Table 2. Proposed Japanese Guidelines for Hypertriglyceridemia

Treatment	Categories		Goal for plasma lipids (mg/dL)		
		Coronary Risk Factors other than LCL-C	Primary LDL-C	Secondary nonHDL-C	HDL-C
Primary Prevention Improving lifestyle as the first line, followed by medication	I (Low Risk Group)	0	<160	<190	≥ 40
	II (Intermediate)	1~2	<140	<170	
	III (High)	≥ 3	<120	<150	
Secondary Prevention Improving lifestyle & medication	Past History of CHD		<100	<130	

Goals for control depend upon categories of LDL cholesterol and non-HDL cholesterol. The primary target in hypertriglyceridemia is LDL-cholesterol. If the goal for LDL-cholesterol in the Japanese Guidelines for Atherosclerosis 2007 is already achieved, nonHDL-C is the secondary target. For the patients with TG>500 mg/dL, potential genetic disorders and the prevention of acute pancreatitis should be considered. Coronary risk factors other than LDL-cholesterol include low HDL cholesterol, aging, diabetes, hypertenstion, smoking, past history of CHD, and obesity (visceral obesity).

dL, respectively. Goals for non-HDL cholesterol in each group are those for LDL cholesterol plus 30 mg/dL. This is based upon our outpatient clinic data that non-HDL cholesterol was 30 mg/dL higher than LDL cholesterol (**Fig. 2**)[21]. ATPIII also recommends using LDL cholesterol goal+30 mg/dL[24]. This also corresponds to the calculated VLDL cholesterol of the cut-off point of normal TGs (150/5 mg/dL). This goal is arbitrarily set and could be modified in the future, especially when the relative atherogenicity of remnants and LDL cholesterol are more precisely determined. In the case of TGs of greater than 500 mg/dL, the risk of pancreatitis should be carefully considered as a potential acute complication.

Treatment of Hypertriglyceridemia Based upon Non-HDL Cholesterol Level

Treatment of patients with hypertriglyceridemia for primary prevention should be initiated with lifestyle modifications, especially reducing weight and increasing physical activity. Lifestyles exacerbating hypertrigylyceridemia, such as overweight, obesity, physical inactivity, cigarette smoking, excess alcohol intake, and very high carbohydrate diets, need to be improved. Other disorders and drugs that cause secondary hypertriglyceridemia, including diabetes, chronic renal filure, nephrotic syndrome, and steroid therapy, should also be treated first. In the event that lifestyle modification for at least three months is not effective to achieve the goal of non-HDL cholesterol, medication should be considered. Currently, due to lack of evidence to fully justify the use of fibrates for high TGs prior to statins, it is recommended to use a statin as the first line choice for high non-HDL cholesterol. If statin therapy is already used to control LDL cholesterol, management of non-HDL should be targeted by increasing the dose of the statin or switching to a stronger form. This is based upon the notion that remnant lipoproteins, as well as LDL, are taken up through LDL receptors that are up-regulated by statins. In the case of type III hyperlipidemia, or if high non-HDL cholesterol is much more prominent than LDL cholesterol because of hypertriglyceridemia, fibrates could be considered as they specifically reduce plasma TGs and are effective against type III hyperlipidemia. However, LDL cholesterol should be carefully monitored since fibrates occasionally raise LDL cholesterol following a decrease in TGs (VLDL cholesterol). In case the goal for LDL cholesterol is not attainable, the addition of cholestimide and/or ezetimibe to statin could be considered, whereas EPA could be considered for hypertriglyceridemia. A positive result from a recent large scale Japanese study using both EPA and pravastatin to estimate the prevention of atherosclerotic events, justifies superimposing EPA on statin therapy, although the contribution of the plasma TG-lowering effect of EPA to the prevention of cardiovascular events is not yet determined[25]. The complexity of the choice of medication for high non-HDL cholesterol is currently inevitable because no agents specifically decrease non-HDL cholesterol. Drug information strongly warns against the use of both statins and fibrates because of increasing the risk of the life-threatening side effect of rhabdomyolysis. Joint use is justified only when the benefit exceeds the risk, which requires expertise in this field; however, considering the very few reports of rhabdomyolysis as a severe side effect in recent post-market studies in Japan, carefully prescribing both agents for high-risk patients such as those with type IIb hyperlipidemia could be re-considered. Joint use might be restricted in the elderly or renal compromised patients. In addition, monitoring mus-

cle symptoms and plasma creatine phosphokinase is necessary in patients prescribed either statins or fibrates.

