pod and the same sample of 100 women resulted in an intraclass correlation of 0.99 ($P < 0.0001$) [65]. Estimating body fat percentage with the Bod Pod is a much more valid strategy than using BMI, as BMI often produces misclassification of overweight and obesity [43].

2.5. Statistical Analysis. Statistical analysis was conducted using the SAS software program, version 9.3 (Cary, NC). Because HOMA values deviated from a normal distribution, they were log-transformed. To simplify reading and to avoid redundancy, log-transformed HOMA values are referred to as HOMA throughout the paper. Bivariate associations were determined using Pearson correlations. The extent to which mean HOMA levels differed across categories of dairy intake was determined using regression analysis and the General Linear Model (GLM) procedure. For these computations, dairy intake was divided into quartiles and the middle-two quartiles were combined forming three categories: low (0 to 0.5 servings of dairy per day), moderate (0.6 to 1.5 servings of dairy per day), and high (1.6 to 6 servings of dairy per day). Dairy intake was also calculated as servings per 4184 kJ (1000 kcal), forming the following categories: low (0 to 0.23 servings per 4184 kJ (1000 kcal)), moderate (0.24 to 0.79 servings per 4184 kJ (1000 kcal)), and high (0.80 to 3.1 servings per 4184 kJ (1000 kcal)). To examine the influence of specific potential confounders, such as age, education, body weight, energy intake, diet, body fat percentage, and physical activity, considered individually and collectively, on the relationship between dairy consumption and HOMA, partial correlations were computed using the GLM procedure. Adjusted means were calculated using the least-squares means procedure.

A power analysis was conducted using the PASS 6.0 statistical software (NCSS, Kaysville, UT, USA) to determine the number of participants needed to achieve 0.80 power with alpha set at 0.05 when evaluating mean differences across three categories (low, moderate, and high) using ANOVA to detect a small effect size of 0.20. Results showed that 240 subjects would be sufficient. Overall, with more than 270 participants, the study had excellent power.

3. Results

A total of 272 women participated in the present investigation. Subjects were primarily Caucasian (~90%), middle-aged (40.1 ± 3.0 years), working either full- or part-time (58%), and married (82%), and about 32% had at least some college education. Table 1 shows additional descriptive characteristics for the study participants, including total physical activity, body fat percentage, weight, fasting glucose, fasting insulin, total energy intake, percent of energy from carbohydrate, protein, and fat, insoluble and soluble fiber intakes per 4184 kJ (1,000 kilo-calories), average servings of dairy consumed per day, HOMA, and log-transformed HOMA. Means, standard deviations, minimum and maximum values, and quartiles are also displayed in Table 1. Average dairy intake for these women was 1.1 ± 1.0 servings per day. Women with low consumption (bottom quartile) averaged 0.2 ± 0.2 servings of dairy per day, while the moderate category had 1.0 ± 0.4 serving per day, and the high dairy participants (top quartile) had 2.4 ± 0.9 servings per day. Mean servings per 4184 kJ (1000 kcal) was 0.6 ± 0.5. Average HOMA was 1.5 ± 1.0 and mean log-transformed HOMA was 0.3 ± 0.6.

Table 2 shows mean differences in the various potential confounding variables across the three dairy consumption categories, low, moderate, and high, including age, body weight, body fat percentage, energy intake, objectively measured physical activity, carbohydrate and fat intake, and fiber consumption, insoluble and soluble. None of these measures differed across the dairy intake categories. However, grams of protein intake per day differed significantly across the dairy categories. Specifically, women with high dairy intake had higher protein intake than those in the moderate or low dairy consumption categories ($F = 7.57$, $P = 0.0006$).

Table 3 displays mean differences in HOMA across the three dairy consumption categories, without and with adjustment for the potential confounders. As shown, when no variables were controlled, significant differences in mean HOMA were seen across the three dairy consumption categories ($F = 6.90$, $P = 0.0091$). Those in the high dairy consumption category had significantly higher HOMA levels (0.41 ± 0.53) than those in the moderate (0.22 ± 0.55) or low consumption categories (0.19 ± 0.58). Differences in the potential confounding factors, including age, weight, body fat percentage, energy intake, total physical activity, education, carbohydrate, protein, and fat consumption, insoluble fiber intake, and soluble fiber intake, considered individually or

Table 1: Descriptive statistics ($n = 272$).

Variables	Mean	SD	Min	Percentile			Max
				25th	50th	75th	
Weight (kg)	66.1	10.0	42.1	58.9	65.2	72.0	95.5
Age (years)	40.1	3.0	34.0	38.0	40.0	43.0	46.0
Activity (counts/week)*	2700.1	781.9	827.8	2103.9	2669.6	3166.6	4945.9
Body fat (%)	31.7	6.9	14.6	27.2	32.2	36.8	44.8
Fasting glucose (mg/dL)	86.7	5.9	73.0	82.0	87.0	90.0	111.0
Fasting insulin (μU/mL)	7.0	4.2	1.2	4.3	6.1	8.3	34.8
Energy intake (kJ/day)	8585.1	1335.0	6293.7	7624.0	8386.4	9332.0	14623.0
Energy intake (kcal/day)	2051.9	319.1	1504.0	1822.1	2004.4	2230.4	3495.1
Carbohydrate (% of total kJ)	55.7	6.2	25.4	51.7	56.0	59.4	73.3
Protein (% of total kJ)	13.8	2.5	8.5	12.3	13.5	15.1	25.5
Fat (% of total kJ)	30.5	5.8	11.6	27.1	30.3	34.5	51.6
Insoluble fiber (g/4184 kJ)†	3.8	1.9	0.5	2.5	3.4	4.7	12.6
Soluble fiber (g/4184 kJ)†	1.7	0.9	0.2	1.1	1.6	2.0	6.3
Dairy intake (serv./day)	1.1	1.0	0.0	0.5	1.0	1.6	6.0
Dairy intake (serv./4184 kJ)	0.6	0.5	0.0	0.2	0.5	0.8	3.1
HOMA‡	1.5	1.0	0.2	0.9	1.3	1.8	8.3
HOMA (log-transformed)	0.3	0.6	−1.5	−0.1	0.3	0.6	2.1

* Average activity counts for 1 week objectively measured using accelerometers, divided by 1000.
† Fiber intake is expressed as g per 4184 kJ (1000 kcal).
‡ HOMA, homeostasis model assessment of insulin resistance.

Table 2: Mean differences in the potential confounders across the dairy intake categories.

HOMA	Dairy consumption categories						F	P
	Low consumption $n = 68$		Moderate consumption $n = 136$		High consumption $n = 68$			
	Mean	SD	Mean	SD	Mean	SD		
Age (years)	39.8	3.2	40.0	2.9	40.7	3.0	1.94	0.1455
Weight (kg)	65.8	11.1	66.8	9.3	64.9	10.2	0.83	0.4352
Body fat (%)	31.8	7.0	31.6	7.0	31.8	6.8	0.04	0.9568
Energy intake (kJ/day)	482.4	85.0	493.7	69.0	491.9	80.2	0.52	0.5958
Physical activity (counts)*	275.2	79.2	266.8	80.8	271.3	72.4	0.28	0.7590
Carbohydrate intake (g)	278.2	65.5	293.6	48.1	295.7	51.4	2.32	0.1000
Protein intake (g)	68.0[a]	16.9	70.8[a]	13.9	77.5[b]	14.4	7.57	0.0006
Fat intake (g)	73.7	17.8	71.5	17.3	66.9	19.9	2.53	0.0814
Insoluble fiber (g/4184 kJ)‡	3.8	2.3	3.6	1.6	4.1	2.0	1.90	0.1512
Soluble fiber (g/4184 kJ)‡	1.7	0.9	1.6	0.9	1.7	0.8	0.22	0.8040

* Activity counts were divided by 10,000 to make the values more manageable. An activity count of 275.2 means that the group had 2.752 million activity counts for the week.
‡ Fiber intake is expressed as grams per 4184 kJ (g per 1000 kcal), as is dairy consumption.
None of the results were statistically significant except protein intake. Means on the same row with different superscript letters were significantly different ($P < 0.05$).
Low consumption included women with dairy intake at or below the 25th percentile. Moderate consumption included those whose dairy intake was between the 25th and 75th percentiles. High consumption included those with dairy intake at or above the 75th percentile. Mean dairy consumption for the low, moderate, and high consumption categories were 0.2 ± 0.2, 1.0 ± 0.4, and 2.4 ± 0.9 servings per day, respectively.
Because "education" was a categorical variable, the relationship between dairy intake and education was analyzed using Chi-square. The results showed no association between the two variables ($P = 0.4524$).

collectively, failed to influence appreciably the relationship between dairy consumption and HOMA (Table 3).

Specifically, the relationship was attenuated slightly after controlling for age ($F = 6.77$, $P = 0.0098$), education ($F = 6.48$, $P = 0.0114$), and percent of calories from protein ($F = 5.87$, $P = 0.0160$), yet it remained statistically significant. Adjusting for differences in energy intake weakened the relationship by 32% ($F = 4.68$, $P = 0.0315$). Controlling for several other variables strengthened the relationship, including body weight ($F = 9.18$, $P = 0.0027$), body fat

Table 3: Mean differences in HOMA by the dairy intake categories, without and with adjustment for the potential confounders.

HOMA*	Dairy consumption categories						F	P
	Low consumption $n = 68$		Moderate consumption $n = 136$		High consumption $n = 68$			
	Mean	SD	Mean	SD	Mean	SD		
Variable controlled								
None	0.19[a]	0.58	0.22[a]	0.55	0.41[b]	0.53	6.90	0.0091
Age (years)	0.19[a]		0.22[a]		0.41[b]		6.77	0.0098
Weight (kg)	0.21[a]		0.21[a]		0.43[b]		9.18	0.0027
Body fat (%)	0.18[a]		0.23[a]		0.40[b]		7.67	0.0060
Energy intake (kJ/day)	0.22[a†]		0.22[a]		0.39[b]		4.68	0.0315
Total activity (counts/week)	0.19[a]		0.22[a]		0.42[b]		7.47	0.0067
Education	0.18[a]		0.21[a]		0.40[b]		6.48	0.0114
Carbohydrate (% of kJ)	0.17[a]		0.23[a]		0.42[b]		7.84	0.0055
Protein intake (% of kJ)	0.19[a]		0.23[a]		0.41[b]		5.87	0.0160
Fat intake (% of kJ)	0.17[a]		0.23[a]		0.43[b]		8.40	0.0041
Insoluble fiber (g/4184 kJ)	0.19[a]		0.22[a]		0.42[b]		7.45	0.0068
Soluble fiber (g/4184 kJ)	0.19[a]		0.22[a]		0.42[b]		7.69	0.0059
All covariates	0.17[a†]		0.19[a]		0.34[b]		4.71	0.0309

*HOMA values were log-transformed.

†Statistically significant at the trend level ($0.05 < P < 0.10$).

Means on the same row with the same superscript letter are not significantly different ($P > 0.05$).

Low consumption included women with dairy intake at or below the 25th percentile. Moderate consumption included dairy intake between the 25th and 75th percentiles. High consumption included dairy intake at or above the 75th percentile. Mean dairy consumption (servings per 4184 kJ) for the low, moderate, and high consumption categories were 0.1 ± 0.1, 0.5 ± 0.2, and 1.2 ± 0.4 servings per day, respectively.

percentage ($F = 7.67$, $P = 0.0060$), total physical activity ($F = 7.47$, $P = 0.0067$), percent of calories from fat ($F = 8.40$, $P = 0.0041$), intake of insoluble fiber ($F = 7.45$, $P = 0.0068$), and intake of soluble fiber ($F = 7.69$, $P = 0.0059$). After controlling for all of the potential confounding factors simultaneously, the dairy and HOMA relationship was weakened, but remained significant ($F = 4.71$, $P = 0.0309$) (Table 2).

With dairy consumption expressed as servings per 4184 kJ (1000 kcal), results were generally weaker, but all models remained statistically significant. With no variables controlled ($F = 5.30$, $P = 0.0220$), women with high dairy intake per 4184 kJ (1000 kcal) had significantly higher HOMA levels (0.39 ± 0.54) than those in the moderate (0.23 ± 0.56) or low consumption categories (0.19±0.55). Likewise, after adjusting statistically for differences in all of the potential confounders ($F = 5.30$, $P = 0.0223$), women with high dairy intake had significantly higher HOMA levels (0.35 ± 0.54) than women in the moderate (0.20 ± 0.56) or low categories (0.16 ± 0.55), calculated as servings per 4184 (1000 kcal).

The Pearson associations between HOMA and the potential confounders including age ($r = 0.02$, $P = 0.7515$), physical activity ($r = -0.09$, $P = 0.1201$), percent of total calories from carbohydrate ($r = -0.09$, $P = 0.1562$), protein ($r = 0.07$, $P = 0.2367$), and fat ($r = 0.06$, $P = 0.3238$), and insoluble fiber intake ($r = -0.06$, $P = 0.3045$) were not statistically significant. However, there were significant bivariate relationships between HOMA and body fat percentage ($r = 0.47$, $P < 0.0001$), body weight ($r = 0.39$, $P < 0.0001$), fasting plasma glucose ($r = 0.45$, $P < 0.0001$), fasting plasma insulin ($r = 0.91$, $P < 0.0001$), total energy intake ($r = 0.23$, $P < 0.0001$), and soluble fiber intake ($r = -0.17$, $P = 0.0040$).

4. Discussion

In the present study, there was a significant and meaningful relationship between dairy consumption, assessed using 7-day weighed diet records, and insulin resistance, measured using HOMA. Specifically, women with high dairy intake (top 25%) had significantly greater insulin resistance than women with moderate or low dairy intake (Table 3). The difference between the upper and lower quartiles produced an effect size of 0.40. The association remained significant after controlling statistically for several potential confounding variables, including age, body weight, body fat percentage, energy intake, total physical activity, education, percent of energy from carbohydrate, protein or fat, insoluble fiber intake, and soluble fiber consumption, considered individually or collectively. Adjusting for differences in energy intake had the strongest effect on the association, but it remained significant. The association also remained statistically significant, with and without control of the potential confounding variables, when dairy consumption was expressed as servings per 4184 kJ (1000 kcal).

Although a very different sample, the present findings are in line with an intervention by Hoppe et al. [40] who studied 24 eight-year-old boys in 2005. HOMA increased significantly after one week in those given a dairy supplement,

but did not change in those given a meat supplement. The researchers did not state that subjects were randomly assigned to groups. The groups seemed to differ on factors other than the milk and meat intervention. It does not appear that energy intake, body composition, or physical activity levels were controlled.

Also in agreement with the present study were findings from Snijder et al. [66] who found that higher dairy consumption was significantly associated with higher fasting glucose levels in a sample from the Netherlands, where dairy consumption is generally high. In addition, Lawlor et al. [67] examined the relationship between milk consumption and insulin resistance and the metabolic syndrome in 4,024 British women. It was observed that women who never drank milk had lower HOMA levels, were less likely to have T2DM, and were less likely to manifest the metabolic syndrome than women who drank milk regularly. Milk consumption was measured nominally (yes milk intake versus no milk intake), thus preventing the determination of a dose-response relationship.

Contradicting the present results, Rideout et al. [42] observed that HOMA levels improved in overweight or obese subjects with higher dairy consumption (4 servings per day of milk or yogurt) compared to lower dairy intake (fewer than 2 servings per day of milk or yogurt) over the course of 12 months in a small crossover trial using 23 adults. Participants were free-living and without energy restriction. Body fat was assessed using dual energy X-ray absorptiometry.

Akter et al. [41] found results conflicting with the present study in a cross-sectional investigation of 496 Japanese adults, where higher intake of full-fat milk or yogurt was associated with lower HOMA. Body composition was indexed and controlled using BMI, physical activity was assessed using a self-reported questionnaire, and dietary intake was assessed using a food frequency questionnaire. Akter et al. [41] point out that this population, in general, consumes considerably less dairy than Western populations, and that only a small percentage regularly consume low-fat or fat-free dairy.

Each of the potential confounding variables influenced the relationship between dairy intake and insulin resistance, some more than others. Adjusting for differences in total daily energy intake weakened the relationship by 32%, more than any other variable. Post hoc analyses showed that energy intake was significantly and positively related to dairy intake ($r = 0.21$, $P = 0.0006$), physical activity ($r = 0.16$, $P = 0.0101$), body weight ($r = 0.40$, $P < 0.0001$), and HOMA ($r = 0.23$, $P < 0.0001$). Thus, women with higher energy intakes were more likely to have higher consumption of dairy products, participate in greater amounts of physical activity, have higher body weights, and be more insulin resistant than women with lower energy intakes.

Adjusting for differences in body fat also weakened the dairy—insulin resistance association. Specifically, if all the women of the present study would have had the same level of body fat, the relationship between dairy and insulin resistance would have been 13% weaker (Table 2). In general, investigations of the relationship between dairy consumption and insulin resistance have largely relied on BMI to index body composition and obesity. Unfortunately, this method tends to produce significant error [43]. Obesity is a strong contributor to insulin resistance [68], even in nondiabetic populations [69]. Consequently, the present study assessed body composition to estimate and control for differences in body fat, rather than using BMI.

Physical activity is another important variable that strongly influences insulin sensitivity and therefore was controlled in the present investigation. It is widely accepted that participation in physical activity reduces risk of insulin resistance and T2DM [70, 71]. Both chronic physical activity and single bouts of exercise have been shown to improve insulin sensitivity [72]. To date, no investigation of the relationship between dairy intake and insulin resistance has measured physical activity objectively and controlled for differences among participants. Questionnaires are typically administered to gather physical activity information. However, subject responses to activity questionnaires are often highly skewed [44, 45]. Consequently, the present study employed accelerometry over a period of seven days to objectively assess each subject's engagement in physical activity. Adjusting for differences in physical activity had little influence on the dairy—insulin resistance relationship of the present study, strengthening it by only 7%, however.

Other variables that strengthened the relationship between dairy consumption and HOMA were body weight (strengthened by 12%), percent of energy from carbohydrate (strengthened by 13%), percent of energy derived from dietary fat (strengthened by 21%), insoluble fiber intake (strengthened by 8%), and soluble fiber intake (strengthened by 8%).

Consistent with the present findings, it has been shown in the literature that dietary fiber intake is associated with improved insulin sensitivity [73–75], particularly higher intake of soluble fiber [74]. There was a significant positive relationship between soluble fiber intake and physical activity ($r = 0.15$, $P = 0.0114$), but soluble fiber was negatively associated with HOMA ($r = -0.17$, $P = 0.0040$). These relationships may partly explain why controlling for soluble fiber intake strengthened the association between dairy consumption and insulin resistance. Namely, women who ate more soluble fiber also participated in greater amounts of physical activity and had lower HOMA levels.

Diets categorized by consistently high glycemic loads tend to predict insulin resistance and subsequent T2DM in both men [76] and women [10], since chronically high insulin requirements to mediate glucose uptake can lead to reduced insulin sensitivity over time. Therefore, consumption of diets with a low glycemic index is recommended to prevent T2DM. Dairy is considered to have a relatively low glycemic index [77], inferring that it may not adversely affect insulin requirements. However, the insulinemic index has been shown to be three to six times higher than expected based on the glycemic index of dairy foods [28, 30], suggesting that there is an insulinotropic component in milk products [28–31]. Thus, while it has been established that chronic hyperglycemia can lead to insulin resistance [78], research indicates that chronic hyperinsulinemia may also lead to reduced insulin sensitivity [33, 34].

High intake of animal protein has been linked to increased risk of T2DM [79]. Elevated levels of amino acids interfere with normal glucose metabolism, particularly in individuals with reduced insulin sensitivity, leading to insulin resistance [79–81]. High consumption of dairy protein could exacerbate insulin resistance. As shown in Table 2, women with high dairy consumption had significantly higher total intakes of protein, which could help explain why those with high dairy intake had the highest level of insulin resistance in the present study.

Beta cell function should be taken into account when discussing insulin secretion. Persistent consumption of foods categorized by either a high glycemic index or a high insulinemic index causes beta cells to release more insulin to initiate glucose uptake into body cells, leading to insulin insensitivity [10, 76]. According to Leahy et al. [78] and Polonsky et al. [82], this could lead to reduced insulin sensitivity and eventual T2DM, as pancreatic beta cells hypersecrete insulin to maintain normal blood glucose levels, leading to beta cell failure, a key feature of T2DM [78, 82].

The hyperinsulinemic response associated with dairy consumption [27–31] may be considered a beneficial and even protective effect for regulating blood glucose levels, particularly in individuals with T2DM [32]. However, this does not mean that the effects of chronic milk and dairy intake on insulin levels in healthy individuals necessarily follow a similar pattern. Similarly, perhaps the short-term benefits of milk and dairy consumption for blood glucose regulation produce adverse long-term effects, including reduced insulin sensitivity.

As is the case with all cross-sectional research, reverse causality is a potential threat. Although the strong relationship between dairy intake and insulin resistance may be a result of dairy consumption causing hyperinsulinemia, leading to insulin resistance over time, it is also possible that women with elevated blood glucose levels chose to consume more dairy, possibly to help control their unhealthy blood glucose levels. Moreover, other factors, "third variables," could be responsible for the relationship between dairy and insulin resistance. However, because about a dozen possible confounding variables were controlled in the present study, the link between dairy and insulin resistance is not likely a function of one of these factors, but other variables not controlled in the present study could account for the results.

An important strength of the present study was its use of high quality, objective measurement methods to control for several potential confounding variables. The Bod Pod was used to estimate body composition rather than BMI. Accelerometry was used to assess physical activity instead of a questionnaire, and 7-day weighed food records were used to quantify dietary intake instead of a questionnaire, thereby reducing the problems associated with diet recall and estimation of serving sizes.

The present investigation was not without weaknesses, however. It was limited by its cross-sectional design, thus preventing the establishment of a cause-and-effect relationship. Also, the focus of the study was on nondiabetic, middle-aged, nonsmoking women, and the sample was largely homogeneous, predominately Caucasian women. Overweight and obesity were not common in the present sample. Hence, generalization of the findings may be limited to populations with similar characteristics.

5. Conclusion

The present study uncovered a significant relationship between dairy consumption and reduced insulin sensitivity in middle-aged, nondiabetic women, suggesting that higher intakes of dairy products may be associated with greater insulin resistance. This relationship was partly explained by differences in body composition, body weight, physical activity, dietary fiber intake, and energy consumption, particularly the latter. However, high dairy consumption remained a significant predictor of insulin resistance after adjusting for all covariates. If a causal relationship was assumed, then high dairy intake may lead to reduced insulin sensitivity over time. Clearly, more research focusing on the relationship between dairy intake and insulin resistance is needed before changes in dairy consumption can be recommended for the prevention of insulin resistance in nondiabetic women.

Conflict of Interests

The authors declare that there is no conflict of interests regarding publication of this paper.

Authors' Contribution

Tucker designed the study, organized and supervised data collection, and analyzed the data; Bruce Bailey and James LeCheminant assisted with data collection; Andrea Erickson and Larry Tucker wrote the paper with assistance from Bailey and LeCheminant. All authors read and approved the final paper.

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Review

Food-Derived Opioid Peptides in Human Health: A Review

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Abstract: World Health Organization data suggest that stress, depression, and anxiety have a noticeable prevalence and are becoming some of the most common causes of disability in the Western world. Stress-related disorders are considered to be a challenge for the healthcare system with their great economic and social impact. The knowledge on these conditions is not very clear among many people, as a high proportion of patients do not respond to the currently available medications for targeting the monoaminergic system. In addition, the use of clinical drugs is also associated with various side effects such as vomiting, dizziness, sedation, nausea, constipation, and many more, which prevents their effective use. Therefore, opioid peptides derived from food sources are becoming one of the safe and natural alternatives because of their production from natural sources such as animals and plant proteins. The requirement for screening and considering dietary proteins as a source of bioactive peptides is highlighted to understand their potential roles in stress-related disorders as a part of a diet or as a drug complementing therapeutic prescription. In this review, we discussed current knowledge on opioid endogenous and exogenous peptides concentrating on their production, purification, and related studies. To fully understand their potential in stress-related conditions, either as a drug or as a therapeutic part of a diet prescription, the need to screen more dietary proteins as a source of novel opioid peptides is emphasized.

Keywords: bioactive peptides; endogenous and exogenous opioid peptides; stress; human health

1. Introduction

Unhealthy lifestyle and the consumption of an unhealthy diet have been major causes of non-communicable diseases (NCD) in recent years. According to a World Health Organization report, 350 million people suffer from depression, in which 15–25% are Western-type communities [1]. Along with depression, anxiety, stress, cardiovascular diseases, and high blood pressure are becoming part of the compelling health problems worldwide [2]. For several years, sleep apnea, stress, and anxiety responses are mutual and related to each other. Stress is a response by the body to restore homeostatic balance, and these responses may cause damage or may lead to disease conditions. Several diseases arise as a consequence of stress having co-morbidity with sleep disorders [3], In particular, prolonged or persistent stress contributes to elevated hormones such as cortisol, the "stress hormone", and decreased serotonin and other neurotransmitters in the brain, including dopamine, which have been correlated with depression. As these chemical systems function properly, they control biological processes such as sleep, appetite, energy, and sex drive, and they enable normal moods and emotions to be expressed. However, if the stress response fails to shut down and reset after a stressful situation has passed, it may lead to depression in susceptible individuals.

Sleep problems lead to issues such as reduction of appetite, a decrease of attention, unstable mood, and fatigue. According to statistics from the Mental Health Services administration (USA), 12 months of major depressive disorder in 2017 was around 13.3% for adolescents and 7.1% for adults [4,5]. It was reported that anxiety and depression disorders are more common in women as compared to men with an approximate 2:1 ratio during women's reproductive years [4,5]. Furthermore, according to a worldwide survey, 45.7% of individuals with long-term major depressive disorders had a lifetime history of one or more than one anxiety disorder [6]. The importance of understanding stress disorders for health especially considering youth should be overemphasized [7].

Food proteins have long been used as good sources of potent bioactive peptides for preventing, managing, and treating human health [8,9]. Food proteins are biomolecules that are involved in different biological functions for improving human health, and bioactive peptides are encrypted in the sequences of proteins and are released at the time of digestion and play important roles in improving health [10,11]. They may have two or several proteinogenic amino acid groups linked with each other by peptide bonds and are released from their native proteins when fermented or treated with enzymes [12–14].

Currently available clinical treatments for stress-related disorders are also associated with various side effects such as vomiting, dizziness, sedation, nausea, and constipation. Therefore, nutraceuticals are becoming a promising target, as they are derived from food naturally and are known as food with medicinal benefits. These foods not only contain basic nutrients but are also rich in probiotics, antioxidants, polyphenols, bioactive compounds, and beneficial fatty acids and are known as functional foods. Their promising therapies and regulatory functions with active compounds may become a powerful tool against synthetic drugs [15], but a lot of studies are still needed to understand their roles. Hence, promoting healthy dietary intake has become a central part of the world health organization's plan of action to prevent and monitor NCDs [8].

Opioid Peptides

Opioids were first identified in 1975 and consist of small molecules of 5–80 amino acids [16]. They diffuse locally and act on another adjacent neuron in much lower concentration as compared with neurotransmitters and give a more prolonged response [17]. Opioid peptides bind with opioid receptors μ-, κ-, and δ- for activation [18]. They are known for a wide range of functions such as neuromodulation, sleepiness [19], and pain modulation [20,21]. Except for casexins and lactoferroxins, which are opioid antagonists, the majority of peptide ligands are opioid agonists [22,23]. Opioid peptides can be categorized into endogenous or exogenous peptides based on their origin: endogenous opioid peptides that are produced by the body itself and exogenous opioid peptides that are produced after the ingestion of food when food proteins are digested by body enzymes [24–26]. Meanwhile, the number of studies on peptides related to stress is also increasing exponentially, which supports our interest in studying bioactive peptides and their roles in human health (Figure 1). Additionally, several attempts in human, as well as translational animal studies, have confirmed the potential of opioids in stress and related condition. The exponential increase in the search for bioactive peptides from food is also due to their low safety concerns and slow clearance from body tissues compared to synthetic drugs [27].

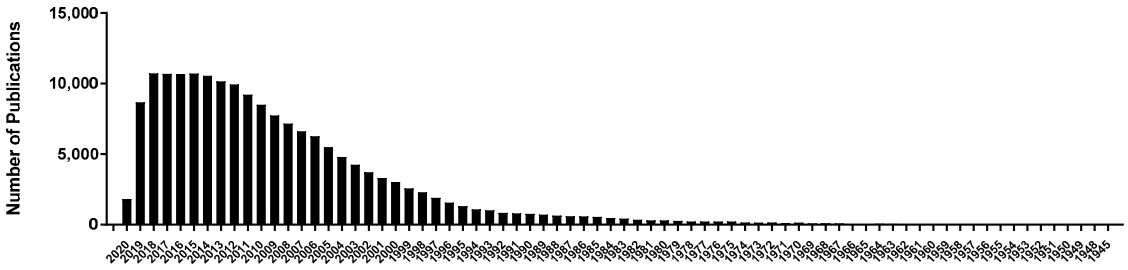

Figure 1. The number of publications on stress-related peptides by year available on PubMed Central as of 30 September 2020.

Here, we will summarize the current literature on opioid peptides, their classification as endogenous and exogenous, production methods, mechanism of action, as well as some opioid-like well-known peptides and their potential roles in stress-related conditions (Table 1).

Table 1. Opioid-like peptides and their roles.

Peptides	Functions	References
Colostrinin	Promotes acquisition of spatial learning in aged rats	[28]
Corticotropin-releasing factor (CRF)	Improves memory retention Enhance learning performance	[17,29]
Neuropeptide Y (NPY)	Neuroprotection as by control of feeding works against neurodegenerative diseases	[17,30]
Substance P (SP)	Improves functional recovery and increases the learning ability	[17,31]
Nociceptin/orphanin FQ (N/OFQ)	Impairs spatial learning in animal models. Facilitate memory	[32–34]
Angiotensin-vasopressin (AVP) and Oxytocin (OT)	Promote social memory and learning behaviors. Deficiency of AVP results in memory impairment	[35,36]
Cholecystokinin (CCK)	CCK peptides improve learning and memory performance in the patients Help in anxiety states Lack of CCK-A receptors cause impaired learning and memory functions. Play a role in conditioned fear stress and anxiety	[29,37,38]
Atrial natriuretic peptide (ANP), Brain-derived natriuretic peptide (BNP), C-type natriuretic peptide (CNP)	Promote action on memory consolidation	[39,40]
Pituitary adenylate cyclase-activating polypeptide (PACAP)	Promote learning (consolidation and retrieval)	[41]
Galanin	Impairs the learning and memory performances overexpression impairs cognition	[42–44]
Bombesin/gastrin-releasing peptide (BN/GRP) and Neuromedin (NM)	Improve memory performance	[45,46]
Hippocampal cholinergic neurostimulation Peptide (HCNP)	Abnormal accumulation and expression associated with memory and learning disorders	[47]
Calcitonin-gene related peptide (CGRP), Substance P(SP) and Neuropeptide Y (NPY)	Enhance memory retention. SP improves functional recovery and increases learning ability. NPY enhances memory	[30]
Insulin	Improves short-term memory	[48]
Orexin-A	Inhibits long-term potentiation (LTP) and retards spatial learning	[49,50]

So far, the potential roles of opioid-like peptides explained in Table 1 in stress-related conditions as well as in human health are well known, but their affinities as opioid peptides are still unclear. However, they are reported to exert opioid-like behaviors, but still, the mechanism of action is not well known. Therefore, further studies should be done to investigate their affinities as well as the mechanism of action as opioid peptides.

2. Classification of Opioid Peptides

They are divided into two categories as endogenous opioid peptides that are self-produced by the body and exogenous opioid peptides that are produced by different food sources such as plants and animals [25,26].

2.1. Endogenous Opioid Peptides

The endogenous opioid peptides are naturally produced in the mammalian system, which can operate as hormones (secreted by the gland and delivered to the target tissues) or neuromodulators (secreted by nerve cells and functioning in the central and peripheral nervous systems) [51]. In 1975 [16], the first endogenous ligand for opioid receptors was discovered and named as Enkephalins, and later, other endogenous peptides named Endorphins, Endomorphins, and Dynomorphins [10,52] were introduced. Opioid peptides that contain the conserved Tyr-Gly-Gly-Phe sequence at their *N* terminus are known as typical opioid

peptides [51]. Therefore, for better understanding, Table 2A,B contains the amino acid sequences of endogenous opioid peptides, along with various binding affinities of endomorphin analogues. As it is well known, there are three opioid receptor types, μ-opioid receptor (MOR), δ-opioid receptor (DOR), and κ-opioid receptor and (KOR), which are responsible for the physiological and pharmacological effects of opioid peptides. Site-directed mutagenesis, receptor chimaera experiments, and NMR data have shown that MOR are the main opioid receptors, and μ-selectivity is primarily characterized by the second and third extracellular loops, as well as the intracellular carboxyl termini and extracellular amino termini of the MOR [53]. Apart from this, very significant features of the μ-selective agonists are the inclusion of Tyr^1, Pro^2, or D-Ala^2 lipophilic residues at the third or fourth position and the amidation at the *C*-terminal [54]. So, with this theoretical basis, it would be possible to determine the conformational changes of the Endomorphins (EMs) belonging to the ligand–receptor binding and, eventually, to conjecture the detailed details of the MOR selectivity mechanism.

Enkephalins opioid peptides are present in the pituitary gland, brain, gastrointestinal tract, and kidney, and they are subdivided into two classes: Met-enkephalins and Leu-enkephalins. Another class of endogenous opioid peptides are endorphins, which are subdivided into four groups: α, β, γ, and σ. All are produced in the hypothalamus, pituitary gland, and in different parts of the nervous system and brain. Among all of them, β-endorphins are the most powerful one and play a crucial role as a neuromodulator [55]; they help to alleviate stress, body pain, and anxiety behaviors [55,56]. Endomorphins consist of types 1 and 2 endomorphins and dynorphins; both are located in the central nervous system and play significant roles in pain and stress-related conditions.

Table 2. (**A**) Endogenous opioid peptides. (**B**) Binding affinities of various Endomorphin analogues.

(**A**)

Opioid Peptide	Amino-Acid Sequence	Protein Precursor	References
endomorphin-1	Tyr-Pro-Trp-Phe-NH_2	pro-endomorphin	[57,58]
endomorphin-2	Tyr-Pro-Phe-Phe-NH_2	pro-endomorphin	[57,58]
met-enkephalin	Tyr-Gly-Gly-Phe-Met	pro-enkephalin	[16],
leu-enkephalin	Tyr-Gly-Gly-Phe-Leu	pro-enkephalin	[16,59]
β-endorphin	Tyr-Gly-Gly-Phe-Met-Thr-Ser-Glu-Lys-Ser-Gln-ThrPro-Leu-Val-Thr-Leu-Phe-Lys-Asn-Ala-Ile-Ile-LysAsn-Ala-Tyr-Lys-Lys-Gly-Glu	pro-opiomelanocortin	[60,61]
dynorphin A	Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-LeuLys-Trp-Asp-Asn-Gln	pro-dynorphin	[62,63]
dynorphin B	Phe-Gly-Gly-Phe-Thr-Gly-Ala-Arg-Lys-Ser-Ala-ArgLys-Leu-Ala-Asn-Gln	pronociceptin	[62,63]

(**B**)

Sequence	IC_{50} (nM)		Ratio of IC_{50} Ratio δ/μ	References
	Affity (μ-Receptor)	Affity (δ-Receptor)		
Endomorphins Modified at First Amino Acid Position				
D-Tyr^1-Pro-Phe-Phe-NH_2	32.1 ± 1.5	4121 ± 1492	128	[64]
Dmt^1-Pro-Trp-Phe-NH_2	0.014 ± 0.003	12.0 ± 4.05	857	[65]
Mmt^1-Pro-Phe-Phe-NH_2	0.132 ± 0.008	528.6 ± 47	4005	[65]
Emt^1-Pro-Phe-Phe NH_2	0.063 ± 0.006	55.7 ± 6.2	884	[65]
Dit^1-Pro-Phe-Phe-NH_2	2.29 ± 0.37	105 ± 16	46	[65]
Det^1-Pro-Phe-Phe-NH_2	0.084 ± 0.006	69.7 ± 5.3	830	[65]
Tmt^1-Pro-Phe-Phe-NH_2	1.111 ± 0.002	593.5 ± 80	5347	[65]
Endomorphins Modified at Second Amino Acid Position				
Tyr-D-Pro^2-Phe-Phe-NH_2	512.4 ± 29	30,641 ± 419	60	[64]
Tyr-Aze^2-Trp-Phe-NH_2	2.3 ± 0.23	3500 ± 360	1500	[66]
Tyr-δAla^2-Phe-Phe-NH_2	34 ± 6.3	710 ± 130	21	[66]
Tyr-3Aze^2-Phe-Phe-NH_2	210 ± 51	6900 ± 1200	32	[66]
Tyr-Aze^2-Phe-Phe-NH_2	5.6 ± 1.2	5100 ± 600	920	[66]

Table 2. *Cont.*

(**B**)

Sequence	IC_{50} (nM)		Ratio of IC_{50} Ratio δ/μ	References
	Affity (μ-Receptor)	Affity (δ-Receptor)		
Endomorphins Modified at Third Amino Acid Position				
Tyr-Pro-D-Phe^3-Phe-NH_2	203.2 ± 83	4230 ± 344	21	[64]
Tyr-Pro-Phe^3-(*p*-NH_2)-Phe-NH_2	185 ± 36	>10,000	>1.9	[67]
TyrProPhe^3(*p*-$NHCOCH_2Br$)-$PheNH_2$	7210 ± 820	>10,000	>1.4	[67]
Tyr-Pro-(2*S*,3*R*)-βMePhe^3-Phe-NH_2	106 ± 9	>10,000	>10	[68]
Tyr-Pro-(2*S*,3*S*)-βMePhe^3-Phe-NH_2	45.3 ± 4.1	179 ± 15	4	[68]
Tyr-Pro-(2*R*,3*S*)-βMePhe^3-Phe-NH_2	4910 ± 328	>10,000	>2	[68]
Tyr-Pro-(2*R*,3*R*)-βMePhe^3-Phe-NH_2	7090 ± 131	6760 ± 865	1	[68]
Tyr-Pro-(F_5)-Phe^3-Phe-NH_2	11.7 ± 0.503	11,700 ± 1010	1000	[69]
Endomorphins Modified at C-TERMINAL Position				
Tyr-Pro-Phe-D-Phe^4-NH_2	45.9 ± 8.6	8159 ± 1569	177	[64]
Tyr-Pro-Phe-(*p*-NH_2)-Phe^4-NH_2	36.7 ± 2.2	>10,000	>270	[67]
TyrProPhePhe^4(*p*-$NHCOCH_2Br$)-NH_2	158 ± 23	1940 ± 310	12	[67]
Tyr-Pro-Phe-(*p*-NCS)-Phe^4-NH_2	345 ± 128	>10,000	>29	[67]
Tyr-Pro-Trp-Dmp^4-NH_2	13.2 ± 1.9	7624 ± 2571	578	[70]
Tyr-Pro-Trp-D-Dmp^4-NH_2	106 ± 20	1765 ± 834	17	[70]
Tyr-Pro-Phe-Phe^4-NH-$(CH_2)_5$-CODap(6DMN)-NH_2	244.5 ± 14	5939 ± 1396	24	[71]
Tyr-Pro-Phe-D-Val^4-NH-Bn	4.97 ± 1.24	3358 ± 414	676	[72]
Tyr-Pro-Trp-D-Val^4-NH-Bn	2.32 ± 0.15	3287 ± 456	1417	[72]

2′,6′-dimethyltyrosine (Dmt), 2′-monomethyltyrosine (Mmt), 2′,3′,6′-trimethyltyrosine (Tmt), 2′-ethyl-6′-methyltyrosine (Emt), 2′,6′-diethyltyrosine (Det), 2′6′-dimethylphenylalanine (Dmp), 6-*N*,*N*-(dimethylamino)-2,3-naphthalimide (6DMN) and 2′,6′-diisopropyltyrosine (Dit).

Using amino acid substitution, addition, deletion, cyclization, or the hybridization of two ligands, endogenous peptides have been modified into semisynthetic analogues to incorporate conformational constraints and make them more potent to be used as clinical analgesics [73]. Endomorphines have been transformed into analogues, which have improved protease stability by the addition of unnatural amino acids accompanied by cyclization [73–75]. Modifications of leu-enkephaline by the substitution, addition, and deletion of amino acids have resulted in a variety of agonists with improved δ receptor selectivity [51]. Extensive research has been done to synthesize analogues with the desired characteristics, which are not addressed in this review, but this information is available in different articles focusing only on therapeutics [20,73].

2.2. Food Derived Exogenous Opioid Peptides

These peptides are also known as exorphins having morphine-like activity, and they are derived exogenously, outside the body via different food sources. Dietary proteins are known as one of the essential sources of opioids because of their structural similarities to endogenous opioids [76]. Accumulating evidence suggests that opioid receptors may recognize peptides derived from the enzymatic hydrolysis of food proteins as they carry amino acid sequences with conserved tyrosine residue at the *N* terminus, and therefore, some of these opioid peptides are presented in Table 3 exhibit opioid activity along with physiological activity [77]. However, bovine α-casein peptides lacking tyrosine at the amino terminus Arg-Tyr-Leu-Gly-Tyr-Leu-Glu have also shown opioid activity [22]. To date, opioid peptides originating from animal proteins have been established as binding to μ receptors and those from plant proteins to δ receptors [78], except for soymorphins. Mostly exogenous opioid peptides are generated in the gastrointestinal tract and are absorbed in the bloodstream. They are also known to resist breakdown by intestinal enzymes such as proteases and can cross the blood–brain barrier to interact with opiate receptors [79]. There are various sources of these food-derived exogenous opioid peptides such as casein from human milk [19], β casein (buffalo milk) [25], and β-casomorphin (parmesan cheese [80], cheddar cheese [81]). Milk protein fermentation with lactic acid bacteria (LAB) is a desirable method for the production of functional foods enriched by bioactive peptides given its low cost and a good nutritional

picture of fermented generated milk [82]. α-casein and β-casein are known as a good source of exogenous opioid peptides [76]. Milk was indicated to have opioid activity in 1979 [83], and morphine was isolated from milk as a drug at concentrations of 200 to 500 ng/L [84]. The opioid activity was attributed to the presence of β-casein [85] and f90-96 (Arg-Tyr-Leu-Gly-Tyr-Leu-Glu) and f90-95 (Arg-Tyr-Leu-Gly-Tyr-Leu) β-casein [86] peptides corresponding to f60-66 (Tyr-Pro-Phe-Pro-Gly-Pro-Ile, β-casomorphin-7). The opioid activity was also shown by the sequence corresponding to f91-96 (Tyr-Leu-Gly-Tyr-Leu-Glu) and f91-95 (Tyr-Leu-Gly-Tyr-Leu) amino acid residues, and Arg-Tyr-Leu-Gly-Tyr-Leu-Glu was the most potent [86]. β-casomorphin-7 from bovine β-casein was the first identified opioid peptide (Tyr-Pro-Phe-Pro-Gly-Pro-Ile) [87] and is known as the most potent opioid peptide in different βb-casomorphins (6, 5, and 4). As a result, β_h-casomorphine-4,-5,-6 and-8 with Tyr-Pro-Phe-amino-termine were tested for opioid activity [57]. Based on the primary structure of human β-casein (β_h-casein) and the sequence comparison with β_b-casein, 10 residual shifted alignment relationships and 47% identity were established [57,88]. Moreover, β-casomorphins (BCM), which are produced by β-casein [89] (region 57–70), have shown potential effects on brain functions [89], calming, and sleep of infants [90] as well as in the modulation of behaviors such as anxiety [91]. Meanwhile, both β_h-casomorphines and β_b-casomorphines bind particularly to μ receptors, with the highest affinity for μ receptors and the lowest affinity for κ receptors [88].

Furthermore, there are various other food sources too that are reported as a source of exogenous opioid peptides such as barley (hordein peptide [79]), wheat (Gluten (gluten exorphins), a major wheat protein complex, Gliadin (gliadorphin), Glutenin (gluten morphin) peptides [55]), while gliadorphin-7 (Tyr-Pro-Gln-Pro-Gln-Pro-Phe) derived from α-gliadin has shown opioid activity [92]. In another study, whey protein consisting of β-lactoglobulin, immunoglobulins, α-lactalbumin, lactoperoxidase, lactoferrin, etc. was reported to exert opioid-like activity [57]. In contrast, soymorphins are known as specific ligands of the μ-opioid receptor, and many soymorphins were isolated from different sources showing opioid activities such as soymorphins 5 (Tyr-Pro-Phe-Val-Val), 6 (Tyr-Pro-Phe-Val-Val-Asn), and 7 (Tyr-Pro-Phe-Val-Val-Asn-Ala) [76]. Out of all three, soymorphin 5 (Tyr-Pro-Phe-Val-Val) has shown the highest opioid activity [93]. In another study, by the amidation or esterification, (by methyl group) of the peptides at carboxyl terminals such as valmuceptin (βh-casein 51–54 amide, Tyr-Pro-Phe-Val-NH_2), morphiceptin (β-casein amide, Tyr-Pro-Phe-Pro-NH_2), α-lactorphin (αh-lactalbumin 50–53 amide, Tyr-Gly-Leu-Phe-NH_2), β-casorphin (β_h-casein 41–44 amide, Try-Pro-Ser-Phe-NH2), β-casomorphin-4 and β-casomorphin-5 amides [94,95], several opioid peptide analogues have been identified. Lactoferroxin A (Tyr-Gly-Ser-Gly-Tyr-OCH_3), B (Arg-Tyr-Tyr-Gly-Tyr-OCH_3), and C (Lys-Tyr-Leu-Gly-Pro-Gln-Tyr-OCH_3) are opioid antagonists derived from methyl-esterified human lactoferrin peptic digest [96]. The opioid activity of some of these peptides derived from food proteins is shown in Table 4.