Conclusions and Future Prospect of the Guidelines

Non-HDL cholesterol containing both LDL cholesterol and remnant cholesterol, is an excellent predictor of atherosclerotic risk, and should be a treatment target. Non-HDL cholesterol is simple, convenient, and free from dietary variations. These advantages are crucial for nation-wide use of the guidelines and health check activity. This simple measurement could also make it possible to re-evaluate previous clinical studies using this parameter to offer a good chance of estimating the usefulness and importance of this marker in a large meta-analytical scale.

In the current study, we propose that LDL cholesterol is the primary target and non-HDL cholesterol should be the secondary target for elevated TG. Considering that non-HDL and LDL cholesterol are partially redundant, non-HDL could replace LDL as the primary target and as a general marker for both elevated cholesterol and TG. As **Table 1** shows, non-HDL cholesterol could be used as a general and convenient lipid marker for type IIb hyperlipidemia.

This proposal still faces the recent problem of selecting lipid markers for the initial assessment for dyslipidemia. The recent GL focus has been on LDL cholesterol rather than TC, while LDL cholesterol has a problem the lower reliability for direct measurement. In addition, a considerable portion of hypertriglyceridemia is not applicable to this equation. For subjects with hypertriglyceridemia, application of this new GL eventually requires all TC, TG, HDL, and LDL cholesterol measurements to assess both LDL and non-HDL cholesterol. Currently, however, the Japanese medical system covers only three out of four lipid measurements as healthcare services provided by health insurance. Further Japanese clinical studies and careful evaluation of the data, as well as technical improvements of reliable LDL cholesterol measurements, are required to determine the most efficient protocol to select lipid measurements as the initial assessment of dyslipidemia to prevent CVD in Japan. Furthermore, guidelines for HDL cholesterol should also be established, although the relative importance and positioning of non-HDL and HDL is yet to be determined.

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Original Article

Long-Term Probucol Treatment Prevents Secondary Cardiovascular Events: a Cohort Study of Patients with Heterozygous Familial Hypercholesterolemia in Japan

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Aim: The POSITIVE study assessed whether long-term treatment with probucol, a potent anti-oxidant and cholesteryl ester transfer protein (CETP) activator, is associated with a lowered risk of cardiovascular events in a very high-risk population: familial hypercholesterolemia (FH).
Methods: The study cohort included 410 patients with heterozygous FH, diagnosed between 1984 and 1999 by cardiovascular and metabolic experts at fifteen centers. Traceable patients were screened using predefined eligibility criteria. The primary outcome measure for comparison between probucol exposure and non-exposure was the time to the first cardiovascular event involving hospitalization.
Results: Analysis revealed significant differences in baseline characteristics and follow-up treatment between exposure and non-exposure. An observed indication bias was the use of probucol in more severe FH at diagnosis, both for primary and secondary prevention. When the multivariate Cox regression procedure was used after adjustment for possible confounding factors, probucol lowered the risk (hazard ratio [HR], 0.13; 95% confidence interval [CI], 0.05–0.34) in secondary prevention (n=74) and was statistically significant (p<0.001), although not significant (HR, 1.5; 95% CI, 0.48–4.67; p=0.49) in primary prevention (n=233). Safety assessment found no specific difference between exposure and non-exposure.
Conclusion: Long-term probucol treatment may prevent secondary attack in a higher cardiovascular risk population of heterozygous FH.

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Key words; Atherosclerosis, Antioxidants, CETP activator, Dyslipidemia

Introduction

Cardiovascular (CV) diseases, including coronary heart disease and stroke, are the leading cause of death in Japan. Prevention of fatal CV events is therefore the final goal as well as the rationale of cholesterol-lowering therapy.