Hydrolysate opioid activity can be assessed using one of many available assays. The naloxone-reversible inhibition of adenylate cyclase activity [97], naloxone-reversible inhibition of electrically induced contraction of isolated organ preparation, either mouse vas deferens and guinea-pig ileum [98], receptor binding assay or radio-receptor assay [99], are widely used assays for food opioid research. The main focus of the standard opioid activity determination tests in vitro was on μ and δ receptor interactions. These experiments are based on the inhibition of electrically evoked contractions of the mouse vas deferens (MVD) and the guinea pig ileum (GPI). The opioid effect in GPI preparations is primarily mediated by μ receptors, whereas the predominant MVD receptors are of the δ type [51]. Saturation and competition studies include receptor binding assays on tissue homogenates. The affinity of various compounds to opioid receptors is defined in saturation binding studies. Competition analyses can be performed subsequently or separately to validate these findings [51].

Exogenous opioid peptides have demonstrated promising effects in various investigations and the effects of administration of these opioid peptides at various doses in different animal models are presented in Table 5. Doses and results are difficult to compare, since various animal models and routes of administration have been used by different researchers. For example, Rubiscolin-6 improves memory consolidation [100], exerts orexigenic (oral administration) [101,102] and anxiolytic

effects [103], and suppresses high-fat consumption [104]. β-casomorphine induces the release of somatostatin and insulin [105] and has been shown to prolong the gastrointestinal transit time [106] as well as modulate intestinal mucus secretion [107]. Apart from the central and peripheral nervous system effects by opioid receptors, β-casomorphin-7 also improved plasma insulin and superoxide dismutase and catalase activity in diabetic rats, thereby shielding them from hyperglycemia and free radical-mediated oxidative stress [108]. Gluten exorphin B5 enhanced the secretion of prolactin [109] and gluten exorphin C enhanced exploratory activity, improved learning, and decreased anxiety [110]; thus, studies show the positive effect of opioid peptides in human health.

Table 3. Exogenous food-derived opioid peptides.

Source	Sequences	Peptide Name	References
Bovine milk β-casein	Tyr-Pro-Phe-Pro Tyr-Pro-Phe-Pro-Gly Tyr-Pro-Phe-Pro-Gly-Pro Tyr-Pro-Phe-Pro-Gly-Pro-Ile	β_b-casomorphin-4 β_b-casomorphin-5 β_b-casomorphin-6 β_b-casomorphin-7	[87]
	Tyr-Pro-Val-Glu-Pro-Phe	Neocasomorphin-6	[111]
Bovine milk α-lactalbumin	Tyr-Gly-Leu-Phe-NH_2	α_b-lactorphin	[22]
Human milk β-casein	Tyr-Pro-Phe-Val Tyr-Pro-Phe-Val-Glu Tyr-Pro-Phe-Val-Glu-Pro-Ile Tyr-Pro-Phe-Val-Glu-Pro-Ile-pro	β_h-casomorphin-4 β_h-casomorphin-5 β_h-casomorphin-7 β_h-casomorphin-8	[22] [57] [88] [22,88]
Human milk lactalbumin	Tyr-Gly-Leu-Phe-NH_2	α_h-lactorphin	[22,94]
Bovine/bovine milk lactoferrin	Tyr-Leu-Gly-Ser-Gly-Tyr-OCH_3 Arg-Tyr-Tyr-Gly-Tyr-OCH_3 Lys-Tyr-Leu-Gly-Pro-Gln-Tyr-OCH_3	lactoferrsoxin A lactoferrsoxin B lactoferrsoxin C	[96]
Soy β-conglycinin	Tyr-Pro-Phe-Val-Val Tyr-Pro-Phe-Val-Val-Asn Tyr-Pro-Phe-Val-Val-Asn-Ala	Soymorphin-5 Soymorphin-6 Soymorphin-7	[93]
Wheat HMW glutenin	Gly-Tyr-Tyr-Pro Gly-Tyr-Tyr-Pro-Thr Tyr-Gly-Gly-Trp Tyr-Gly-Gly-Trp-Leu	gluten exorphin A4 gluten exorphin A5 gluten exorphin B4 gluten exorphin B5	[78,112]
	Tyr-Pro-Ile-Ser-Leu	gluten exorphin C	[78,113]
Spinach RuBisCo	Tyr-Pro-Leu-Asp-Leu Tyr-Pro-Leu-Asp-Leu-Phe	rubiscolin-5 rubiscolin-6	[93,114]

Table 4. The opioid activity of exogenous peptides (IC50 in μM).

Opioid Peptide	Opioid Activity (IC50 in μM)		μ/δ Ratio	Reference
	Mouse (vas Deferens) (δ)	Guinea-Pig (ileum) (μ)		
rubiscolin-5	51	1110	21.8	[114]
rubiscolin-6	24.4	748	30.7	[114]
β_b-casomorphin-4	84	22	0.26	[87]
β_b-casomorphin-5	40	6.5	0.16	[87]
β_b-casomorphin-6	>150	27.4	<0.18	[87]
β_b-casomorphin-7	>200	57	<0.29	[87]
β_h-casomorphin-4	750	19	0.025	[94]
β_h-casomorphin-5	ND	14	ND	[94]
β_h-casomorphin-6	350	25	0.071	[94]
β_h-casomorphin-8	540	25	0.047	[94]

Table 4. *Cont.*

Opioid Peptide	Opioid Activity (IC50 in μM)		μ/δ Ratio	Reference
	Mouse (vas Deferens) (δ)	Guinea-Pig (ileum) (μ)		
gluten-exorphin A4	70	>1000	ND	[112]
gluten exorphin A5	60	1000	60.7	[112]
gluten exorphin B4	3.4	1.5	0.44	[112]
gluten exorphin B5	0.017	0.05	2.9	[112]
gluten exorphin C	30	110	3.7	[113]
soymorphin-5	50	6	0.12	[93]
soymorphin-6	32	9.2	0.287	[93]
soymorphin-7	50	13	0.26	[93]
Human milk lactalbumin (α-lactorphin)	>1000	50	ND	[94]
Bovine milk lactoferrin (lactoferrsoxin)	4.38	5.68	0.77	[96]

ND—not determined, IC_{50} is the 50% inhibitory concentration.

Table 5. Trials of exogenous opioid peptides in animal models.

Opioid Peptide	Animal Model	Dosage	Administration Route	Effect	Time Duration	Reference
rubiscolin-5	Mice	3 nM/mouse	i.c.v	antinociception	Effects observed up to 30 min post-injection	[114]
rubiscolin-6	Mice	1 nM/mouse	i.c.v			
rubiscolin–6	Mice	100 mg/kg 3 nM/mouse	Oral i.c.v	enhancement in memory consolidation	Effects observed up to 24 h post-injection	[100]
β-casomorphin-4,-5,-6,-7	Rat	60–2000 nM	i.c.v	analgesic, naloxone reversible	Effects observed up to 30–40 min post-injection	[115]
β-casomorphin-5	Mice Rat	1mg/kg 166 nM	i.p i.v	improvement in learning and memory, analgestic	Effects observed up to 30-min post-injection Significant analgesia l0-min after injection up to 60 min post-injection	[116,117]
β-casomorphin-7	Rat	0.1–20 nM	i.c.v	food-intake stimulation	Effects observed up to 6 h post-injection	[118]
gluten exorphin C	Mice	5mg/kg	i.p	improvement in learning and behavior, decreased anxiety	Effects observed up to 15–20 min post-injection	[110]
Gluten-exorphin B5	Rat	3 mg/kg	i.v	stimulated prolactin secretion	Effects observed after 20 min post-injection	[109]
soymorphin-5, 6, and 7	Mice	10–30 mg/kg or 3 mg/kg	Oral i.p	anxiolytic effect	Oral—Effects observed up to 20–25 min post-injection i.p—Effects observed <30min of post-injection	[93]
soymorphin-5 and 7	Mice	30 mg/kg or 48 μ mol/kg	oral	reduced food intake and showed anorexigenic activity	Effects observed after 2 h of oral administration	[119]
soymorphin-5 amide	Rat	5 mg/kg	i.p	decreased anxiety	Effects observed after 30 min of administration	[120]

i.c.v—intracerebroventricular; i.p—intraperitoneal; i.v—intravenous; nM—nano mol.

The IC_{50} value is the concentration that would inhibit the electrically-evoked maximal contractions of the organ by 50%. As explained earlier, the opioid effect in GPI is primarily mediated by μ receptors, whereas in MVD receptors are of the δ type. So, the values in Table 4 represent the minimum concentration required for the activity with their selective receptors. For example, in soymorphins 5, 6, and 7, the IC_{50} value is lower in GPI assay as compared to values obtained by MVD assay, which shows there selectivity toward μ opioid receptors for activity.

Some current knowledge on the biological effects observed upon the intracerebroventricular, intraperitoneal, and oral administration of exogenous opioid peptides in the animal models are highlighted in Table 5. The fact that food-derived peptides can cross the small intestine and be present in blood and tissues has been appreciated for a long time [121–123]. In contrast, some pieces of evidence also suggests that [124] the degree of peptide absorption decreases with increasing chain length, so peptide length is also a point of consideration.

Although these studies (Table 5) provide useful information on the possible roles of the exogenous opioid peptides, still there are shortcomings in their research: for example, the exogenous peptides applied (similar to their endogenous counterparts) are unstable and are hydrolyzed into shorter forms, which typically have distinct activities after hydrolyzation from the parent peptide administered, leading to difficulties in the prediction of outcomes. So, technologies such as probe/radiolabeling will be effective here for the administration of parent peptides before hydrolysis inside the animal or human body. However, peptides can be secured against enzymatic cleavage by inserting a structure that induces a tail probe [125], by lactam bridge [126], by stacking or clipping peptide sequences [127], or by cycling [128].

For better understanding, deeper research with an explored signal cascade mechanism at the cellular and molecular level is needed to explore food-derived opioid peptides as therapeutic mediators, functional foods, or nutraceuticals for human health promotion.

3. Production of Opioid Peptides from Food Proteins

There are various emerging improvements in the methodologies for the analysis and the development of food-derived peptides. The main methods for their discovery can be classified as a traditional, in silico approach, or integrated approaches from the present literature.

3.1. The Traditional Approach

The traditional approach focuses on enzyme selection and production of the peptide through protein hydrolysis, purification of peptides, and peptide identification.

This is an extensively used method for recovering bioactive peptides from different food sources and comprises a collection of certain protein sources of interest. The proteins were usually digested with the help of food-grade enzymes (proteases, proteinases, or peptidases) to hydrolyze proteins into peptide fragments [129–132]. In addition to enzymes, various microorganisms may be used to ferment the proteins to enhance peptide breakdown [133–135]. The use of fermentation is possible because of the many proteolytic enzymes they possess for degrading proteins and to satisfy their nitrogen demands. Subsequently, the fermented peptides are extracted and purified according to their structural chemistry. The fractions undergo in vitro testing to evaluate their potential health effects. In an earlier study, β-casomorphin-7 was detected for the first time in the fermented sample [136]. In another study, gluten and exorphins were identified in a hydrolysate of pepsin and thermolysin [112] as well as in hydrolysate of pepsin–trypsin–chymotrypsin [113]. Gluten exorphins A5 with a weight around 0.747–2.192 mg/kg and C of 3.201–6.689 mg/kg have recently been found in bread and pasta following the simulation of in vitro GI digestion (using pepsin–trypsin–chymotrypsin) [137]. Similarly, casomorphin releases were analyzed in milk and its products after simulated GI digestion by different enzymes [138].

The hydrolysates with beneficial health effects are purified, and the active peptides were identified. Methods such as high-pressure liquid chromatography joined with mass spectrometry, reverse-phase liquid chromatography joined with mass spectrometry, and liquid chromatography-electrospray

ionization along with quantitative time-of-flight tandem mass spectrometry have been used in studies to identify the bioactive peptides present in the sample [139–141]. This classical approach is effective in discovering new bioactive peptides from various protein substrates.

3.2. The In Silico Approach

The in silico methods comprise the use of information gathered from databases to find out the occurrence frequency of encrypted bioactive peptides in the primary food protein structure. The protein sequences can be accessible from databases for the analysis of different bioactive peptides (Table 6). As the existence of these peptides does not naturally mean the liberation of encrypted peptides, some bioinformatics software has been created that mimic proteolytic enzyme specificities to produce silico peptide profiles. Then, these proteases can be utilized for the hydrolysis of food protein, and their potential health effects were tested to build their efficacy. This method helps differentiate the identified peptides from unknown sources of protein (Figure 2). Most in silico platforms can predict the bioactive potential of the identified peptides, and the bioactivity can be confirmed through experiments in vitro and in vivo. As the in silico approach is rapid, cost-effective, and a greater number of options are available, various researchers are using this approach [142–144] to identify potent bioactive peptides. Proteolysis tools such as ExPASy Peptide Cutter (http:/web.expasy.org/peptidecutter), BIOPEP, and PoPS (http://pops.csse.monash.edu.au) are used to classify the specificities of different enzymes for releasing target peptide from food. By using this approach, sequences of cereal proteins (wheat, oat, barley, and rice) display high concentrations of peptides with dipeptidyl peptidase-inhibitory, anti-thrombotic, angiotensin-converting enzyme-inhibitory, antioxidant, hypotensive, and opioid activity [145]. In recent years, these computer-based databases have been used to predict the existence of bioactive peptides in food proteins [24,146–148]. Yet, more research is required to better understand this approach for the prediction of various food-based bioactive peptides.

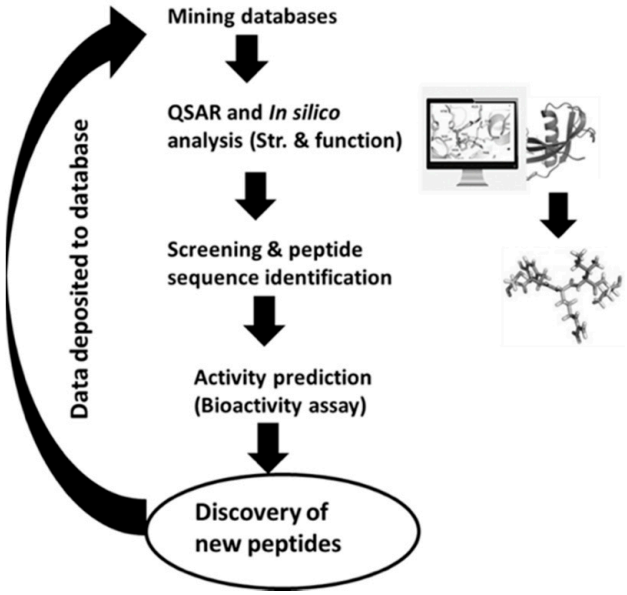

Figure 2. In silico approach to identify peptides from unknown sources of protein. QSAR: quantitative structure–activity relationship, Str: Structure.

Table 6. In silico databases and online tools for analysis of bioactive peptides.

Databases Name	Address	Role
NeuroPIpred	https://webs.iiitd.edu.in/raghava/neuropipred	Neuropeptide database
NeuroPP	http://i.uestc.edu.cn/neuropeptide/neuropp/home.html	Neuropeptide database
BIOPEP (Bioactivity) (digestion) (Protein) (toxicity)	http://www.uwm.edu.pl/biochemia/index.php/en/biopep	Prediction for precursors of bioactive peptides,
ToxinPred (Toxicity)	http://crdd.osdd.net/raghava//toxinpred/	Prediction of toxicity of peptides
I-TASSER (Protein Structure)	https://zhanglab.ccmb.med.umich.edu/I-TASSER/	Structure and function prediction
NCBI (Protein Database)	https://www.ncbi.nlm.nih.gov/	Protein sequences information
AlgPred (Toxicity)	http://crdd.osdd.net/raghava//algpred/	Prediction of toxicity of peptides
ProtParam (phytochemical)	http://web.expasy.org/protparam/	Compute GRAVY (grand average of hydropathicity)
UniProtKB (Protein database)	http://www.uniprot.org/	Structure and sequences information
APD (Peptide database)	http://aps.unmc.edu/AP/main.html	Bioactive peptide prediction
AntiBP2 (Bioactivity prediction)	http://crdd.osdd.net/raghava//antibp2/	Antibacterial peptide prediction
PEPstrMOD (Peptide database)	http://osddlinux.osdd.net/raghava/pepstrmod	Prediction of tertiary structures

3.3. Chemical Synthesis Approach

The key chemical approaches for peptide synthesis are solution-phase synthesis (SPS) and solid-phase peptide synthesis (SPPS). SPS is generally performed by coupling single amino acids in solution. Long peptide synthesis is feasible by synthesizing short fragments of the target peptides first and compressing them to produce long peptides [149]. This SPS approach is called the fragments condensation process. In the SPS method, it is possible to deprotect and purify intermediate products to achieve high purity of the target peptide [150,151]. SPS is economical and efficiently extensible, but the long reaction time remains a drawback. Meanwhile, the SPPS approach requires peptide synthesis using resin as a support for a growing peptide chain. An amino acid's reactive side chain and α-amino group are first covered (mostly using the fluorenylmethoxycarbonyl protecting group (Fmoc) or tert-Butoxycarbonyl (Boc)) and the amino acids *C*-terminus is bound to the resin [152]. The *N*-terminal protecting group is normally removed (or cleaved) by using trifluoroacetic acid (Boc) or by 20% piperidine in *N*-*N*-dimethylformamide (Fmoc); then, the resin is washed before the introduction of subsequent amino acids. The peptide is separated off the resin after the required sequence is completed [149]. Presently, SPPS is widely used for therapeutic peptide synthesis because of its lower manufacturing costs and advancements in chromatographic equipment [149]. Long peptide or protein chains can also be synthesized by the chemical ligation approaches. The Native Chemical Ligation (NCL) is an efficient process for ligating peptides. A non-protected peptide segment containing an *N*-terminal cysteine is reacted to ligate peptide fragments with another unprotected peptide to form a thioester-linked intermediate, which is later reconstructed into a peptide bond. This process enables the formation of peptides of high molecular weight such as multivalent peptide-based non-symmetric dendrimer [153] and collagen-like polymers [154]. The advantages of this process are the high strength of the starting materials in NCL, the well-established chemical methods to manufacture peptide thioesters, and the high chemo-selective nature of the peptides. In addition, various researchers have used this approach such as Hartman et al. [155] for the treatment of pathological conditions of oxidation, Meisel et al. [156,157] for the oral administration of chemically synthesized peptides to mice in inflammation and atherosclerosis, González-García et al. [158] to transform bioactive peptide from waste to valuable product, Agyei et al. [159] to produce bioactive peptides to get large-scale recovery in pharmaceuticals, and Kim et al. [160] for the purification of bioactive peptides in food industries.

3.4. The Integrated Approach

An integrated bioinformatics approach may be utilized in the detection of bioactive peptides because of limitations associated with the previous approaches. The strengths of both classical and bioinformatics approaches (Figure 3) can be combined to advance the analysis and need for peptides in health benefits and functional foods. The bioactive peptides determined in food proteins via the in silico method could be chemically synthesized, and this approach will lead to the detection of new peptides from new sources [161]. However, this is possible only if the whole sequence of a protein and the functional activities of the peptides are already known. Nonetheless, various important bioactive peptides of low concentration can further be missing if have not already been identified in the database. Therefore, alternative techniques such as peptide display methodologies were being used for the search of bioactive peptides at present.

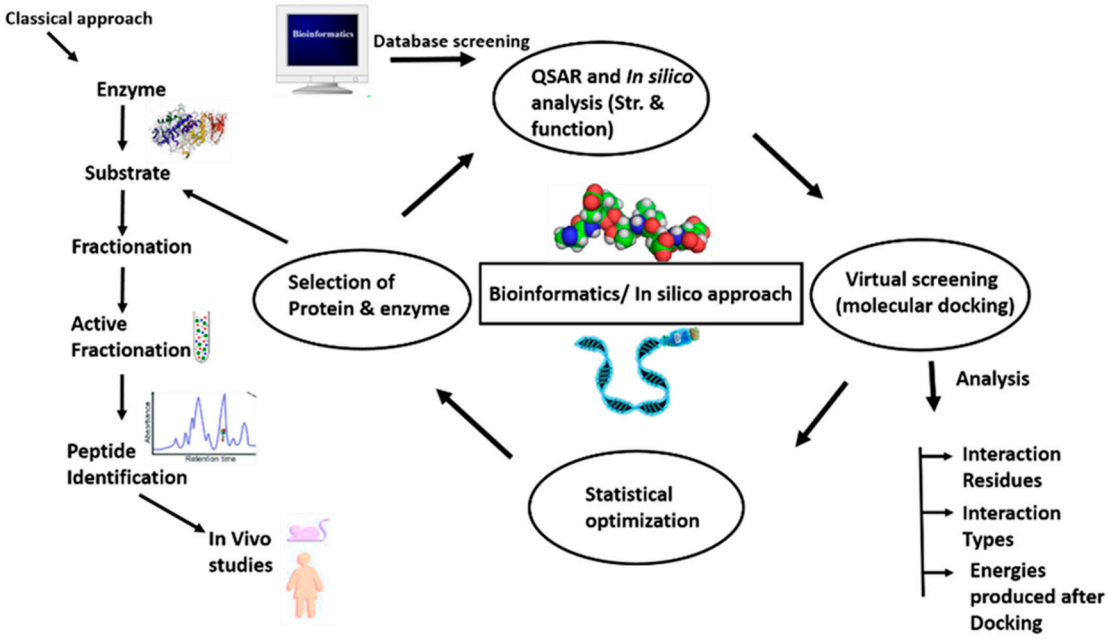

Figure 3. Representation of integrated approach (conventional/classical and bioinformatics) for the identification of food-derived bioactive peptides.

3.5. Screening for Bioactive Peptides

After fermentation or enzymatic hydrolysis, it is usually very hard to identify particular bioactive peptides in a sample. Chemically synthesizing all of the peptides that could be found in a peptide digested for the screening purposes is costly and very laborious (if not impossible). Hence, many researchers use the bioassay fractionation approach, in which liquid chromatography separates protein digests into fractions. The fractions are analyzed, and potent fractions are further fractionated before mass spectrometry [162]. The peptides encrypted in the fractions are eventually recognized. While the approach has been successful and efficient, it typically omits the activity of lower concentrated fragments.