Probucol, a conventional cholesterol-lowering drug, originated with the report by Barnhart in 1970 [1]. The drug has been used clinically in Japan since 1985. Nearly 60,000 Japanese patients still take probucol; western countries discontinued probucol use after

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the original manufacturer's withdrawal notice to the United States FDA in 1995 after 18 year's use of the drug. Probucol's cholesterol-lowering mechanism has not yet been clearly established, but it is thought to increase catabolic excretion of cholesterol into bile[2]. Later studies[3-5] have described new mechanisms of probucol, including anti-atherogenic and anti-oxidant actions. Another controversial and anti-atherogenic feature of probucol is its paradoxical effect of lowering high-density lipoprotein cholesterol (HDL-C). This action reflects, most likely, its molecular mechanisms: promoting cholesterol efflux, and enhancing reverse cholesterol transport by activation of cholesteryl ester transfer protein (CETP)[6-8] and class B type 1 scavenger receptor[9, 10]. Matsuzawa and his colleagues reported an observed close correlation between the extent of regression in Achilles' tendon xanthoma and probucol-induced decrease in HDL-C levels in patients with familial hypercholesterolemia (FH)[11].

No large-scale, randomized, double blind comparative study has been conducted to justify the use of probucol in the prevention of CV events or diseases. however, clinical studies as well as pre-clinical data have been accumulating evidence of the clinical worth of probucol in arteriosclerotic diseases. Numerous clinical results, including a reduction in Achilles' tendon xanthoma thickness after long-term treatment for FH[12, 13], reduced rates of restenosis after angioplasty[14-16], and a decrease in carotid artery intima-media thickness[17, 18] support the therapeutic and preventative effects of probucol on arteriosclerotic lesions and plaque. To evaluate the risk and benefit of long-term probucol treatment, we conducted a cohort study to determine whether probucol treatment is associated with the risk reduction of CV events in patients with heterozygous FH, a very high-risk population.

Methods

Study Cohort

We registered patients with FH who received treatment between January 1, 1984 and December 31, 1999 at 15 centers specializing in CV and metabolic diseases, including FH, nationwide. Patients were traceable by medical recend and met the diagnostic criteria for heterozygous FH under the Japan Atherosclerosis Society Guidelines (2002) for the Diagnosis and Treatment of Atherosclerotic CV Diseases[19]. Definite heterozygous FH was defined as having at least two of the major features: total cholesterol (TC) of 260 mg/dL and above; tendon xanthoma or xanthoma tuberosum; reduced or abnormal receptor activity noted by LDL receptor analysis. Probable heterozygous FH was defined as having at least one each of the major (as above) and minor features: palpebral xanthoma; arcus juvenilis (< 50 years); juvenile (< 50 years) ischemic heart disease. For other eligibility criteria, we excluded patients with possible homozygous FH or with severe ventricular arrhythmias (polymorphic premature ventricular contractions). Possible homozygous FH was defined as having any one of the clinical features: defect of homozygous or hetero-polymeric LDL receptors confirmed by gene analysis; no LDLR activity observed by receptor analysis, severe elevation of plasma TC higher than 500 mg/dL; xanthoma or atherosclerotic vascular lesions including symptoms of juvenile ischemic heart disease; hypercholesterolemia confirmed in both parents; history of ischemic heart disease confirmed in both parents; or poor response to any 3-hydroxy-3methyl-glutaryl-coenzyme A reductase inhibitor (statin).

During the study period between June, 2004 and September, 2005, we collected anonymous case report forms with the patients' baseline data, including medical history, findings at clinical examination, medication data, and laboratory data. The investigators transcribed the data on to case report forms (identified by a code) from the stored medical charts of the patients. The observation period was the period for which each patient's clinical course could be traced. The longest observation period exceeded 20 years for patients on stable doses of probucol.

We required a sample size of 200 in both the probucol exposure and non-exposure groups, supposing a difference of 10% in the incidence of CV events for 5 years (15% in exposure and 25% in non-exposure). A least 400 subjects were needed to detect the difference with 80% power and a type I error of 5% at the 5% significance level with two-sided log-rank test based on normal approximation. The study protocol was approved through the process of ethics committee or institutional review board at each center.