Using in silico platforms alone to predict bioactivity may be easy, but it may not be very accurate, as not all of the peptides predicted may be bioactive. Consequently, other researchers correlate their data collected from HPLC-MS along with many house databases that enhance the detection of less concentrated peptides [14]. However, several essential new low-concentration bioactive peptides can still be lost. In such a situation, the recombinant peptide libraries related to the coding sequence (peptide display) of these peptides can be used to identify dynamic bioactive peptides as an effective tool. Peptide display methodologies have been used as an effective research tool to track protein interactions at high throughput [163,164]. Phage display has been widely applied among most

available molecular display techniques such as covalent antibody display, yeast and bacterial display, mRNA display, ribosome display, and CIS display. Phage display is a peptide selection approach that comprises the fusion of a peptide or protein with a protein coat of bacteriophage displayed on the virus surface [165]. The random peptide libraries displayed in a phage provide a functional approach to biopeptides, distinguishing peptides binding from those which are nonbinding peptides via affinity purification. The phage displayed random peptide library identification is an effective way of detecting peptides that can bind and control target molecular behaviors. This method has been practiced in identifying receptor-bound bioactive peptides [166], disease-specific antigen mimics [166,167], cell-specific peptides, non-protein-bound peptides [168], or organ-specific peptides [169], as well as in the designing of peptide-mediated drug delivery systems [165]. Consequently, analysis for bioactive peptides applying phage display technology is an advantageous approach that can be used in basic research. This way, the bioactive peptides achieved can be cloned and overexpressed to increase their quantity.

4. Mechanism of Action

A receptor is a protein that binds with a chemical messenger and brings out an intracellular response. In the case of the opioid peptide, they either bind to a receptor or are converted into smaller peptides or amino acids (Figure 4). The receptors present are G-proteins coupled receptors (GPCRs); they are known as the largest gene family among all receptors, consisting of around 1000 different genes in human and other mammals [170].

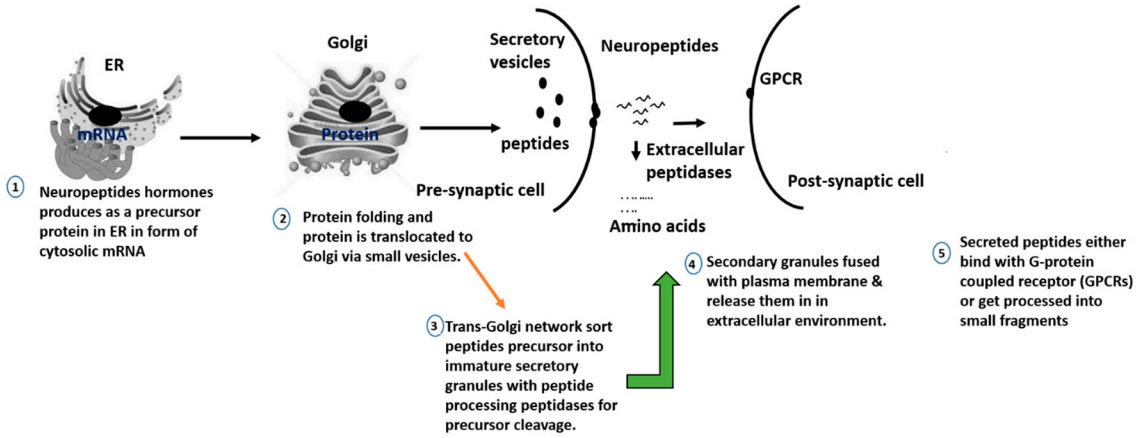

Figure 4. An overview of neuropeptide biosynthesis.

When an opioid peptide binds to a receptor molecule, several intracellular changes of molecules occur along with other changes such as enzyme activation, the opening of ion channels, and the transcription of genes [170]. G-protein coupled receptors are also known as heptahelical receptors, as they consist of seven-transmembrane spanning receptors, and their signaling is conducted via G-proteins. Opioid peptides when functioning through GPCRs bind to the external surface of the receptor, which changes the conformation of the receptor proteins, leading to intracellular changes of the G proteins with the receptors [170]. The G-protein consists of 3 subunits α, β, and γ; when G-proteins are in an inactive state, the α-subunit binds with GDP (Guanosine diphosphate) along with other two subunits, β and γ, forming a G-protein complex. After receptor activation, the G-proteins attached GDP molecule is released and converted to a GTP (Guanosine triphosphate) molecule, and the GTP-α-subunit complex is dissociated from $\beta\gamma$ subunits, which remain attached. The Gα-subunit is now able to modulate the activity of the effector molecules such as phospholipase or adenylyl cyclase [170]. Whereas it was also seen that in some cases, $\beta\gamma$ subunits are also able to modulate some effector activities [170]. As soon as GTP bound with the α-subunit is hydrolyzed by cellular proteins, GTP hydrolyzed to GDP [170]. Thereafter, the free α-subunit again reassociates to form

a heterotrimeric complex with βγ subunits, and this complex couples with the receptor for the next cycle of G-proteins upon activation (Figure 5). There are different subfamilies of G-proteins based on α-subunit Gi, Gs, Gq, and Go [76]. Everyone has multiple members who work via different pathways. The Gαs subunits activate adenylyl cyclase, whereas Gαo and Gαi subunits inhibit the adenylyl cyclase enzyme [76]. These activations lead to a series of reactions as adenylyl cyclase bring about cAMP(Cyclic adenosine monophosphate) formation from ATP, which activates protein kinase A. Earlier protein kinase A phosphorylates various intracellular substrates that result in biological modulations [76]. Moreover, Gαq subunits activate phospholipases that produce diacylglycerol and inositol 1,4,5-triphosphate [171]. Diacylglycerol activates protein kinase C for the phosphorylation of various molecules, whereas inositol 1,4,5-triphosphate activates receptors present on the endoplasmic reticulum for the opening of the Ca^{2+} channel [171]. Gα12/13 leads to the activation of guanine-nucleotide factors such as RhoGTPases for the exchange of GDP to proteins leading to biological changes [170].

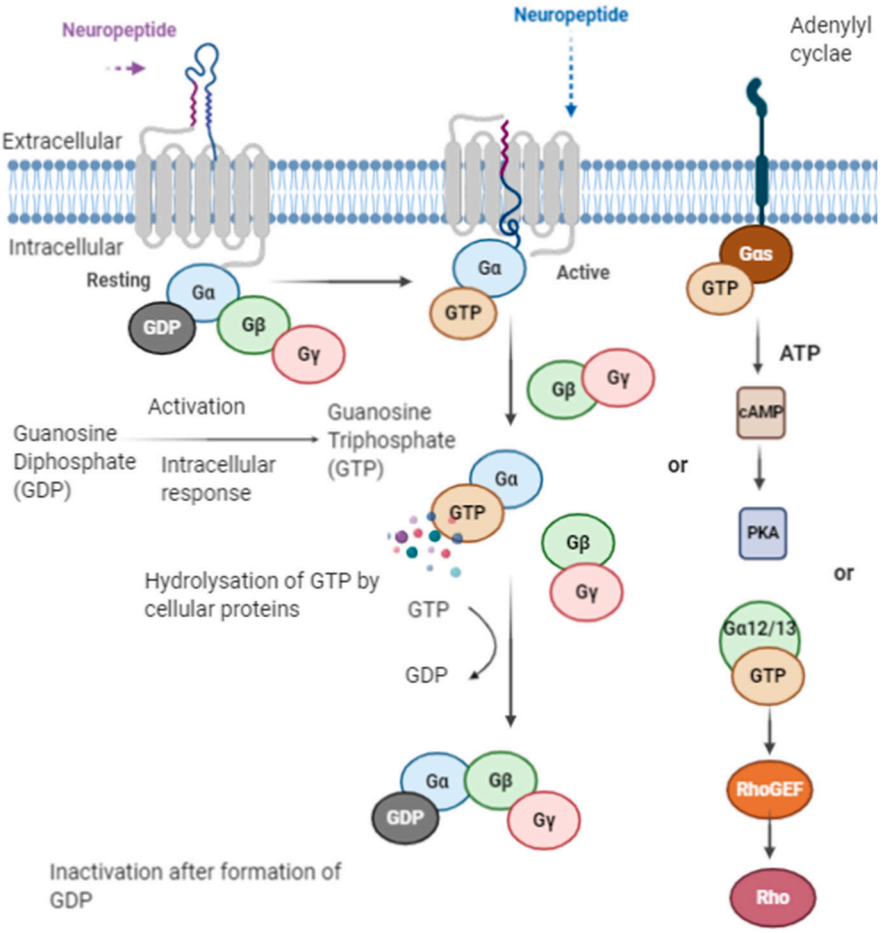

Figure 5. Mechanism of action.

These opioids peptide signaling systems are very complex as compared with some conventional neurotransmitters where no multiple receptor binding occurs and only a single ligand works with different receptors such as for acetylcholine [170]. Whereas most of them bind with multiple receptors but as an addition, they are also able to bind with receptors subtypes, creating more complexity in the signaling pathways. Yet, with the complexity of receptors and mechanism of action in these bioactive peptides, they still provide a promising and successful role in various clinical trials [170].

Transport of Opioid Peptides in the Body

During digestion, digestive enzymes hydrolyze the food proteins to peptides and amino acids. Several factors influence the GI tract's transport and absorption of peptides, including pKa, peptide size, and pH microclimate. Gastric emptying and the intestinal transit affect the location where the peptide is present along the GI tract and thus influence absorption. Peptides larger than di-tripeptides are found not easily absorbed in healthy people except in the conditions such as stress or disease when intestinal permeability is increased [172]. It has been seen that there is no absolute barrier to the intestinal mucosa and different peptides, including gluten exorphins A5 and A4, can cross the intestinal epithelium [57,137], whereas the mechanism of transfer is still not clear. In another study, it was observed that in a mammalian system, four different peptide transport systems PTS-1, PTS-2, PTS-3, and PTS-4 were involved that can transfer peptides, including food-derived peptides, from the peripheral circulation to the central nervous system through the blood–brain barrier [173]. In contrast, all four PTS-1 systems carry opioid peptides, including Tyr-MIF-1 met-enkephalin and leu-enkephalin [173]. Food-derived opioids absorbed in the gastrointestinal tract initially interact with receptors that are present on the enteric nervous system (ENS) and thus influence GI functions. The ENS is a network of nerve cells located in the GI tract wall, which control motility and secretion and regulate digestion, absorption, and immunomodulation [174]. In contrast, the glycosylation of peptides has also shown promising results in the transportation of peptides via the GLUT1 glucose carrier, along with the glycosylated analogues of dermorphin and met-encephalin [175]. However, due to peptidase activity, the half-life of opioid peptides in the blood is low. Endogenous opioids leu-enkephalin and dynorphin-A (1–13) have 6.7 min and 1 min half-lives [176,177]. In contrast, dermorphin exhibits a longer half-life than Enkephalins [178], and the half-life can be extended by binding these peptides to carrier proteins such as transferrin [179] or albumin [180]; some of the half-life stability data of opioid peptides are shown in Table 7. Therefore, in vivo half-life investigations of exogenous opioid peptides in blood need investigation for future research benefits.

Table 7. Stability half-life of opioid peptides.

Sequence	Half-Life (Mouse Brain) [min]	Reference
[D-Ala^2, *p*-Cl-Phe^4]EM-1	>300	[181]
[Dmt^1,Nip^2]EM-1	30.9 ± 3.29	[182]
[(*2S,3S*)β-MePhe^4]EM-2	35.8 ± 1.8	[68]
[(*1S,2R*)ACHC^2]EM-1	>12 h	[183]
Guanidino-[D-Pro^{2}Gly^3, *p*-Cl-Phe^5]EM-1	187.3 ± 24	[181]
[(*1S,2R*)ACPC^2]EM-2	>12 h	[183]
[Dmt^1,Nip^2]EM-2	10.7 ± 0.3	[182]
Guanidino-[D-Pro^2-Gly^3]EM-1	111.8 ± 19.2	[181]
Guanidino-[Sar^2]EM-1	43.9 ± 2.4	[181]

EM-1—endomorphin 1, EM-2—endomorphin 2, cis-/trans-2-aminocyclopentanecarboxylic acid (ACPC), cis-/trans-2-aminocyclohexanecarboxylic acid (ACHC), piperidine-3-carboxylic acid (Nip), 2′6′-dimethyltyrosine (Dmt).

5. Clinical and Animal Studies Related to Exogenous Opioid Peptides in Stress-Related Conditions

There are various preclinical and clinical studies available to support the role of several bioactive peptides in animals as well as in humans. Interestingly, as of now, opioid peptides are known as a potential target for the development of various new therapies related to stress disorders [184,185]. Here, we highlighted some pieces of evidence to support the role of food-derived peptides in stress-related conditions.

Stress, Anxiety, and Depression

After the discovery of endogenous opioid peptides, experiments were performed to investigate exogenous peptides and their role in animal models. Studies related to exogenous opioid peptides have shown positive impacts on human health.

For instance, Lister et al. [91] had used various nociceptive models, and intracerebral (i.c.) or intracerebroventricular (i.c.v) routes were tested in which it was observed that various β-casomorphins (-3, -4, -5, -6, and -7) had shown anxiolytic effects. Limit et al. have found the effect of peptides isolated from soybean on brain functions [186]. Similarly, Bernet et al. explained the role of fish hydrolysates (Gabolysat PC60) on the levels of GABA and their sympathoadrenal activity leading anxiolytic effects in rats [187].

Additionally, rubiscolin-6 isolated from spinach RuBisCo was administered orally in mice and found to have an anxiolytic effect arbitrated by dopamine receptors [103] as well as a reduction of nociception in mice [188].

It was also seen that certain milk-based β-casomorphins interact with the receptors of opiates to affect the absorption of food in different conditions such as stress and anxiety [189]. Yin et al. has reported the protective role of β-casomorphins against oxidative stress [108]. In another, Chesnokova et al. [120] and Kaneko et al. [119] have shown the potential role of soymorphin-5 in the management of anxiety in mice.

On the other hand, soymorphin-5 demonstrated anxiolytic effects in mice, showing an improvement in the time mice remained in the open arms during an elevated plus-maze test [190]. In a human study, α-lactalbumin from full whey protein induced anxiolytic effects [191]. So, by going through different studies, we found that food-derived opioid peptides play a promising role in stress-related conditions as well as in human health.

6. Conclusions

In this review, we provided a detailed discussion on types of opioid peptides along with their sources, structures generated by enzyme hydrolysis of the food proteins, and their clinical pieces of evidence related to stress, anxiety, and depression. The opioid peptides have been discovered in the 1970s, and to our understanding, they hold great promise as valuable functional ingredients in healthy diets. In the modern generation, stress, anxiety, and depressive disorders are becoming a major issue, and food-derived opioid peptides showing anxiolytic and anti-stress effects can be a beneficial food substrate in maintaining a healthy population. The fermentation and enzymatic hydrolysis of food proteins are widely used in varying quantities and bioactivity to release these peptides. The amino acid profiles of various plants and animal food proteins suggest that these can become a great potential source for the production of bioactive peptides. Their effects are also dependent on their stability in blood, binding affinities, and half-lives as well as the capability of crossing the blood–brain barrier. The relative activity of these opioids depends on their affinity to the receptors, μ, δ, and κ. To date, opioid peptides from animal proteins tend to attach primarily to μ receptors, whereas those from plant proteins bind with δ receptors, except soymorphine. Most food opioid investigations were focused on widely used tissue preparations, mouse vas deferens, and guinea-pig ileum, which are unique to μ and δ receptors, respectively. It is not known whether the receptor of food-derived opioid peptides binds to the κ receptor. There is no proof in the literature of the use of rabbit vas deferens, which is known to be rich in κ opioid receptor, to confirm whether food opioids bind to this receptor [59].

On the other hand, various studies such as meta-analysis, animal model, and clinical examinations have shown the impact of these peptides on the nervous system (analgesia, antinociception, and improved memory), GI functions (increased intestinal transit time, increased appetite, and suppression of high fat intake), and increased β-oxidation and energy consumption, indicating the possibility of their use as nutraceuticals for pain relief, stress reduction, blood sugar, and obesity.

Still, to validate their roles, future consideration is needed to understand the stability of these peptides during digestion in animal and humans by in in vitro and in vivo studies as well as the health-related effects they generate across the gut–brain axis. Therefore, further research is required

to understand and develop methods for the development of opioid peptides from food proteins in substantial quantities for pharmaceutical, beverage, and food use and system strategies to ensure their targeted distribution.

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Review

Low Vitamin B12 and Lipid Metabolism: Evidence from Pre-Clinical and Clinical Studies

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Abstract: Obesity is a worldwide epidemic responsible for 5% of global mortality. The risks of developing other key metabolic disorders like diabetes, hypertension and cardiovascular diseases (CVDs) are increased by obesity, causing a great public health concern. A series of epidemiological studies and animal models have demonstrated a relationship between the importance of vitamin B12 (B12) and various components of metabolic syndrome. High prevalence of low B12 levels has been shown in European (27%) and South Indian (32%) patients with type 2 diabetes (T2D). A longitudinal prospective study in pregnant women has shown that low B12 status could independently predict the development of T2D five years after delivery. Likewise, children born to mothers with low B12 levels may have excess fat accumulation which in turn can result in higher insulin resistance and risk of T2D and/or CVD in adulthood. However, the independent role of B12 on lipid metabolism, a key risk factor for cardiometabolic disorders, has not been explored to a larger extent. In this review, we provide evidence from pre-clinical and clinical studies on the role of low B12 status on lipid metabolism and insights on the possible epigenetic mechanisms including DNA methylation, micro-RNA and histone modifications. Although, there are only a few association studies of B12 on epigenetic mechanisms, novel approaches to understand the functional changes caused by these epigenetic markers are warranted.

Keywords: vitamin B12 (B12); lipid metabolism; cardiovascular disease (CVD); obesity; metabolic syndrome (MetS); type 2 diabetes mellitus (T2D)

1. Introduction

Obesity is currently a worldwide epidemic that results in higher insulin resistance and increases the risk of developing metabolic disorders like type 2 diabetes mellitus (T2D), hypertension and cardiovascular diseases (CVDs) [1], thus, posing a critical public health concern [2]. Approximately 2.1 billion (30%) humans worldwide are reported to be obese or overweight, contributing to 5% of global mortality. If sustained, the prevalence rate of obesity is likely to increase to 50% of the global adult population by 2030 [3]. In addition, studies from the global hunger index have reported that, approximately 2 billion people globally are affected by deficiency of micronutrients. The world health organization (WHO) is specifically concerned about the levels of vitamin B12 (B12) and folate (B9) due to increasing prevalence of their deficiencies across the populations [4]. Epidemiological studies have also clearly shown the association of these nutritional metabolites and manifestations of metabolic risk [5–8].

Dyslipidemia is a key risk factor for atherosclerosis and CVD. Studies have also shown the association of low B12 with obesity, hypertension, T2D and metabolic syndrome (MetS) in diverse populations. Low B12 may also be associated with adverse lipid profile and CVDs [9]. In pre-clinical studies, low B12 levels might increase lipid accumulation in adipocytes and trigger dyslipidemia in mice [10], suggesting that low B12 and dyslipidemia might be causally related.

Obesity could be developed principally, as a result of excessive macronutrient intake and/or reduced energy expenditure, which in-turn triggers disruption in lipid and glucose homeostasis. However, the contribution of low levels of micronutrients, such as B12 to the pathogenesis of obesity and dyslipidemia has not been fully explored. This review is aimed at summarizing the current knowledge and latest evidence of the effect of low B12 on lipid metabolism, particularly the clinical studies and epidemiological observations from pregnant women, adolescents, and adults. It will also summarize the pre-clinical evidence from cell-lines, animal models and the possible molecular and epigenetic mechanisms involved.

The current review has been produced following a detailed search of literature using the MEDLINE/PubMed [11] and Global Health (CABI) [12] databases. Medical subject headings (MeSH) and keywords such as "vitamin B12 deficiency", "insufficiency of B12", "vitamin B12" or "cobalamin" were used. Combinations of these keywords with "pregnancy", "vegetarians", "lipid metabolism" and "metabolic disorders" were achieved using Boolean operators (OR, AND).

2. B12: Biochemical Structure, Sources, Bioavailability, Cellular Uptake and Metabolism

B12 (cobalamin) is classified as an essential vitamin as it is entirely obtained from diet. It is also synthesized naturally by some large intestine-resident bacteria in humans [13]. However, the site of absorption in the small intestine is significantly distant from the site of synthesis, accounting for non-bioavailability of the naturally synthesized B12 in humans [14,15]. B12 was first isolated in the year 1948 [16], following the discovery of using liver extract as a source of therapy for pernicious anemia [17]. Using x-ray crystallography, B12 was structurally described as a massive organometallic compound with its size ranging between 1300 to 1500 Da [18]. The vitamin is uniquely composed of a central cobalt atom linked to six ligands, with four of the latter structurally reduced to form a corrin ring that connects and encircles the former through direct nitrogen linkages. Directly below the central cobalt is an α-axial 5,6-dimethylbenzimidazole (DMB) ligand which, through a phosphoribosyl moiety, links to the corrin ring and confers a high specificity on the vitamin for intrinsic factor (IF) binding in the lower gastrointestinal tract [19]. The β-axial ligand (R-ligand), positioned above the corrin ring, may differ in diverse forms such as methyl, 5′–deoxyadenosyl, hydroxo, acquo or cyano groups. These are, therefore, named as methylcobalamin, deoxyadenosylcobalamin, hydroxycobalamin, acquocobalamin and cyanocobalamin, respectively.

B12 is predominantly absent in plants, however, it is reported that traces of B12 could be obtained in dried purple and green lavers and some edible algae [20]. Natural sources are predominately present in animals and animal products such as meat, eggs and milk. The loss of fat and moisture associated with cooking of beef has been reported to account for an average loss of 27–C33% B12 per unit nitrogen, suggesting that extensive cooking might decrease the levels of B12 in food [21]. The bioavailability of B12 in eggs is relatively lower (<9%) compared with other sources such as meats from chicken (61–66%), sheep (56–89%) and fish (42%) [20]. It is also presumed that in healthy adults, with non-defective gastrointestinal tract (GIT), the bioavailability of dietary B12 is about 50%. In addition, the GIT-resident microorganisms possess the enzymes needed for B12 biosynthesis [22,23]. The absorptive capability of the GIT of an individual undergoes adverse alteration with age, affecting the bioavailability of B12 in humans [24]. However, the absorption of B12, aided by availability of calcium, occurs at terminal ileum where precise receptors (cubam) are expressed on the microvilli on the intestinal endothelium. These receptors bind the IF-B12 complex followed by B12 delivery into the peripheral blood [24,25].