Definitions and Endpoints

The primary outcome measure was the time to the first CV event, defined as acute myocardial infarction (MI), angina pectoris (AP), heart failure (HF), stroke, transient ischemic attack (TIA) or arteriosclerotic peripheral artery diseases (PAD) leading to hospitalization or death as well as sudden death within 24 hours of an observed intrinsic event. The obtained baseline data at the first visit of each patient included demographic characteristics: sex, date of diagnosis at the participant medical center, age, height, weight, and habits of smoking and drinking. Body mass index (BMI) was calculated as weight in kilograms divided

by the square of height in meters. The other collected characteristic factors at diagnosis were the presence of xanthoma and its location, prior CV event, onset date if any prior CV event, treatment for the event, and other possible risk factors for CV events, including the presence of hypertension, diabetes, ventricular arrhythmia, and PAD. We collected data on cholesterol-lowering therapy (with or without probucol) and other concomitant therapy with anti-platelet, antihypertensive or diabetic drugs. Dates of drug initiation, discontinuation, re-administration, and termination were entered as elemental information. Treatment period was defined as the length from initiation until medication termination, or until the occurrence of the defined CV event, whichever came first. A lipid profile of TC, triglyceride (TG), low-density lipoprotein cholesterol (LDL-C) and HDL-C, blood pressure, level of fasting blood sugar (FBS), hemoglobinA_{1c} (HbA_{1c}), and thickness of tendon xanthoma in both feet were variables of interest, seen as potential predictors of CV events. We obtained measurements of those variables on a yearly basis after each patient was diagnosed. LDL-C levels were calculated from TC and HDL-C measurements with the Friedewald formula in TG < 400 mg/dL. For TG of 400 mg/dL and more than 400 mg/dL, the expression of 0.16 X TG was applied in stead of 0.2 X TG[20]. Most patients had fasted compliantly at periodic checkups of their lipid levels. We set a follow-up period of 10 years for the measurements.

Statistical Analyses

The primary objective of analysis was a comparison between probucol exposure and non-exposure to evaluate whether treatment with probucol (500 mg to 1,000 mg daily) for FH provided CV benefits. The analysis was based on intent-to-treat principles. The secondary objective was to assess whether changes in the lipid profile after probucol treatment predicted CV events in the cohort. Event-free survival, defined as the time from diagnosis to the first CV event, was determined as a response variable. Statistical analysis was performed to evaluate clinical outcomes separately for secondary and primary prevention groups; that is, patients with or without a history of CV events at diagnosis.

Baseline characteristics of each group were explored to detect risk factors for CV events because potential confounders, including indication bias, were anticipated. For baseline comparison, Wilcoxon's rank sum test and Fisher's exact test were used for continuous variables and categorical variables respectively. For detection of risk factors, univariate Cox proportional hazards regression with a baseline variable as covariate was used as a screening step to determine the relationship with CV events. Variables that achieved significance at the level of 20% in univariate analysis were subsequently included in a multivariate Cox proportional hazards regression using backward variable selection. Variables proving significant at the 10% significance level were selected as risk factors to be adjusted. Consequently, probucol treatment effect was evaluated using the multivariate Cox model with adjustment for the selected baseline variables. Finally, the other observed treatment factors: cholesterol-lowering drugs other than probucol, LDL-apheresis, anti-platelet drugs, anti-hypertensive drugs, and diabetic drugs were entered into that model to assess their effects.

For the association between changes in lipid profile after probucol treatment and the risk of CV events, pre-treatment values of TG, LDL-C, HDL-C as well as TC, and each lipid reduction ratio after treatment were used as covariates. Multivariate analyses of time from probucol start to the first CV event used multivariate Cox's proportional hazards models. Statistical analysis was peerformed with SAS version 8.2.

Results

Patient Characteristics

We collected data from the medical records of 541 patients, and excluded the data of 131 patients that did not meet eligibility predefined in the protocol.