In humans, the plasma proteins transcobalamin (TC) and haptocorrin (HC) are proposed to be crucial in the transport of B12 through circulation. TC, demonstrating a relatively increased selectivity

for B12 in humans, forms a TC–B12 complex called holo-transcobalamin (holo-TC) which is reported as the biologically active proportion of B12 within circulation [26]. The precise role of HCs in humans is unclear, although they bind the greater proportion (80%) of circulating B12. HCs are reported to express a relatively lower selectivity for B12, compared to TC, suggesting that they might either play a role in the clearance of damaged forms of B12 or serve as B12 reservoirs in humans [26]. Generally, within all body cells including hepatocytes (Figure 1), B12 is believed to undergo processing into key cytosolic (MeCbl) and mitochondrial (AdoCbl) co-enzyme forms. A significant amount of the biologically active water soluble B12 is believed to accumulate in the liver of healthy humans. In liver biopsy samples from healthy humans, the average B12 content per gram of wet liver tissue is 1.94 µg (range: 1.41 to 2.58 µg) [27]. It is reported, however, that B12 content of the human liver may be lower in cases of cirrhosis, viral hepatitis, fatty liver and or obstructive jaundice [27]. Moreover, the human body is known to eliminate a total of about 2 to 5 µg of B12 daily, primarily, via feces and minimal amounts in urine [28].

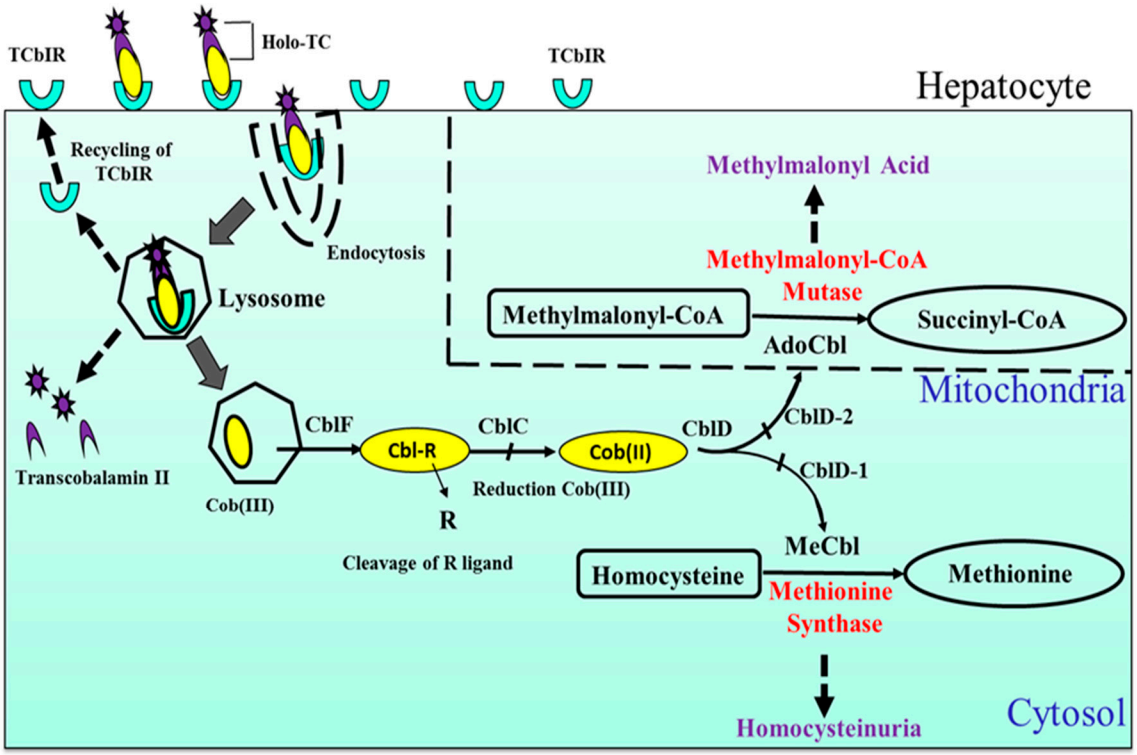

Figure 1. Cellular (hepatocyte) uptake and metabolism of vitamin B12 (B12): Cells generally internalize B12-bound transcobolamin (holo-TC) with the aid of transcobalamin receptor (TCR) TCbIR/CD320 via endocytosis and fused into lysosomes. Within this organelle, B12 is liberated from the TC with the latter (Apo-TC) subjected to degradation whilst the former (B12) is transported to the cytosol and further processed to its catalytic forms, methyl-cobalamin (MeCbl) and 5'-adenosyl cobalamin (AdoCbl), acting in the cytosol and mitochondria as co-enzymes in the methyl malonyl CoA mutase (MCM) and methionine synthase (MS) pathways, respectively. The transcobalamin receptors (TCbIR) are however recycled back to the cellular surface membrane.

3. Vitamin B12 Deficiency: Diagnostic Markers and Risk Factors

There is no international consensus on the lower limit of B12 to define B12 deficiency in adults and pregnancy. But there is some agreement that it should be between 120 and 220 pmol/L (higher threshold in pregnancy) with the upper limit between 650 and 850 pmol/L [29]. Other parameters, such as holo-TC, methyl malonic acid (MMA) and homocysteine (tHcy), are better markers of tissue level B12 deficiency, especially when the serum B12 levels are borderline (150–220 pmol/L) [5]. In the expanded

newborn screening, estimation of metabolites such as propionylcarnitine, MMA and tHcy in samples of newborn dry blood spot, using the tandem mass spectrometry, can facilitate the early diagnosis of infant B12 deficiency resulting from maternal low B12 [30]. Since elevation of the metabolites MMA and tHcy is associated with congenital disorders of B12 (as shown in Table 1), these also should be considered in the differential diagnoses [31].

Currently, there is limited data on the prevalence of B12 deficiency globally, based on tHcy MMA levels [32]. The risk of developing B12 deficiency is higher in vegetarian populations such as in India [33]. However, it is not uncommon in other populations, ranging between 10 and 30% [29]. And is higher in pregnant populations [29]. Some of the causes of B12 deficiency are shown in Table 1.

Table 1. Causes of B12 deficiency [34].

(a) Reduced B12 Intake i. Malnutrition ii. Vegetarian diet iii. Alcohol abuse iv. Old age (>75 years)
(b) An Impairment of B12 Bioavailability Via Gastric Wall Damage (with a Decrease in Intrinsic Factor)
i. Total (in certain stomach cancers) or partial gastrectomy including bariatric surgery.
ii. Atrophic autoimmune gastritis (such as pernicious anemia) or other gastritis (e.g., *Helicobactor pylori*).
(c) Impairment of Absorption Via the Intestines
i. Blind-loop syndrome
ii. Overgrowth of bacteria, giardiasis and tapeworm infections
iii. Ileal resection iv. Crohn's disease
(d) Inherited (Congenital) Disorders of B12 Deficiency
i. Defect of the intrinsic factor receptor such as in ImerslundGräsback syndrome
ii. Juvenile pernicious anemia—Congenital intrinsic factor (IF) deficiency
iii. Cobalamin mutation (C-G−1 gene)
iv. Deficiency in Transcobalamin (TC) v. Methylmalonic acidemia, homocystinuria and combined methylmalonic acidemia and homocystinuria [31]
(e) Increased B12 Requirements
i. Hemolytic anemic conditions
ii. HIV infection
(f) Drugs
i. Metformin ii. Proton pump inhibitors iii. Prolonged use of histamine receptor 2 (H_2) blockers (especially >12 months)

4. Association of Low B12 with Cardiometabolic Risk

4.1. Evidence from Pre-Clinical Studies

Currently, pre-clinical evidence shows that the effect of low B12 on cardiometabolic risk has been demonstrated mainly using animal models (Table 2). Emerging studies have begun testing some of these hypotheses on some human cell line and primary cell models. Potential mechanisms underlying the low B12-cardiometabolic risk associations are being elucidated at the pre-clinical level. In animal studies, low or deficient B12 level was defined based on plasma levels of B12 in animals fed with specified diet whereas in in vitro studies, based on the levels of B12 present in the culture media. The pre-clinical evidence of low B12 that have a role on cardiometabolic risk are provided below.

4.1.1. Vitamin B12 and Obesity

A study in adult Wistar rats that were fed a B12 restricted diet (control−0.010 mg/kg B12 vs. B12 restricted–0.006 mg/kg B12) during maternal or postnatal period, predicted higher visceral adiposity and resulted in alteration in the metabolism of lipids in the offspring [35]. Low plasma B12 (277 pg/mL B12) resulting from B12-restricted diet or a combination of B12- and B9-restricted diet (219 pg/mL B12) for a period of three months in pre-pregnant Wistar rats resulted in increased body weight compared to control (1164 pg/mL B12) [35]. B12-deficient rats had increased total body fat whereas B9-deficient rats presented with elevation in visceral fat mass [35]. Likewise, in female C57 BL/6 mice, severe decrease in plasma B12 (138 pg/mL), but not moderate (208 pg/mL) (compared to 406 pg/mL control B12) was reported to induce higher adiposity and altered lipid profile in their offspring [10].

4.1.2. Vitamin B12 and Insulin Resistance

Restriction of maternal B12/folate/methionine (control vs. restricted: B12 (1000.5 pM vs. 198 pM), folate (6.90 nM vs. 4.42 nM), methionine (39.1 μM vs. 30.8 μM)) at the stage of conception in sheep models, showed increased resistance to insulin and elevated blood pressure in its offspring [36]. In addition, the adult male offspring had higher adiposity and altered functioning of their immunity. This evidence was explained by the observation of male-specific demethylation of the affected loci, therefore, providing convincing reasons for the these differences observed in the phenotypes of the offspring [36]. Finally, the study concluded that the decreased methylation of DNA could be explained by lower availability of s-adenosyl methionine (SAM), a well-known methyl donor, which is crucial in epigenetic modulations underlying the development of resistance to insulin [36,37].

4.1.3. Vitamin B12 and Dyslipidemia

Mother Wistar rats with low plasma B12 (277 pg/mL B12 compared to 1164 pg/mL control) produced offspring which had increased levels of adiposity, triglycerides (TG) and total cholesterol as well as decreased leptin and adiponectin, compared to control offspring, showing a dysregulated metabolism of lipids [35]. C57 BL/6 mice with deficient plasma B12 levels at 12 weeks (145 pg/mL vs. 548 pg/mL control) and 36 weeks (123 pg/mL vs. 522 pg/mL control) had increased plasma TG, cholesterol and pro-inflammatory markers comprising tumor necrosis factor-alpha (TNFα), interleukin−1 b, interleukin−6, as well as lower adiponectin concentrations [38]. B12 deficiency inhibited beta oxidation of fatty acids and lipolysis in hepatic tissues of rat offspring born to B12 deficient mothers (227 pg/mL B12 vs. 1164 gp/mL control) [39]. However, in these studies, B12 supplementation during parturition resulted in the restoration of both pathways [39]. In our recent study, targeting the elucidation of the cellular mechanism induced by low B12 in human adipocyte cell line (Chub-S7 (0.15 nM compared to 500 nM control)), elevated levels of cholesterol [40] and TG [41] were observed in low B12 cells compared with controls. This was explained by a reduction in the methylation potential and the SAM: SAH ratio in low B12 conditions [40]. Validation of the above findings was further endorsed in primary human adipocyte models, demonstrating that B12-deficient primary human adipocytes had significantly elevated levels of total cholesterol, tHcy and mRNA expression of key genes that regulate the biosynthesis cholesterol, as compared to controls [40].

4.1.4. Vitamin B12 and Cardiovascular Diseases

Adult male Sprague–Dawley rat model treated with testosterone enanthate (0.5 mg/100 gm) and B12 (500 μg/kg) demonstrated significant changes with peripheral cortisol and association with vascular dysfunction [42]. Another study involving four-weeks simultaneous therapy with 10 mg/kg folate and 500 μg/kg B12 in Wistar rats, aiming to assess the effect of B12 and folate supplementation on myocardial infarction (MI) in rats presenting with hyperhomocysteinemia, there was a significant reduction in the elevated heart rate and blood pressure as well as attenuation of severe cardiac histopathological alterations [43]. The study suggested that aggravation of MI risk might result from

hyperhomocysteinemia, however, B12 and/or folate administration may reduce MI risk and tHcy levels, accounting for reduced harmful consequences associated with hyperhomocysteinemia [43].

Table 2. The effect of low B12 on components of cardiometabolic risk in pre-clinical studies.

Obesity	Insulin Resistance	Dyslipidemia	Cardiovascular Diseases
a. Increased visceral adiposity in Wistar rats [35].	Increased resistance to insulin and elevated blood pressure in sheep [36,37]	a. Increased adiposity, TG and total cholesterol levels in Wistar rat models [35].	a. Disruption of androgen testosterone levels associated with vascular dysfunction in Sprague–Dawley rat model due to low B12 [42]
b. Higher adiposity in female C57 BL/6 mice [10].		b. Increased plasma TG, cholesterol and some pro-inflammatory markers [38]	b. Reduction in myocardial infarction (MI) risk and tHcy levels due to B12 and/or folate supplementation in rat models [43]
c. Increased body weight in Wistar rats [35,38]		c. Increased cholesterol levels in human adipocyte cell line (Chub-S7) [40]	
d. Higher total body fat in Wistar rats [35,38]		d. Increased TG in human adipocyte cell line (Chub-S7) [41].	

4.2. Evidence from Clinical Studies

Most observational and epidemiological evidence on the effects of B12 on metabolic risk are from an Asian population but there are few studies in Western populations. As previously stated, the term "low B12" in all clinical evidence reported in this review refers to plasma/serum B12 levels ≤120–300 pmol/L (Table 3).

4.2.1. Vitamin B12 and Obesity

An association between maternal body mass index (BMI) at early stage pregnancy and plasma B12 and/or B9, in a recent cross-sectional study, was observed in obese women compared to women with normal BMI [44]. Sukumar et al. reported that pregnant women presenting with low B12 at the first trimester had increased BMI compared with those with normal B12 levels [45]. In India, low maternal level of B12 was suggestive of contributing to an increased likelihood of developing higher adiposity and insulin resistance in the offspring [46]. Likewise, the mean B12 concentration was significantly lesser in children with obesity, compared to healthy volunteers, and was negatively associated with the severity of obesity [47]. In a recent systematic review of clinical data, although not conclusive, lower concentrations of B12 were observed in obese individuals compared to overweight and normal weight individuals [48].

4.2.2. Vitamin B12 and Insulin Resistance

In White Caucasian pregnant women without gestational diabetes mellitus (GDM), an inverse relationship between B12 level and insulin resistance was reported [49]. Low level of B12 in the plasma of mothers at early but not late pregnancy, was linked with significant increase in the resistance to insulin expressed by homeostasis model of insulin resistance (HOMA-IR) in their children [50]. However, there was no relationship established between B9 status in the mothers and insulin resistance in their children [50]. Similarly, within school children of Nepal, high insulin resistance was observed as a result of low maternal levels of B12 [51]. In obese adolescents with low or borderline levels of B12, there was an association between low B12 status and insulin resistance and obesity [52]. In support of these clinical studies, a study from our group in low B12 pregnant women demonstrated that altered circulating micro-RNAs derived from adipose tissues could possibly mediate adipogenic and insulin-resistant phenotypes as a precursor to obesity [41]. In addition, studies from other population

groups such as women with polycystic ovarian syndrome, adults (males and females) in a primary care setting, and obese adolescents (males and females) also showed low B12 levels were associated with higher insulin resistance [29,53,54].

4.2.3. Vitamin B12 and Dyslipidemia

Clinical studies involving three independent cohorts of women; (i) at child-bearing age, (ii) in early pregnancy, and (iii) at delivery, revealed that low levels of B12 were associated with elevated levels of low-density lipoprotein (LDL)-cholesterol, total-cholesterol and cholesterol-to-high-density lipoprotein (HDL) ratio [40]. The study also showed that the women had high prevalence of low B12 (14% in child bearing age, 45% in early pregnancy, 40% during delivery) [40]. Maternal subcutaneous adipose tissues (ScAT) obtained from mothers with low circulating B12 showed upregulation of genes involved in the biosynthesis of cholesterol, such as 3-hydroxy−3-methylglutaryl-CoA reductase (HMGCR), sterol regulatory element binding protein 2 (SREBF2), low-density lipoprotein receptor (LDLR) and sterol regulatory element binding protein 1 (SREBF1) [40]. These studies implicate that low B12 status might be causally linked to increased levels of low-density lipoprotein cholesterol (LDL-C), total cholesterol, cholesterol-to-high-density lipoprotein (HDL) ratio and subsequent insulin resistance. Further evidence from other studies have shown that babies born to low B12 mothers are 'thin-fat', a term describing phenotypically lean individuals with increased fat accumulation in their bodies as well as decreased lean mass [46,55]. This may consequently lead to elevated resistance to insulin as well as increased risk of developing CVD in adulthood [46,55]. In addition, studies in patients with non-alcoholic fatty liver disease (NAFLD) and non-alcoholic steatohepatitis (NASH) showed that low B12 level significantly increased the levels of triglycerides, cholesterol and blood glucose levels in these patients [56]. A study in adult population from North India showed that low B12 level was observed to be significantly associated with both low HDL and hyperhomocysteinemia, whereas after controlling for the confounder tHcy, B12 was found to be associated with all lipid indices including HDL [57]. A negative correlation was also observed between B12 levels and the prevalence of MetS in euthyroid participants [58].

4.2.4. Vitamin B12 and Cardiovascular Diseases

B12 plays a crucial role in the generation of methionine from tHcy via re-methylation [59]. Therefore, low B12 is associated with an elevation of circulatory tHcy which is known to associate with the risk of CVD [60]. In Chinese subjects, a study showed a correlation between the highest circulating levels of B12 and the lowest tHcy levels in patients presenting with CVD [61]. Similarly, in Japanese subjects, evidence of an inverse relationship was observed between dietary folate (B9) and B6 intake and the incidence of heart failure mortality in men as well as mortality resulting from stroke, coronary artery disease (CAD) and total CVD in women [62]. This was similar to the evidence in an American population study in which therapy for about 7.3 years with a B12–B6–folate combination pill resulted in a significant decrease in tHcy levels [63]. Other studies suggested that supplementation with 250 µg B12 and 5 mg folate resulted in 32% reduction of fasting plasma tHcy levels following 12 weeks of therapy in patients with CAD [64]. A study by Setola et al. [65] demonstrated that folate and B12 treatment in patients with MetS improved resistance to insulin and endothelial dysfunction as well as decreased tHcy levels, suggesting the beneficial effects of these vitamins on CVD risk factors. Recent evidence has shown that elevated tHcy and decreased circulatory B12 levels in women, demonstrated a strong association with higher risk of all-cause and CVD deaths in the elderly population [66]. Meta-analysis of several prospective studies shows reliable evidence of a correlation existing between plasma tHcy and elevated CVD risk [67]. For instance, in different randomized control trials (RCTs) involving an established renal disease or CVD, B12 supplementation using a dosage range of 0.4–1.0 mg B12 per day accounted for a significant reduction in the risk of developing stroke [67]. Meanwhile, studies have proposed that hyperhomocysteinemia and cardiovascular risk in patients may precede the development of end-stage renal disease (ESRD), chronic kidney disease (CKD) and dialysis [68–70].

Although CKD patients demonstrate an impairment in tissue uptake of B12 resulting in functional deficiency [71], the current evidence remains unclear to consider altered B12, folate and elevated tHcy levels as markers for CVD and cardiovascular mortality risk in ESRD and CKD individuals [72].

Table 3. The effect of low B12 on components of cardiometabolic risk in clinical studies.

Obesity	Insulin Resistance	Dyslipidemia	Cardiovascular Diseases
a. Low B12 (<150 pmol/L) was associated with increased adiposity with higher T2D risk in pregnant women [46]	b. Low B12 (<180 pmol/L [49]) was associated with increased resistance to insulin in White Caucasian pregnant women without GDM.	a. Low B12 (<148 pmol/L) was associated with elevated levels of LDL-cholesterol, total-cholesterol and cholesterol-to-HDL ratio in pregnant and non-pregnant women at childbearing age [40]	a. B12 was negatively correlated with tHcy levels in Chinese-CVD patients ≥ 65 years of age (median low B12 = 4.19 pmol/L) [61].
b. Low B12 (median−203 pmol/L [44] and <148 pmol/L [48]) levels were associated with increased BMI in maternal and general and/or clinal populations respectively	b. Prediction of higher risk of resistance to insulin in children born to low B12 (<150 pmol/L [46], 148 pmol/L [50]) mothers	b. Low B12 (≤220 pg/mL) was associated with both low HDL and hyperhomocysteinemia in North Indian population [57]	b. Low B12 (<148 pmol/L) and high tHcy levels were associated with higher risk of all-cause and CVD deaths in aged women [66]
c. Low B12 (102–208 pmol/L interquartile range) was associated with obesity in children compared to healthy volunteers [47].	c. Low B12 (≤150 pmol/L [29], <148 pmol/L [52], <200 pg/mL [53], <178 pg/mL [54]) levels were associated with increased IR in adult patients, polycystic ovarian syndrome women and obese adolescents	c. B12 negatively correlated with markers of MetS such as low HDL and high TG levels in erythroid patients (Low B12 range 180–301 pmol/L) [58]	c. B12 supplementation reduced the risk of stroke in patients with CVD and/or renal disease [67].

5. Molecular Mechanisms Regulating Low B12 and Lipogenesis

Environmental factors such as nutrition may affect lipid metabolism by targeting at the transcriptional level, thereby, regulating the expression of genes and subsequently the phenotype without altering the sequence of nucleotides by the process of epigenetics [73]. B12 plays a crucial role in humans by serving as a cofactor in the reaction pathways essential for maturation and preservation of cells [74,75]. In the cytosol, B12 is a cofactor for methionine synthase (MS) (Figure 2), which is actively involved in the biosynthesis of methionine via re-methylation of tHcy and in turn generation of s-adenosyl methionine (SAM), a potent donor of methyl groups for methylation of several biochemical processes. Currently, the well-known markers of epigenetic mechanisms that regulate the metabolism of lipids and resistance to insulin are DNA methylation, microRNAs, chromatin remodeling as well as modification of histones [76,77]. B12 acting as a co-enzyme for a source of methyl groups (SAM), regulates these epigenetic mechanisms and several transcriptional as well as post-translational factors involved in the process of de novo lipogenesis. In the mitochondrial based propionate metabolism pathway, B12 serves as a cofactor in the conversion of methyl malonyl-CoA to succinyl-CoA by methyl malonyl-CoA mutase (MCM) (Figure 2) [78]. Succinyl-CoA is utilized as substrate in the Krebs cycle for ATP synthesis essential for the sustenance of cellular metabolisms (reviewed by [79]) and utilized for hemoglobin synthesis in red blood cell production. Low B12 levels, therefore, results in a reversible increase in methyl malonyl-CoA leading to accumulation of MMA. MMA, in turn, acts as a potent inhibitor of the rate limiting enzyme carnitine palmitoyl transferase 1 (CPT1), critical for the breakdown of long chain fatty acids in the beta oxidation pathway. This inhibition of CPT1 may build up fatty acids and TGs (reviewed by [80]) accounting for higher lipogenesis and insulin resistance [81] which might result in dyslipidemia.

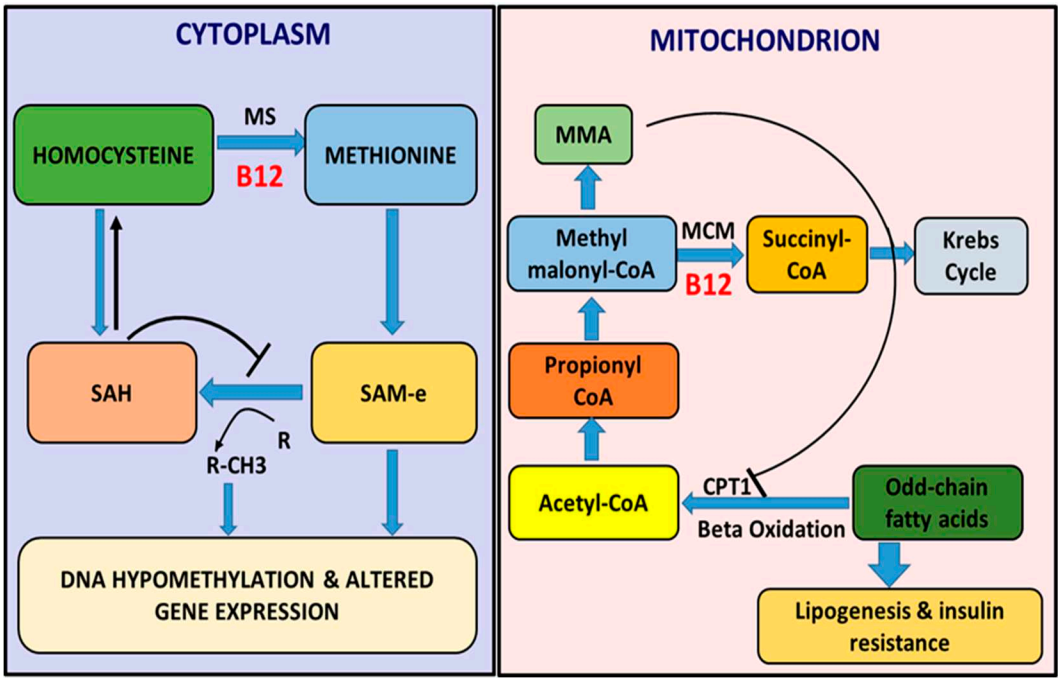

Figure 2. Cellular role of B12 in lipogenesis. There is a reduction in the production of methionine as well as the methyl donor s-adenosyl methionine (SAM) within the cell's cytosol, resulting from B12 deficiency, leading to hyperhomocysteinemia as well as reversible increase in s-adenosyl homocysteine (SAH) which is known to be an inhibitor of DNA methyl transferases (DNMTs). The inhibition of DNMTs together with low levels of SAM results in hypomethylation of DNA and altered gene expressions. Beta oxidation of fatty acid is inhibited by generation of methyl malonic acid (MMA) from methyl malonyl-CoA within the mitochondria due to insufficiency of B12, a cofactor for methyl malonyl-CoA mutase (MCM) enzyme required for the biosynthesis of succinyl-CoA from methyl malonyl-CoA in the propionate metabolism pathway.

6. Epigenetic Mechanisms Underlying Low B12 Levels in Lipid Metabolism

There is currently, emerging evidence suggesting that abnormalities associated with one-carbon metabolites due to low B12, might possibly exert their modulations through epigenetic mechanisms. Some preliminary findings have indicated that induction of alterations in normal methylation of DNA is due to changes in the levels of one-carbon metabolites. However, it was suggested that in order to elucidate the molecular mechanism underlying these effects, there is a need for future studies to target combinations of gene expression assays with epigenetic studies [79]. Studies in animals are likely to be more informative as clear elucidation of molecular mechanisms in humans might be difficult due to tissue-specificity of the epigenetic phenomena [79].

6.1. DNA Methylation and Regulation of Lipid Metabolism through Low B12

Methylation generally involves the process of transferring a methyl group to substrates such as enzymes, proteins, amino acids and DNA in diverse cells and tissues [82]. In the process, guanine is normally bound with 5-methylcytosine following its development from cytosine [83]. Usually, hypermethylation of global DNA and 5′-cytosine-phosphate-guanine−3′ (CpG) islands located within the promoters of genes involved in lipid synthesis, accounts for the stability of the genome and silencing of the lipogenic genes, respectively [84]. This is greatly dependent on the availability and sufficiency of SAM synthesized with the aid of methyl donors such as B12 and folate [76]. Low B12 independently impairs the MS action [85] affecting SAM/SAH ratio which is a principal determinant of the methylation potential of cellular DNA, thereby inducing dysregulation of gene expressions [86,87]. Demethylation

associated with DNA of the genome, persists as one of the earliest evidences of epigenetic modifications triggered by diets deficient in methyl donors, contributing to hepatocarcinogenesis in animals [88].

A study in zebrafish showed that micronutrient-deficient diet (without 1-carbon nutrients B12, B9, B6, methionine and choline supplementation compared to control), fed to parents affected the hepatic methylation of DNA and gene expression of lipogenesis leading to lipid accumulation in the F1 offspring [87]. High global methylation of DNA and promoter region methylation of lipid genes are critical to the maintenance of normal physiology of metabolism [89,90] by ensuring stability of the genome and silencing of abnormal lipogenic genes [84]. A study in an adipocyte model showed that hypomethylation in promoter regions led to higher expressions of LDLR and SREBF1 genes and cholesterol biosynthesis in low B12 (0.15 nM B12 media compared to 500 nM control) [40]. Increased genome incorporation with uracil subsequent to hypomethylation was observed in B12-deficient rats (50 μg/kg B12 fed to Sprague–Dawley rats) [91], whereas fatty acid supplementation in low B12 conditions could not restore global methylation [92]. Evidence of hypomethylation, as seen in low B12, was observed in the CpG sites of some liver-derived genes involved in the pathogenesis of T2D due to low folate levels [93]. Methyl donor supplementation in rat obesogenic models ameliorated hypomethylation near the promoter of genes such as acylglycerol−3-phosphate-O-acyltransferases 3 (AGPAT3), SREBF2 and ESR1 (estrogen receptor−1) resulting in the reversion of higher fatty acid, TG and cholesterol accumulation in the liver [94]. These studies thus implicate that methyl donor deficiency compromises methylation capacity, resulting in the dysregulation of lipid metabolism [87,95,96] which can be reversed by supplementation of methyl donors.

6.2. MicroRNAs (miRNAs) and Regulation of Lipid Metabolism through Low B12

MicroRNAs (miRNAs), formerly termed as small temporal RNAs (stRNAs), are the class of small RNAs comprising 21–25 nucleotides of single-stranded RNA which are highly conserved and engaged in regulating the expression of genes at the transcriptional and post-translational levels [97]. MiRNAs physiologically express diversified patterns and engage in the regulation of certain genes underlying the metabolism of lipids and inflammation [98,99]. MiRNAs are proposed to be engaged in the modulation of adipocyte differentiation accounting for development of insulin resistance, T2D and dyslipidemia [100,101]. Genes regulating hepatic metabolism of fatty acids and/or insulin signaling may be repressed by some miRNAs regulating the levels of HDL, TG and insulin [102]. About 100 different miRNAs are expressed differentially in cases of NASH in humans [103] and alterations in miRNA-29c, miRNA-34a, miRNA-200b and miRNA-155 are due to methyl-donor deficiencies [104]. In humans, miRNA-122 is largely expressed in hepatocytes but under expressed in NASH [104] and therefore is proposed to be the potential target for treatment of dyslipidemia [105] and high cholesterol [106]. Using adipocyte models, our group reported that twelve different adipocyte-derived miRNAs targeting peroxisome proliferator-activated receptor gamma (PPARγ) (miRNA-31, miRNA-130b and miRNA-23a), adipocyte differentiation (miRNA-143, miRNA-145, miRNA-146a, miRNA-125b, miRNA-222 and miRNA-221), CCAAT/enhancer-binding protein alpha (miRNA-31) and pathways of insulin resistance (miRNA-107 and miRNA-103a) were significantly altered due to low B12, thereby modulating adipocyte differentiation and physiology [41]. In addition, B12 levels were positively associated with seven different circulating miRNAs (miRNA-27b, miRNA-130, miRNA-103a, miRNA-107, miRNA-125b, miRNA-23a, miRNA-221 and miRNA-222). Of these, four (miRNA-107, miRNA-27b, miRNA-23a and miRNA103a) were inversely and independently associated with maternal BMI, similar to low B12 after adjusting for likely confounders (such as age, parity, smoking, supplement use, glucose and insulin). The reduction in effect size between B12 and BMI on additional adjustment for these four miRNAs, highlights that some of the negative effects of B12 on BMI may be mediated by these miRNAs [41].

6.3. Modifications of Histones and Regulation of Lipid Metabolism through Low B12

The mechanistic effect of B12 on hepatic lipid metabolism regulation via modulation of histones is primitive. However, mechanisms including methylation, acetylation, phosphorylation as well as

ubiquitylation are proposed to be involved in ubiquitylation modification of histones that contribute to lipid metabolism regulation in the liver. The most intensively understood mechanism directly involved in dysregulation of hepatic lipid metabolism is histone acetylation which is normally catalyzed by histone acetyltransferases (HATS) and histone deacetylases (HDACs). In female C57 BL/6 mice, chronic deficiency of B12 (580 pmol/L B12 compared to 2331 pmol/L control) showed a dysregulated histone-modifying enzyme expression in the brain and abnormal behavioral anomalies [107]. Recent evidence from pilot data indicated that low folate level in the liver was associated with higher levels of methylation in H3 K4 [108]. Reduced methylation of histone has also been shown in liver X receptor-alpha (LXRα) in rats, following consumption of a high-fat diet after the third generation in rats [109]. Histones such as H3 lysine 4 (H3 K4) and H3 lysine 9 (H2 K9) are known to be subjected to regulation by methylation of the histones in association with DNA methylation [110]. Evidence of deficiency in the methyl donor choline in C57 BL/6 mice at the 12th–17th day of gestation resulted in alterations in the methylation of histone H3, whereas choline after undergoing conversion to betaine, enhanced re-methylation to tHcy [111]. Convincing evidence shows the link between NAFLD [73], resistance to insulin and obesity with modification of histone such as demethylation of H3 at lys9 [112]. Conditions of hyperlipidemia and obesity were observed in mouse models following the functional loss of Jhdm2 a, the H3 K9-specific demethylase, which implies that the status of methylation of H3 K9 is very essential and may tend to regulate the expression of metabolic genes [113]. Further studies are recommended to determine the exact role of B12 on the metabolism of lipids via modulation of histones.

7. Impact of One-Carbon Micronutrient Supplementation on Lipid Metabolism

In recent times, although agents that potentially resolve insulin resistance appear promising, the general therapy for correcting dysregulation of lipogenesis has not been established. However, current treatment may be reliant on alterations in lifestyle such as diet, weight reduction and/or exercise [114]. It is generally suggested that interventions involving fortification of foods, supplementation with B12 during preconception period and provision of adequate education could collectively be the most effective ways to enhance improvement in the levels of B12 in infants as well as mothers [115]. Supplementing with a diet rich in one-carbon nutrients such as vitamin B6, B9 and B12, is the principal way to significantly influence methylation of DNA because of their contribution to the synthesis of SAM. A similar micronutrient, betaine, obtained from food functions as a methyl donor and has been identified as a potent alleviator of fatty liver. Betaine was shown to facilitate the export of triglycerides from the liver as a way of attenuating steatosis, especially in cases of NAFLD [116]. Post-supplementation with only B12 was associated with significant alteration in the methylation of DNA in which 589 CpGs demonstrated differential methylation in addition to 2892 regions. Supplementation with both B12 and folate resulted in differential methylation of 169 CpGs and 3241 regions, thereby influencing the expression of genes associated with T2D [117]. Evidence of higher percentage of transmethylation to methionine and reduced transculturation to cysteine were observed as a result of supplementation with both B12 and milk protein compared with only milk supplementation in women. Therefore, among Indian women presenting with low B12 in early pregnancy, supplementation with B12 and energy-protein balance plays a crucial role in enhancing the optimum function of the methionine cycle especially at the third trimester of pregnancy [118]. Similarly, B12, folate, choline and betaine-rich methyl donor supplements were used to regress the accumulation of fats in the liver induced by high-fat-sucrose probably by changing the levels of methylation of CpG areas in the promotor regions of SREBF2, estrogen receptor 1 (ESR1) and/or acylglycerol−3-Oacyltransferase−3 (AGPAT3) [96]. Hypermethylation of DNA in fatty acid synthase (FAS) gene expression was estimated after supplementation with methyl donors that apparently boosted the retrogression of NAFLD-induced by high fat diet [94]. Normalization of histochemical indices in hepatic lobules was achieved in an animal-model study where the accumulation of lipids towards the center of hepatic lobules was brought under control subsequent to treatment with methionine

in combination with vitamin B12 rather than B15 [119]. Finally, oral B12 supplementation during pregnancy resulted in higher plasma B12 levels in infants and reduction in MMA and tHcy levels [120].

8. Conclusions and Prospects

In summary, the global incidence of metabolic disorders is increasing with advancement in urbanization in addition to several environmental and genetic factors. The pathogenesis of MetS, T2D and CVD have been associated with deficiencies in micronutrients. The effect of low B12 on the pathogenesis of several metabolic disorders such as obesity, insulin resistance, T2D and CVD, has been studied at the pre-clinical and clinical levels. Clinically, low B12 status in children, adolescents and pregnant mothers was associated with higher adiposity and lipids, as well as increased risk of insulin resistance, T2D and CVD. Babies born to low B12 mothers via adverse maternal programming seem to have a high accumulation of adiposity and insulin levels at birth which may predispose them to a higher risk of developing cardiometabolic disorders in later life. This review provides compelling evidence that the regulation of lipids and increased adiposity are associated with low B12 via epigenetic mechanisms. Future studies with a critical emphasis to establish causality by understanding the functional relevance of these epigenetic changes induced by low B12 are required.

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Abbreviations

A	
AdoCbl–5′Adenosyl cobalamin	Adenosyl cobalamin
AGPAT3	Acylglycerol−3-phosphate O-acyltransferases 3
B	
BMI	Body mass index
C	
CAD	Coronary artery disease
CHD	Coronary heart disease
CKD	Chronic kidney disease
CpG	5′cytosine-phosphate-guanine-3′
CPT1α	Carnitine palmitoyl transferase I alpha
CVD	Cardiovascular diseases
D	
DAG	Diacylglycerol
DGAT 1&2	Diacylglycerol acyltransferase 1&2
DKA	Diabetic keto acidosis
DM	Diabetes mellitus
DMB	5, 6-dimethylbenzimidazole
DNMTs	DNA methyl transferases
E	
ESRD	End-stage renal disease
ESR1	Estrogen receptor 1
ETC	Electron transport chain

F	
FAO	Fatty acid oxidation
FAS	Fatty acid synthase
FFA	Free fatty acids
G	
GDM	Gestational diabetes mellitus
GIT	Gastrointestinal tract
GPAT	Glycerol-3-phosphate acyltransferase
H	
HATS	Histone acetyltransferases
HC	Haptocorrin
HDACs	Histone deacetylases
HDL-C	High-density lipoprotein cholesterol
HFD	High fat diet
tHcy	total homocysteine
HMGCR	3-hydroxy-3-methylglutaryl-CoA reductase
HMGCS	3-hydroxy-3-methylglutaryl-CoA synthase
HoloTC	Holotranscobalamin
HOMA-IR	Homeostasis model of insulin resistance
I	
IDF	International Diabetes Federation
IF	Intrinsic factor
IR	Insulin resistance
L	
LCFAs	Long-chain fatty acids
LDL-C	Low-density lipoprotein cholesterol
LDLR	Low-density lipoprotein receptor
LXR-α	Liver X receptor alpha
M	
MCM	Methyl malonyl CoA mutase
MeCbl	Methyl-cobalamin
MetS	Metabolic syndrome
MI	Myocardial infarction
MMA	Methyl malonic acid
MS	Methionine synthase
MP	Methylation potential
miRNAs	MicroRNAs
N	
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
P	
PPAR-γ	Peroxisome proliferator-activated receptor gamma
S	
SAM	S-adenosyl methionine
SAH	S- adenosyl homocysteine
ScAT	Subcutaneous adipose tissues
SREBF1&2	Sterol regulatory element binding protein 1 and 2
stRNAs	Small temporal RNAs
T	
TCA	Tricarboxylic acid
TC	Transcobalamin
TCR or TCbIR	Transcobalamin receptor
T2D	Type 2 diabetes mellitus
TG	Triglyceride/Triacylglycerol
TNFα	Tumor necrosis factor-alpha

V	
VLDL	Very low-density lipoprotein
W	
WHO	World Health Organization

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Tumor necrosis factor-α directly stimulates the overproduction of hepatic apolipoprotein B100-containing VLDL via impairment of hepatic insulin signaling

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Qin B, Anderson RA, Adeli K. Tumor necrosis factor-α directly stimulates the overproduction of hepatic apolipoprotein B100-containing VLDL via impairment of hepatic insulin signaling. *Am J Physiol Gastrointest Liver Physiol* 294: G1120–G1129, 2008. First published March 27, 2008; doi:10.1152/ajpgi.00407.2007.—Insulin-resistant states are commonly associated with both increased circulating levels of tumor necrosis factor (TNF)-α and hepatic overproduction of very low density lipoproteins (VLDL). Here, we provide evidence that increased TNF-α can directly stimulate the hepatic assembly and secretion of apolipoprotein B (apoB) 100-containing $VLDL_1$, using the Syrian golden hamster, an animal model that closely resembles humans in hepatic VLDL-apoB100 metabolism. In vivo TNF-α infusion for 4 h in chow-fed hamsters induced whole-body insulin resistance on the basis of euglycemic hyperinsulinemic clamp studies. Immunoprecipitation and immunoblotting analysis of livers from TNF-α-treated hamsters indicated decreased tyrosine phosphorylation of insulin receptor (IR)-β, IR substrate-1 (Tyr), Akt (Ser^{473}), p38, ERK1/2, and JNK but increased serine phosphorylation of IRS-1 (Ser^{307}) and Shc. TNF-α infusion also significantly increased hepatic production of total circulating apoB100 and VLDL-apoB100 in both fasting and postprandial (fat load) states. Ex vivo experiments, using cultured primary hepatocytes from hamsters, also showed TNF-α-induced VLDL-apoB100 oversecretion, an effect that was blocked by TNF receptor 2 antibody. Unexpectedly, TNF-α decreased the sterol regulatory element-binding protein-1c mass and mRNA levels but significantly increased microsomal triglyceride transfer protein mass and mRNA levels in primary hepatocytes. In summary, these data provide direct evidence that TNF-α induces whole-body insulin resistance and impairs hepatic insulin signaling accompanied by overproduction of apoB100-containing VLDL particles, an effect likely mediated via TNF receptor 2.

TNF-α; liver; insulin resistance; lipid; lipoprotein; apoB

THERE IS GROWING EVIDENCE suggesting a strong link between systemic inflammation and type 2 diabetes mellitus (type 2 DM) (13, 21). Tumor necrosis factor (TNF)-α, a major proinflammatory cytokine, has been implicated in metabolic disorders, such as obesity and insulin resistance (33, 34), indicating that perturbations of TNF-α metabolisms may affect the onset of type 2 DM and play a role in the development of cardiovascular disorders. Plasma TNF-α concentration has been shown to positively correlate with very low density lipoprotein (VLDL) triglyceride in healthy middle-aged men (56) and patients who are postinfarction (38) and is associated with early atherosclerosis. Elevated levels of lipoproteins containing apolipoprotein B (apoB) are widely thought to contribute to the development of atherosclerosis in human and experimental animals, even in the absence of other risk factors (25). ApoB100 is the major protein component of plasma lipoproteins and is required for the synthesis and secretion of triglyceride-rich circulating lipoproteins such as VLDL (41). Numerous studies have shown that the insulin resistance observed in type 2 DM is associated with an overproduction of apoB100-containing lipoproteins and reduced VLDL fractional catabolism (10, 35). Furthermore, the excess production of hepatic $VLDL_1$ particles is the main abnormality of lipoprotein metabolism in type 2 DM (2, 3, 23).

Although a great deal of information on hepatic lipogenesis and VLDL production has been obtained in TNF-α-treated normoglycemic and diabetic rats (15–17), data available are conflicting and are limited to VLDL-triglyceride (TG) production; also rodent models employed have limitations when attempting to delineate the effect of TNF-α on VLDL synthesis and secretion in humans. TNF-α has been shown to induce an increase in in vivo VLDL-TG production in rats (15, 17), which was thought to be the result of decreased lipoprotein lipase activity and increased hepatic lipogenesis (15). However, these studies did not investigate the role of TNF-α on hepatic VLDL-apoB production, nor did they explore the underlying mechanisms. In addition, rat hepatocytes synthesize VLDL particles containing either apoB48 or apoB100, unlike human hepatocytes, which synthesize VLDL containing solely apoB100 (39). There are also conflicting data published on the effect of TNF-α on hepatic lipid metabolism in rat models. In contrast to the above studies, in vivo continuous TNF-α infusion of supraphysiological doses has been reported to reduce plasma TG levels in rats (58). Consequently, further investigation in this area is clearly needed to delineate the link between TNF-α and hepatic VLDL metabolism and explore the underlying mechanisms.

In the present study, we hypothesized that TNF-α can potentially induce hepatic VLDL overproduction via induction of hepatic insulin resistance. Systemic TNF-α infusion during hyperinsulinemic-euglycemic clamp has been shown to induce insulin resistance (46); however, no direct study exists on the link between TNF-α, hepatic insulin signaling, and hepatic lipoprotein production. In the present report, we examined the

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effects of TNF-α, hepatic-insulin signaling, and hepatic apolipoprotein production by using the Syrian Golden hamster, a model that closely resembles humans in hepatic VLDL metabolism. We present both in vivo and ex vivo evidence that TNF-α not only induces hepatic insulin resistance, but also stimulates fasting and postprandial overproduction of hepatic apoB100-containing $VLDL_1$ particles.

MATERIALS AND METHODS

Experimental animals. Male Syrian golden hamsters (*Mesocriceus auratus*) weighing between 130–150 g were obtained from Charles River (Montreal, QC, Canada). All animals were housed individually and given free access to food and water. Following a 1-wk acclimatization period, the animals either underwent the in vivo protocol or were euthanized for isolation of hepatocytes for the ex vivo protocols. All animal protocols were approved by the animal ethics committee at the Hospital for Sick Children, University of Toronto.