The flow diagram (**Fig. 1**) gives reasons for the exclusion. A substantial fraction of probucol-exposed patients, 80.0% and 93.2%, took probucol within two years after diagnosis for in primary and secondary prevention groups, respectively. Baseline characteristics at diagnosis are given for each group (**Table 1, 2**). The secondary prevention group (**Table 2**) had prior diseases of AP, MI, stroke, HF, and TIA. This group was found to have significant higher proportions of men (60.2%, $p<0.01$), smokers (50.0%, $p<0.01$), hypertension (40.9%, $p<0.001$) diabetes (15.9%, $p=0.02$), and older median age (52 years, $p=0.01$) than the primary prevention group. Moreover, the group tended to have hypo-HDL cholesterolemia of median 42 (20–90) mg/dL, and to receive combined treatments with anti-platelet drugs (56.8%), anti-hypertensive drugs (53.4%), and LDL-apheresis (14.8%).

Comparison between probucol-exposed and non-exposed groups revealed significant differences in some baseline characteristics and treatments, which showed a confounding indication that patients with more severe FH took probucol. For baseline characteristics, the exposed group for primary prevention had more

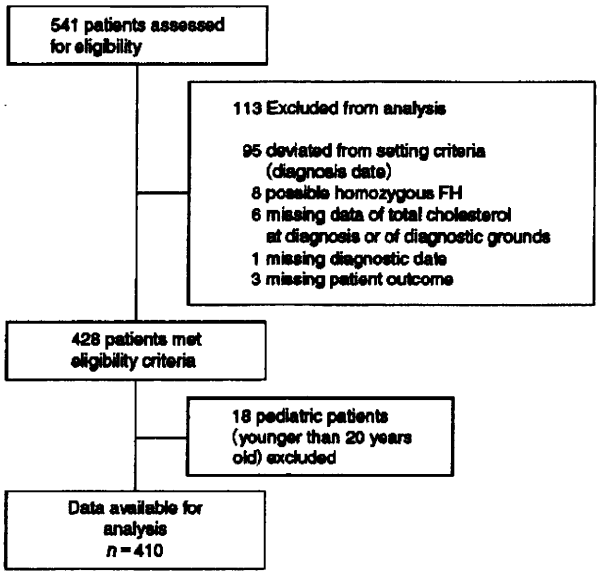


Fig. 1. Patient Flowchart.

We collected data from the medical records of 541 patients, and excluded the data of 131 patients who did not meet the eligibility predefined in the protocol. The flow diagram gives reasons for the exclusion.

palpebral xanthoma (13.4%, $p=0.05$), thicker median measurement of tendon xanthoma (12.5 mm, $p<0.01$), higher median HbA_{1c} (5.8%, $p=0.03$), and more use of antihypertensive drugs (25.3%, $p<0.01$). Their lipid profile was more severe with a higher median baseline TC (325 mg/dL, $p=0.001$), a higher median LDL-C level (253 mg/dL, $p<0.001$), and a lower HDL-C level (47 mg/dL, $p<0.001$) than the unexposed group. The exposed group for secondary prevention had a higher prevalence of post-MI (44.6%, $p<0.01$) than the unexposed group. Observed medications were also significantly different between the exposed and unexposed groups. The exposed group used anti-hypertensive drugs concomitantly at a higher rate (25.3% vs. 11.2%, $p<0.01$) for primary prevention.

Descriptive analysis of baseline characteristics and treatments during observation implies that in both primary and secondary prevention, the exposed groups tended to include patients with more severe FH at diagnosis. Arguably, patients considered more severe at diagnosis would receive more intensive treatment, including probucol.

Outcomes

We present the absolute number of CV events requiring hospitalization by prevention group with details of the events (**Table 3**). The incidence of CV events without consideration of confounding factors was 11.6% in the exposed group and 4.5% in the unexposed group for primary prevention. For secondary prevention, the incidence was 27.0% in the exposed group and 64.3% in the unexposed group. The event-free survival curve of the secondary prevention group is given (**Fig. 2**).