Euglycemic-hyperinsulinemic clamp study. The right jugular vein and the left carotid artery were exposed under isoflurane anesthesia, and hamsters were inserted with catheters encased in silastic tubing that were exteriorized to the back of the neck (48, 49). Following an overnight recovery period and a 16-h fast, a 4-h infusion of 0.9% normal saline (vehicle) or TNF-α (0.5 μg/kg per h) was administered by the venous catheter. With the infusion of saline or TNF-α continuing, a baseline blood sample was drawn at the 2-h time point. The hamster was then submitted to a euglycemic clamp procedure for the final 2 h to assess the whole-body insulin sensitivity. The venous catheter was then used for the infusion of glucose (20%) and insulin (3 mU/kg per min). TNF-α was dissolved in saline and administered at a dosage shown to be sufficient to effectively inhibit insulin action (66, 67). During the euglycemic clamp, blood glucose concentrations were measured every 10 min, and the euglycemia was maintained at around 4.2 ± 0.1 mM of baseline. The glucose infusion rate (GIR) was calculated every 10 min (48). Finally, the liver tissues were excised and stored at −80°C until analyses.

In vivo Triton WR1339 infusion and preparation of VLDL-apoB100 fractions. Hamsters were fasted overnight for 16 h, and the catheters were inserted as described (48). Following a 4-h recovery period, hamsters were infused with saline or TNF-α (0.5 μg/kg per h) for 4 h. After the first 2-h and 20-min infusion, an intravenous bolus of Triton WR1339 (0.5 g/kg) was administered. Triton WR1339 effectively blocks the activity of lipoprotein lipase in vivo and therefore blocks the VLDL particle clearance, such that the secretion rate of VLDL-apoB is proportional to the rate of increase in plasma VLDL-apoB over time (57). After Triton WR1339 administration, 300 μl of blood were collected (as baseline). An additional blood sample was taken at the 90-min time point following Triton WR1339 treatment. Studies performed in the postprandial state were as described above except that hamsters were manually administered a 200-μl olive oil (1 g polyunsaturated, 2 g saturated, 11 g unsaturated; 120 kcal) load via oral gavage. The bolus of Triton WR1339 was administered without prior fasting, and blood samples were drawn as described above.

To isolate the VLDL fraction, the serum samples were fractionated by rate flotation ultracentrifugation. A serum sample (100 μl) was mixed with 4 ml of 1.10 g/ml NaBr solution and loaded onto the bottom of a Beckman SW41 centrifuge tube. The sample was overlaid with 3 ml each of 1.065, 1.02 g/ml, and 2 ml of 1.006 g/ml NaBr solutions. After ultracentrifugation at 151,000 *g* at 4°C for 70 min, the top layer (1 ml) containing the triglyceride-rich lipoprotein (TRL) was removed, and 1 ml of 1.006 g/ml NaBr solution was added and centrifuged at 151,000 *g* at 4°C for 18 h. The top 2-ml layer was further fractionated to isolate VLDL ($VLDL_1$ and $VLDL_2$) particles, essentially as described (40). After immunoprecipitation with antiserum against hamster apoB antibody, samples were resolved in SDS-PAGE and subjected to immunoblotting using hamster apoB antiserum. ApoB bands were visualized and quantified using an imaging densitometer. To determine the total serum apoB100, the serum samples were diluted (200-fold) and then treated as described above.

Hamster primary hepatocytes. Hamsters were anesthetized by isoflurane. After achieving complete general anesthesia, the liver was perfused and hepatocytes were isolated as described (59, 60).

Preparation of nuclear extracts from primary hepatocytes. Crude nuclear extracts were prepared from primary hepatocytes, essentially as described (62). The hepatocytes are washed twice in 2 ml cold PBS and lysed in 500 μl of cold buffer A (10 mM HEPES pH 7.9, $MgCl_2$ 1.5 mM, KCl 10 mM, DTT 0.5 mM, aprotinin 100 μg/ml, leupeptin 5 μg/ml, pepstatin 1 μg/ml, and PMSF 0.5 mM). After a 15-min incubation on ice, 0.5% NP-40 in final concentration was added to the homogenates and the tubes were vortexed for 10 s. The nuclei were pelleted at 6,500 rpm (tabletop microfuge) for 20 s. The nuclear pellet was suspended in 150 μl of cold buffer B (20 mM HEPES pH 7.9, $MgCl_2$ 1.5 mM, NaCl 420 mM, EDTA 0.2 mM, glycerol 25% vol/vol, aprotinin 100 μg/ml, leupeptin 5 μg/ml, pepstatin 1 μg/ml, and PMSF 0.5 mM) and incubated on ice for 30 min. Nuclear extracts were recovered after centrifugation for 10 min at 12,000 rpm at 4°C. Protein concentration was determined by Bradford assay, and the aliquots were stored at −80°C until analysis.

Immunoprecipitation and immunoblotting. Hepatocytes were lysed, and the immunoprecipitation/immunoblotting protocols were performed as described (49, 59, 60).

Metabolic labeling of primary hepatocytes and measurement of VLDL-apoB secretion. Hamster primary hepatocytes were used for pulse-chase experiments as described (59) with some modifications. In brief, hepatocytes were first treated with TNF-α (10 ng/ml) in methionine/cysteine-free minimal essential medium at 37°C for 1 h and then used for pulse-chase experiments. After labeling with 100 μCi/ml [^{35}S]methionine/cysteine for 45 min, the pulse-labeled cells were treated with TNF-α (10 ng/ml) in the chase medium. Following the pulse, the cells were washed with PBS and the radiolabel was chased by the addition of attachment media supplemented with 10 mM methionine. At various chase times, media were collected and cells were harvested and lysed in solubilization buffer. Radiolabeled apoB100 was immunoprecipitated from media and cell lysates with an anti-hamster apoB antibody, analyzed by SDS-PAGE and fluorography, and the radiolabeled apoB was quantified by scintillation counting as described (59).

To measure VLDL-apoB secretion, hepatocytes were preincubated in methionine/cysteine-free MEM and TNF-α (10 ng/ml) for 1 h and pulsed chased for 2 h with 100 μCi/ml [^{35}S] protein-labeling mix. Culture media were collected for VLDL isolation, and cells were harvested and lysed. The density of the culture media was adjusted to 1.006 g/ml, and VLDL was isolated by ultracentrifugation (18 h, 151,000 *g*). The VLDL fraction was collected and solubilized. Radiolabeled VLDL-apoB100 was immunoprecipitated, analyzed by SDS-PAGE/fluorography, and quantified as detailed above.

Effects of anti-TNF-receptor-1/2 antibodies on TNF-α-induced apoB100 secretion ex vivo. Hepatocytes were pulse labeled as described (50) with minor alterations. Briefly, hepatocytes were pretreated with the antibodies against the p55 TNF receptor (TNFR)-1 and p75 TNFR-2 for 30 min and then stimulated with TNF-α (10 ng/ml) for 60 min in methionine-free DMEM at 37°C. Cells were [^{35}S] methionine/cysteine labeled for 2 h. The cells were harvested and lysed, and apoB100 was immunoprecipitated as described (50, 59). Media were then fractionated using discontinuous KBr gradient ultracentrifugation as detailed above.

SREBP-1c, and MTP mRNA abundance. RT-PCR analyses were carried out as described (49). Hepatocytes were treated with TNF-α (10 ng/ml) at 37°C for 24 h. The mRNA levels were assessed by real-time quantitative RT-PCR using an ABI Prism 7700 sequence detector. The primers used for PCR were as follows: sterol regulatory element-binding protein (SREBP)1c primers: 5′-GCGGACG-CAGTCTGGG-3′ and 5′-ATGAGCTGGAGCATGTCTTCAAA-3′; mi-

crosomal triglyceride transfer protein (MTP) primers, 5′-GTCAGGAAGCTGTGTCAGAATG-3′ and 5′- CTCCTTTTTCTCTGGCTTTTCA-3′; and 18S primers, 5′-TAAGTCCCTGCCC TTTG TACACA-3′ and 5′-GATCCGAGGGCCTCACTAAAC-3′.

Other laboratory methods. Measurement of glucose, serum insulin, cholesterol, and TG levels were performed as described (49).

Statistical analysis. Statistical significance was calculated with a two-tailed paired Student's *t*-test analysis or one-way ANOVA. *P* values <0.05 were considered significant.

RESULTS

Effect of TNF-α infusion on whole-body insulin sensitivity. Normal, chow-fed hamsters were used to perform euglycemic-hyperinsulinemic studies following TNF-α infusion. Table 1 summarizes metabolic profile of hamsters during clamp studies. Four-hour TNF-α infusion induced a significant decrease in GIR compared with the saline-treated controls. The cholesterol and TG levels were significantly increased by 90-min Triton WR1339 treatment (Table 2), compared with baseline levels in control hamsters, in both fasting and postprandial conditions ($P < 0.001$, respectively). There was a trend for TNF-α infusion-treated hamsters to have higher cholesterol and TG levels, but, perhaps because of high inter-animal variability, the differences were not statistically significant ($P = 0.13$ and $P = 0.09$, respectively). TNF-α treatment significantly increased serum TG levels ($P < 0.05$) compared with controls in the postprandial state at the end of the 90-min Triton treatment, but the change in cholesterol levels was not significant ($P = 0.11$). Additionally, TNF-α infusion did not significantly affect serum insulin concentrations in fasting or postprandial state (Table 2).

Effect of TNF-α infusion on hepatic signal transduction. The effects of TNF-α on hepatic insulin signaling after euglycemic clamp are shown in Fig. 1. The total protein mass of hepatic insulin receptor (IR)-β, IR substrate (IRS)-1, Shc, Akt, p38, ERK1/2, and JNK did not differ significantly between saline- and TNF-α-treated hamsters. The tyrosine phosphorylation levels of hepatic IR-β and IRS-1 in the TNF-α-treated hamsters were significantly lower, however, when compared with the controls (Fig. 1, *A* and *B*, 60 ± 17% and 64 ± 16% of saline, $P < 0.05$, respectively). Likewise, significantly reduced phosphorylation of Akt (Ser^{473}), p38, ERK1/2 (44/42 kDa), and JNK (55/46 kDa) was observed in the TNF-α-treated groups (Fig. 1, *D*, *F*, *G*, and *H*, 57 ± 7%, 65 ± 3%, 57 ± 4%, and 75 ± 14% of saline, $P < 0.05$, respectively). In addition, there were significant increases in the phosphorylation of IRS-1 (Ser^{307}) and Shc (52/46 kDa), both of which have been linked to insulin resistance (Fig. 1, *C* and *E*, 170 ± 18%, 130 ± 6% of saline, respectively, $P < 0.05$).

Table 1. *Body weights, the blood glucose and serum insulin levels, and GIR during the euglycemic clamp procedure*

Euglycemic Clamp Study	Control ($n = 4$)	TNF-α ($n = 5$)
Body weight, g	138±3	139±4
Base plasma glucose, mM	4.1±0.1	4.1±0.2
Clamp plasma glucose, mM	4.3±0.2	4.5±0.3
Base serum insulin, pM	215±22	220±31
Clamp serum insulin, pM	1400±49	1320±175
GIR, ml/kg per min	10.4±0.3	8.1±0.4*

Data are means ± SE. *$P < 0.05$ vs. control. GIR, glucose infusion rate.

TNF-α infusion increases the accumulation of apoB100-containing lipoproteins in both fasted and postprandial states. To investigate the effect of TNF-α on the accumulation of circulating apoB100-containing lipoproteins, fasted hamsters were infused 4 h with either saline or TNF-α, and serum was collected at baseline and 90 min after Triton WR1339 treatment. Total and VLDL-apoB100 were significantly increased at the 90-min time point in both groups compared with baseline ($P < 0.001$). There was significantly higher accumulation of both total serum apoB100 (Fig. 2*A*) and VLDL-apoB100 (Fig. 2*B*) in the TNF-α-infused group, compared with controls at 90-min time points ($P < 0.05$). We also compared the effect of TNF-α on $VLDL_1$ versus $VLDL_2$ distribution; the result suggests that $VLDL_1$-apoB100 was 80% higher at the 90-min time point compared with the control ($P < 0.05$, Fig. 2*C*). Mean $VLDL_2$-apoB100 did not significantly change in either group.

The in vivo postprandial hepatic lipoprotein production was also assessed in TNF-α-treated hamsters following an oral fat load. Following Triton infusion, the levels of total serum apoB100 (Fig. 2*D*) and VLDL-apoB100 (Fig. 2*E*) were significantly higher in the TNF-α-infused group at 90 min ($P < 0.05$), compared with the controls, suggesting that TNF-α infusion results in increased hepatic secretion of apoB-containing VLDL lipoproteins in the postprandial state (following an oral fat load). $VLDL_1$-apoB100 was 93% higher in TNF-α-treated hamsters at the 90-min time point compared with the control ($P < 0.05$, Fig. 2*F*), but no change was observed in $VLDL_2$-apoB100.

TNF-α stimulates hepatic apoB secretion ex vivo. To investigate whether TNF-α directly affects the production of apoB100-containing lipoprotein, primary hepatocytes were freshly isolated from chow-fed hamsters and then incubated ex vivo with TNF-α. Cells were then subjected to pulse-chase labeling experiments to assess the stability and secretion of apoB. The hepatocytes were treated with TNF-α for 1 h and radiolabeled, and the radiolabel was chased for 1-h and 2-h periods. Radiolabeled apoB100 was immunoprecipitated from cells and media. Figure 3 shows quantitation of radiolabeled apoB100 in cells and media (Fig. 3*A*) and total (cells + media) radiolabeled apoB100 recovered (Fig. 3*B*) (apoB remaining at each chase time expressed as a percent of the labeled apoB at *time 0*). The radiolabeled apoB (counts per minute) at the zero time point was not significantly different between control and TNF-α-treated hepatocytes (6,533 ± 145 vs. 6,676 ± 176, $P = 0.29$). Percent apoB remaining in TNF-α-treated hepatocytes was significantly higher at both 1-h and 2-h chase times, compared with untreated control cells ($P < 0.05$, respectively). The data suggest that intracellular degradation of apoB100 in cells treated with TNF-α was less pronounced than that in basal conditions, perhaps due to increased apoB100 stability.

TNF-α stimulates hepatic VLDL-apoB secretion ex vivo in primary hepatocytes. To determine the effects of TNF-α on VLDL-apoB secretion, we performed ex vivo steady-state labeling experiments in which hepatocytes were treated with TNF-α for 1 h and then radiolabeled for 2 h. Culture media containing secreted lipoprotein particles were then collected and subjected to ultracentrifugation to isolate VLDL. Figure 3*C* shows the immunoprecipitable VLDL-apoB secreted by vehicle and TNF-α-treated hepatocytes. There was a significant elevation in the amount of VLDL-apoB secreted into the media in TNF-α-treated hepatocytes (1.72-fold of control, $P < 0.05$). The result suggests the secretion of a considerably

Table 2. *The serum TG, cholesterol, and insulin levels, before and after Triton WR-1339 treatment in the fasting and fat loading studies*

	Fasting						Postprandial					
	TG (mM)		Chol (mM)		Insulin (pM)		TG (mM)		Chol (mM)		Insulin (pM)	
Groups	Base	Triton	Base	Triton	Base	Triton	Base	Triton	Base	Triton	Base	Triton
Control (n = 6)	0.99±0.12	6.93±0.34†	1.15±0.03	2.08±0.07†	168±5	133±7	1.05±0.19	11.80±0.53†	1.25±0.09	2.86±0.12†	158±5	179±7
TNF-α (n = 6)	1.13±0.14	8.51±0.53	1.20±0.06	2.36±0.09	165±7	129±9	1.20±0.20	16.80±1.20*	1.30±0.11	3.43±0.18	162±6	182±9

Data are means ± SE. *$P < 0.05$ vs. control; †$P < 0.001$ vs. baseline. TG, triglyceride; Chol, cholesterol.

higher number of VLDL particles by TNF-α-treated hamster hepatocytes. Taken together, these data suggest an overall increase in apoB100 stability that is translated into an increase in apoB-containing VLDL secretion.

Effects of TNF-α on mass and mRNA expression of MTP and SREBP-1c in hepatocytes. MTP is an essential factor for lipid transfer to nascent hepatic of apoB-containing VLDL. Mass and mRNA levels were therefore measured to determine whether changes in MTP were responsible for the changes observed in VLDL secretion. Our results show that the MTP mass increased by 163 ± 17.8% (Fig. 4*A*) and mRNA increased by 130 ± 10% TNF-α (Fig. 4*B*) in hepatocytes treated with TNF-α ($P < 0.05$, respectively).

To assess whether the observed changes in MTP expression in TNF-α-treated hepatocytes might be paralleled by changes in SREBP-1c, we also measured SREBP-1c mass and mRNA levels. Surprisingly, hepatocytes treated with TNF-α had a significantly decreased mass of mature and immature forms of SREBP-1c compared with control [Fig. 5*A*, 61.5 ± 9.3% (immature); 67.2 ± 5.3% (mature); $P < 0.05$]. Also mRNA expression of SREBP-1c decreased by 54 ± 5% (Fig. 5*B*, $P < 0.05$). TNF-α treatment did not affect apoB mRNA levels in hepatocytes (data not shown).

TNF-α stimulates apoB100 production in hepatocytes through p75 TNFR. To identify the receptor that mediates TNF-α-induced apoB100 overproduction, primary hamster hepatocytes were first incubated with specific blocking antibodies against TNFR-1 and TNFR-2. As shown in Fig. 6, only TNFR-2 antibody significantly blocked TNF-α-induced enhancement in both cellular (Fig. 6*A*) and secreted apoB100

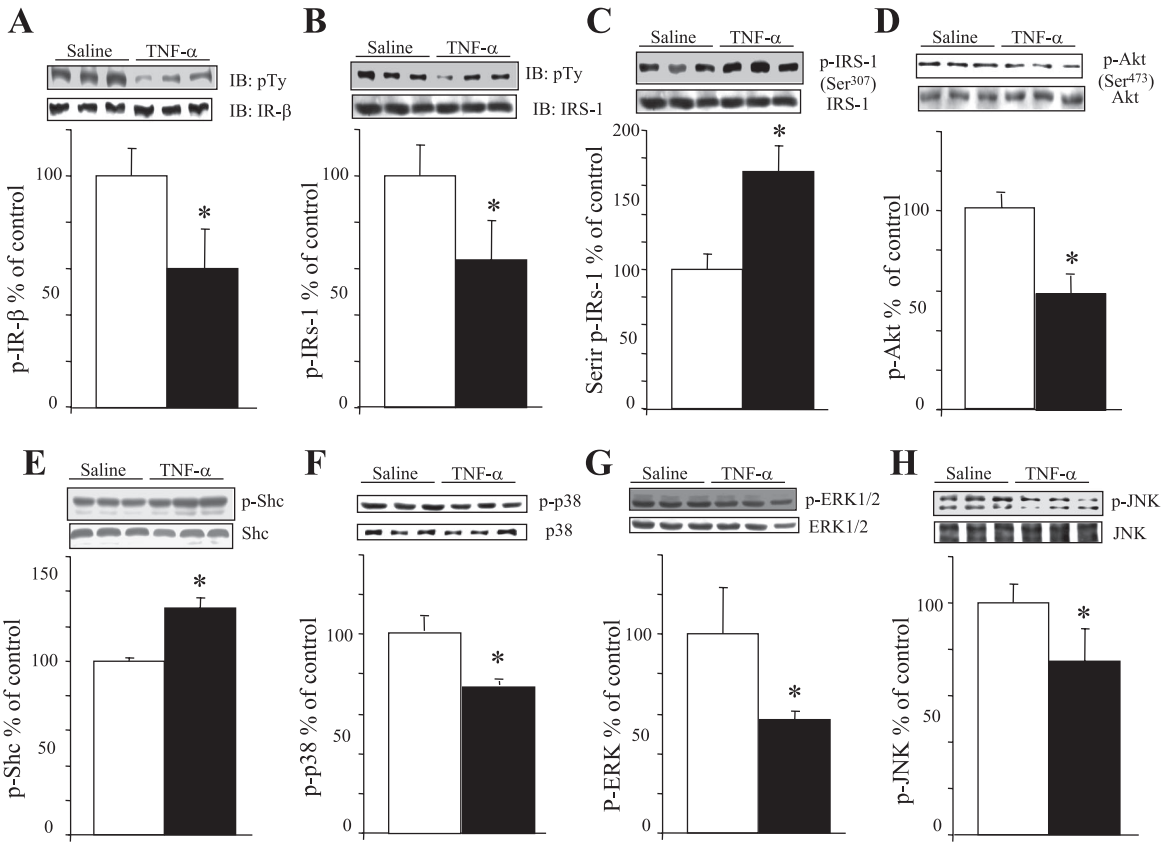

Fig. 1. Effect of tumor necrosis factor (TNF)-α infusion on in vivo hepatic insulin signaling cascade during euglycemic-hyperinsulinemic clamp. *A–H*: effects of TNF-α infusion on the mass and phosphorylation of insulin receptor (IR)-β, IR substrate (IRS)-1 (Tyr), IRS-1 (Ser^{307}), Shc, Akt (Ser^{473}), and MAPKs in the liver tissue of hamsters from the euglycemic clamp study. Cell lysates were analyzed by immunoblotting with the corresponding antibodies against either phosphorylation or total mass. Data are means ± SE (n = 4 or 5); open bars, saline; shaded bars, TNF-α, *$P < 0.05$ and **$P < 0.01$ vs. control. IB, immunoblotting; pTy, phosphotyrosine; p, phosphorylation.

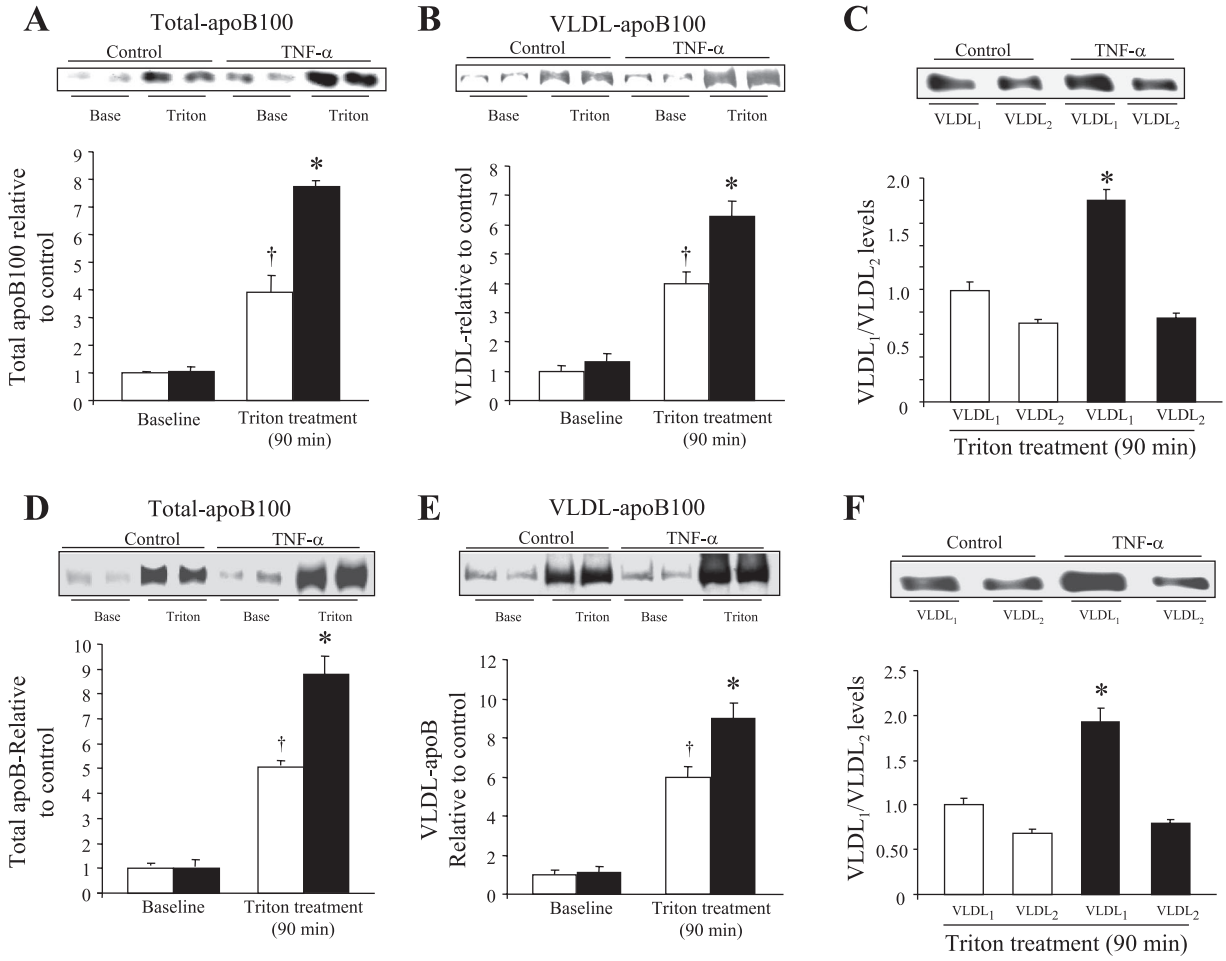

Fig. 2. Effect of TNF-α infusion on fasting and postprandial apolipoprotein B (apoB)100-containing lipoproteins. The total serum- and very low density lipoproteins (VLDL)-apoB100 as well as $VLDL_1$ and $VLDL_2$-apoB100 were immunoblotted using anti-hamster apoB primary antibody. Immunoblots were analyzed using densitometry and expressed as a proportion of *time 0*. *A–C*: representative experiments on the total serum- (*A*) and VLDL-apoB (*B*) and the distribution of $VLDL_1$- vs. $VLDL_2$-apoB100 (*C*) in the fasting state. Results of densitometric analysis of replicate experiments are shown (means ± SE, $n = 6$, respectively). *D–F*: representative experiments on the total serum- (*D*) and VLDL-apoB (*E*) and the distribution of $VLDL_1$ and $VLDL_2$-apoB100 (*F*) in the postprandial state (after fat loading). The values given are means ± SE and are representative of several replicate experiments ($n = 6$, respectively). Open bars, saline; shaded bars, TNF-α; †$P < 0.001$ vs. baseline; controls *$P < 0.05$ vs. controls.

(Fig. 6*B*) or VLDL-apoB100 secretion (Fig. 6*C*). No significant effects were observed with TNFR-1 antibody, suggesting that TNFR-2 is likely to mediate the stimulatory effect of TNF-α on apoB100 production. TNFR-1/2 antibodies had no effect on total apoB100 and VLDL-apoB100 secretion in normal/control hepatocytes without TNF-α stimulation (data not shown).

DISCUSSION

Low-grade systemic inflammation is a prevalent feature of obesity and insulin resistance (12), and TNF-α may be a key mediator linking inflammation and dysregulation of lipid and glucose metabolism (6, 28). Development of insulin resistance is also known to be clearly associated with metabolic dyslipidemia (19, 51). However, mechanisms linking inflammatory cytokines with specific perturbations in hepatic lipoprotein metabolism have not been directly examined. In the present report, we found that TNF-α infusion over a 4-h period induced whole-body insulin resistance in hamsters leading to overproduction of VLDL-apoB100 in both fasting and postprandial states. TNF-α stimulated $VLDL_1$-apoB100 production but had no effect on $VLDL_2$ apoB production, which is consistent with the observation that acute insulin infusion inhibits the production of large buoyant VLDL1 particles (47). These abnormalities resemble those seen in type 2 DM in humans, in which increased $VLDL_1$ production is the main abnormality of lipoprotein metabolism (2, 3, 24). Additionally, we observed that TNF-α significantly stimulates postprandial $VLDL_1$-apoB100 and hypertriglyceridemia after a fat load. Recent evidence (63, 68) suggests that, in the postprandial state, the accumulation of apoB48 TRL may not only lead to the enhanced delivery of exogenous lipids to liver, which will result in an increased production of hepatic lipoproteins, but also delay in the clearance of hepatic TRLs by competing for clearance pathways. Therefore, the overproduction of postprandial TRL-apoB48 induced by TNF-α (49) may play an important role to stimulate postprandial VLDL production.

TNF-α-induced perturbations in insulin signaling have been well documented in insulin-responsive tissues such as muscle and adipose (7, 8, 33). Previously, TNF-α infusion has been

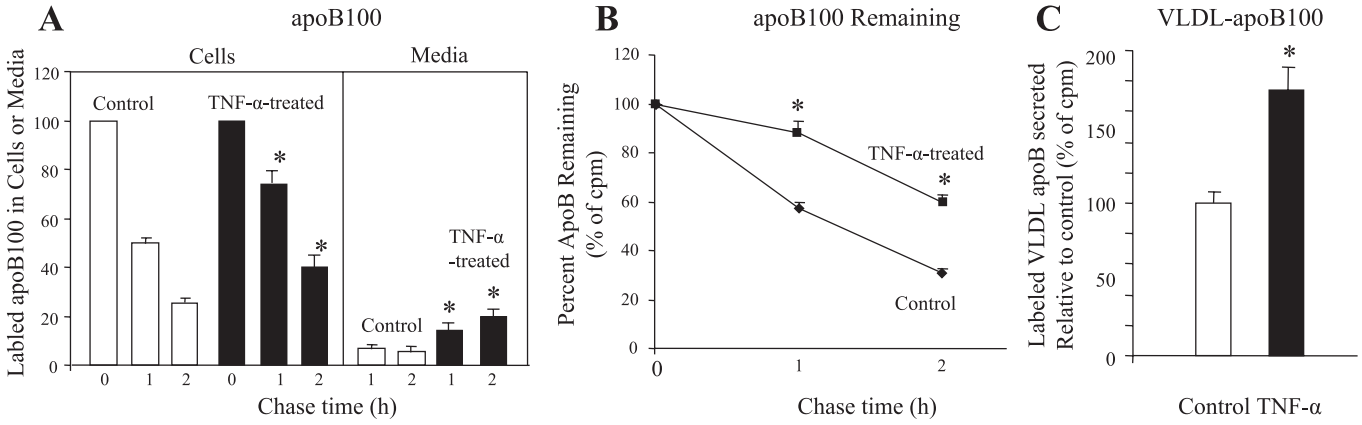

Fig. 3. Ex vivo effect of TNF-α on the synthesis and secretion of apoB100 in primary hepatocytes. Primary hepatocytes were pretreated with saline or TNF-α (10 ng/ml) for 1 h at 37°C, then were pulsed with 100 μCi/ml [^{35}S] methionine and chased for 0, 1, and 2 h in the chase medium supplemented with TNF-α (10 ng/ml). The media and cell were collected at each chase time point, and these were subjected to immunoprecipitation using anti-hamster apoB antibody and then analyzed by SDS-PAGE and fluorography; to investigate the effect of TNF-α on VLDL-apoB production, primary hepatocytes were pretreated with TNF-α (10 ng/ml) for 1 h, and pulsed for 2 h with 100 μCi/ml [^{35}S] methionine. Media was collected and subjected to the ultracentrifugation protocol described in MATERIALS AND METHODS. The VLDL was collected and immunoprecipitated using anti-hamster apoB antibody. *A* and *B*: distribution of labeled apoB100 in cells and media as well as total recovered apoB100 (cell + media). *C*: secreted levels of VLDL-apoB100 in control and TNF-α-treated hepatocytes, respectively. Data are means ± SE; open bars, saline; shaded bars, TNF-α; *$P < 0.05$ vs. controls.

shown to induce hepatic glucose output, despite hyperinsulinemia in rats (46), suggesting hepatic insulin resistance. However, a direct effect of TNF-α on hepatic insulin sensitivity has not been previously examined. In the hamster model, we examined the effects of TNF-α infusion on hepatic insulin signaling after insulin clamp. TNF-α infusion was found to alter the phosphorylation of a number of insulin-signaling molecules in the liver of hamster. Hotamisligil et al. (32) were the first to demonstrate that TNF-α lowers tissue insulin sensitivity by promoting serine phosphorylation of IRS-1, which in turn causes the serine phosphorylation of insulin receptor. This prevents the normal tyrosine phosphorylation of the insulin receptor in adipocytes and thus interferes with phosphorylation of Shc and the downstream activation in vascular smooth muscle cells (27). Our results suggest that TNF-α inhibits hepatic IR-β and IRS-1 tyrosine phosphorylation. In contrast, TNF-α markedly increased the phosphorylation of Ser^{307} IRS-1 and Shc. A previous study (26) has shown that TNF-α treatment, like insulin treatment, induces a transient phosphorylation of Shc expression in vascular smooth muscle cells, and pretreatment with TNF-α led to a transient suppression of insulin-induced tyrosine phosphorylation of Shc; after exposure to TNF-α for 90 min, insulin-stimulated Shc phosphorylation was restored. Furthermore, Li and Goldstein have shown that reducing IRS-1 serine phosphorylation results in increased IRS-1 tyrosine phosphorylation and decreased Shc tyrosine phosphorylation in liver cells (44). Therefore, it appears that Shc expression may differ in in vivo and in vitro conditions, as well as in different cell types. We also examined the impact of TNF-α infusion on phosphorylation of Ser^{473}Akt,

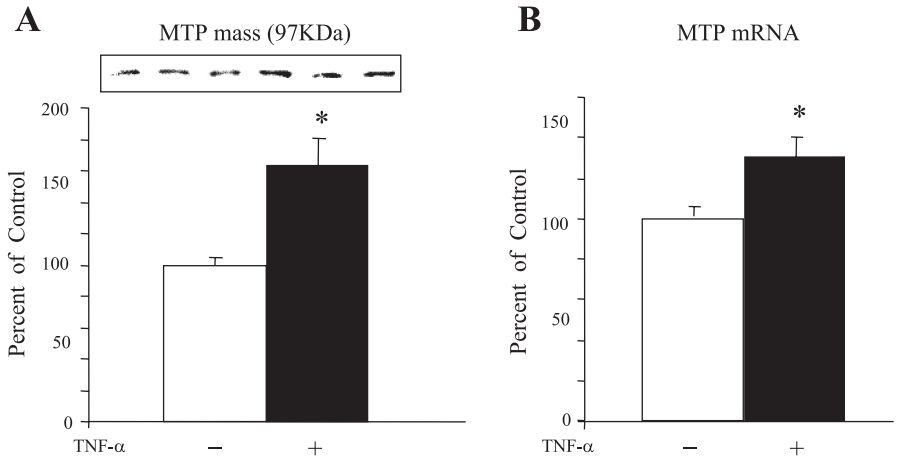

Fig. 4. Effect of TNF-α on the mass and mRNA levels of microsome triglyceride transfer protein (MTP) in primary hamster hepatocytes. *A*: hepatocytes were treated with TNF-α (10 ng/ml) at 37°C for 24 h; then the cells are washed twice in 2 ml cold PBS, and the cell lysates were prepared as described previously (49, 59), subjected to SDS-PAGE, immunoblotted with an anti-mouse MTP antibody (BD Biosciences), washed, and then incubated with a secondary antibody conjugated to peroxidase. Proteins were detected by commercially available ECL kit (Amersham Biosciences). *B*: MTP mRNA levels of 24-h TNF-α (10 ng/ml)-treated hepatocytes at 37°C were determined with RT-PCR using cDNA made from 10 ng total RNA as template. The mRNA levels were normalized using the 18S rRNA level in each sample. Values are means ± SE and are presented as a percentage of control ($n = 3$); open bars, saline; shaded bars, TNF-α. *$P < 0.05$ vs. controls.

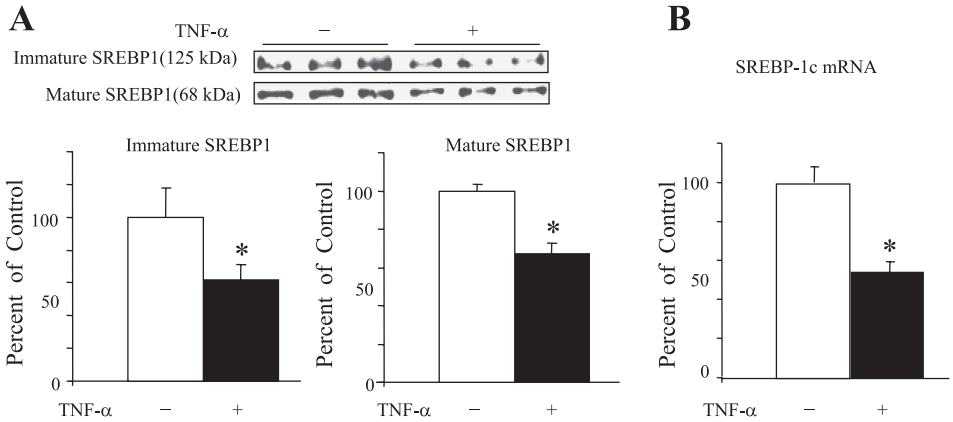

Fig. 5. Effect of TNF-α on the mass and mRNA levels of sterol regulatory element-binding protein (SREBP)-1c in primary hamster hepatocytes. *A*: effects of TNF-α treatment on the mass of SREBP-1c in primary hepatocytes. Nuclear proteins were separated on 8% SDS-PAGE and transferred to nitrocellulose membrane, blocked in 5% milk, and incubated in SREBP-1c primary antibody (Santa Cruz Biotechnology) overnight at 4°C. After being washed, blots were probed with rabbit IgG secondary antibody. Proteins were detected by commercially available ECL kit, (Amersham Biosciences). *B*: MTP mRNA levels of 24-h TNF-α (10 ng/ml) treated hepatocytes at 37°C were determined with RT-PCR using cDNA made from 10 ng total RNA as template. The mRNA levels were normalized using the 18S rRNA level in each sample. Data are means ± SE ($n = 3$ for each group); open bars, saline; shaded bars, TNF-α. *$P < 0.05$ vs. control.

a key signaling factor downstream of phosphatidylinositol 3-kinase. In the liver from TNF-α infusion-treated hamsters, insulin-stimulated phosphorylation of Ser^{473}Akt was significantly reduced, suggesting reduced Akt activity with TNF-α, most likely due to the observed decrease in tyrosine phosphorylation of upstream molecules of the insulin-signaling cascade. Our observations are in agreement with the findings that Akt phosphorylation was impaired in muscle and adipocytes of insulin-resistant diabetic subjects (42, 52).

We also observed that TNF-α markedly inhibited the phosphorylation of p38 MAPK, ERK1/2, and JNK. MAPKs are known to be affected by inflammation and have been implicated in the induction of insulin resistance (14, 22, 32) and diabetes-associated dyslipidemia (4). We initially expected that TNF-α infusion would lead to activation of molecules of the hepatic inflammatory signaling cascades such as JNK in the liver; however, hepatic JNK phosphorylation was found to be reduced. A previous study (29) reported that TNF-α induces insulin resistance in adipocytes and leads to a transient phosphorylation of JNK at 15 min; conversely, after TNF-α treatment for 4 h, the phosphorylation of JNK levels were significantly decreased, compared with the controls. Consequently, the regulation of phospho-JNK by TNF-α may be different under various experimental conditions. In our study, it appears that TNF-α may have initially induced pJNK, but after 4 h of treatment, JNK phosphorylation was actually decreased.

Increasing evidence (2, 59–61, 69) suggests that insulin regulates the assembly process of VLDL and impaired insulin signaling results in increased VLDL production. Our results suggest that TNF-α infusion induces whole-body insulin resistance and impairs hepatic insulin signaling, which might play key roles for downregulation of hepatic-VLDL apoB and VLDL-TG production (1). Insulin has been shown to acutely inhibit hepatic assembly and secretion of VLDL particles likely via mechanisms involving an increase in apoB degradation and a decrease in MTP expression (11, 45). In contrast to insulin, our ex vivo data suggest that TNF-α can markedly increase the intracellular stability of newly synthesized apoB100, suppressing its intracellular degradation and promoting its extracellular secretion. This stimulation of apoB secretion may be mediated by reduced insulin sensitivity of hepatocytes to the inhibitory effects of insulin on the VLDL assembly process. Present

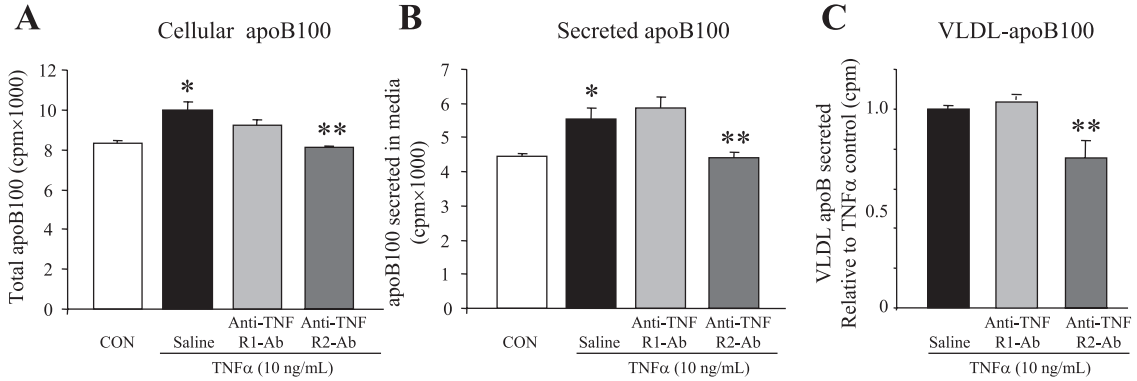

Fig. 6. TNF receptor p75 mediates the effects of TNF-α on apoB100 and VLDL-apoB100 secretion. Anti-TNF receptor p75 antibody inhibits apoB100 oversecretion induced by TNF-α. The hepatocytes were pretreated with antibodies against the TNFR-1/2 (1:1,000) for 30 min and treated with TNF-α (10 ng/ml) for 60 min, followed by 2-h pulse labeling. VLDL fractions were isolated by ultracentrifugation as described. Labeled apoB was immunoprecipitated from VLDL fractions, subjected to SDS-PAGE, and analyzed by fluorography. Values given are representative of 2 independent experiments performed in duplicate. Data are means ± SE; open bars, saline; shaded bars, TNF-α; *$P < 0.05$ vs. control; **$P < 0.05$ vs. TNF-α alone.

studies were not performed to examine the effect of TNF-α on different pathways involved in apoB degradation. Further experiments are necessary to investigate these mechanisms. Another important factor is likely to be MTP, which is rate limiting for the production of apoB-containing VLDL. MTP catalyzes the transfer of lipids to newly synthesized apoB within the endoplasmic reticulum, facilitating secretion of nascent lipoproteins (36). Hepatic expression of MTP is increased in the obese and hypertriglyceridemic rat (43) and in obese diabetic mice (5). Our previous studies have shown that an increased expression of MTP contributes to the overproduction of hepatic apoB100 lipoproteins from the fructose-fed hamster model of insulin resistance (9, 59); amelioration of hepatic insulin resistance in this model resulted in normalization of MTP expression and reduction of the overproduction of apoB100-containing lipoproteins (9). Furthermore, both cell and animal studies demonstrate that apoB secretion is decreased in a dose-dependent manner upon treatment with specific MTP inhibitors (37, 64). In this study, we observed that TNF-α treatment significantly increased hepatic MTP mass and mRNA. The increased MTP expression may potentially lead to upregulation of VLDL assembly and increase of apoB100 production.

Additionally, SREBP-1c, an important regulator of lipogenesis, seems to have a crucial role in the regulation of TG accumulation in the liver (65), and decisive evidence exists for the insulin-induced expression of SREBP-1c (20). Consistent with a previous study (53) that mRNA levels of SREBP-1c are decreased following TNF-α treatment of adipocytes, we found that hepatocytes treated with TNF-α had a significantly reduced mass and mRNA expression of SREBP-1c. Moreover, Shimomura et al. (55) reported that hepatic TG levels increased by 60% and SREBP-1c mRNA levels were reduced by 80% shortly (42 h) after a single high-dose streptozotocin injection. Interestingly, this appears to be in contrast to increased hepatic SREBP-1c mRNA levels in the livers of mice with type 2 DM (54) and of streptozotocin-treated mice with hyperglycemia (5). It is plausible that, although acute treatment with TNF-α reduces SREBP-1c mRNA, chronically increased circulating levels of TNF-α may be associated with increased SREBP-1c mRNA expression as observed in animal models of type 2 DM.

The activation of the TNF-α/TNFR pathway has been shown to be associated with several metabolic abnormalities, such as insulin resistance, increased hepatic VLDL secretion, and free fatty acid output by adipose tissue (30, 38). In the present study, we examined the effects of blocking TNFR function on apoB100-lipoprotein secretion in ex vivo experiments and observed that blocking of the TNFR-2 inhibited TNF-α-induced oversecretion of VLDL-apoB100 in radiolabeling experiments. In contrast, similar experiments with TNFR-1 demonstrated no significant effects. This is consistent with the observation that the adipose tissue of obese subjects exhibits increased expression of TNF-α and TNFR-2 but not TNFR-1 mRNA, which is accompanied by elevation in plasma soluble TNFR-2 (18, 31). Further experiments will still be necessary to elucidate the relative role of each receptor, as well as their respective action in regard to hepatic insulin resistance.

In summary, increased circulating TNF-α levels may interfere with hepatic insulin signal transduction and lead to significant overproduction of VLDL and hypertriglyceridemia. The stimulatory effects of TNF-α appear to be mediated via TNFR-2 and involve downregulation of SREBP-1c and upregulation of MTP, enhancing apoB100 protein stability, resulting in an increased rate of $VLDL_1$ assembly and secretion. Further research is underway to examine the link between the inflammatory pathways induced by TNF-α and the process of $VLDL_1$ assembly and secretion.

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