To identify risk factors for CV events, we determined the relationship between the incidence and every baseline variable using univariate Cox regression at a significant level of 20%. Variables proving significant at the 10% significance level in multivariate Cox regression were selected as risk factors to be adjusted. We estimated the effect of treatment after adjusting the selected risk factors. We calculated hazard ratios (HRs) with 95% confidence interval (CI) for binary variables, BMI ≥25 vs BMI <25, drinking vs no drinking, for example, and the indicated HRs corresponded to a 1 standard deviation increase for continuous variables, including TC. Estimated results are given (**Table 4**).

In the primary prevention group, significant variables were BMI ≥25 (HR 1.86, 95% CI 0.87–3.98; $p=0.11$), drinking (HR 2.17, 95% CI 1.02–4.63; $p=0.05$), tendon xanthoma (HR 2.17, 95% CI 0.76–6.23; $p=0.15$), prior diseases other than CV events (HR 1.87, 95% CI 0.87–3.99; $p=0.11$), PAD (HR 5.23, 95% CI 0.70–39.2; $p=0.11$), diabetes (HR 2.27, 95% CI 0.79–6.50; $p=0.13$), TC (HR 1.37, 95% CI 0.99–1.89; $p=0.06$), HDL-C (HR 0.75, 95% CI 0.50–1.12, $p=0.16$), SBP (HR 1.48, 95% CI 1.00–2.18; $p=0.05$), and the thickness of tendon xanthoma (HR 1.50, 95% CI 1.06–2.14; $p=0.02$). Three of these variables, drinking, TC, and PAD were selected for adjustment at the 10% significance level as a result of a multivariate Cox regression with backward variable selection. After adjustment for these three baseline variables, we found no significant effect by probucol at the 5% significant level. The estimated hazard ratio of probucol use for CV events was 1.50 (95% CI 0.48–4.67; $p=0.49$).

In the secondary prevention group, significance variables were drinking (HR 1.74, 95% CI 0.80–3.79; $p=0.17$), presence of palpebral xanthoma (HR 5.34, 95% CI 2.26–12.61, $p<0.001$), TIA (HR 4.16, 95% CI 0.54–32.21; $p=0.17$), history of coronary artery bypass graft (HR 0.31, 95% CI 0.11–0.90; $p=0.03$), hypertension (HR 0.58, 95% CI 0.26–1.28; $p=0.18$), diabetes (HR 2.89, 95% CI 1.30–6.42; $p<0.01$), and fasting blood sugar (HR 1.31, 95% CI 0.91–1.89; $p=0.15$). Two of these variables, palpebral xanthoma and diabetes, were selected for adjustment at the 10% sig-

Table 1. Baseline characteristics of patients in primary prevention group[†]

Characteristics	Primary prevention No. (%) of patients			*p*
	All *n*=322	Exposed *n*=233 (72.4)	Unexposed *n*=89 (27.6)	
Age, mean (range)	49 (27-74)	50 (20-74)	47 (20-72)	0.18
Men, No. (%)	134 (41.6%)	96 (41.2%)	38 (42.7%)	0.90
BMI ≥25	71 (22.5%)	49 (21.4%)	22 (25.6%)	0.45
Smoker	99 (33.2%)	74 (34.1%)	25 (30.9%)	0.68
Drinker	124 (42.2%)	93 (43.7%)	31 (38.3%)	0.43
Xanthoma	259 (80.7%)	190 (81.9%)	69 (77.5%)	0.43
Tendon xanthoma	245 (76.3%)	181 (78.0%)	64 (71.9%)	0.30
Nodular xanthoma	28 (8.7%)	22 (9.5%)	6 (6.7%)	0.51
Palpebral xanthoma	36 (11.2%)	31 (13.4%)	5 (5.6%)	0.05
PAD	4 (1.2%)	1 (0.4%)	3 (3.4%)	0.07
Hypertension	54 (16.8%)	40 (17.2%)	14 (15.7%)	0.87
Diabetes	22 (6.9%)	17 (7.3%)	5 (5.6%)	0.81
Lipid profile, mg/dL				
TC[§]	320 (188-493)	325 (188-493)	307 (194-464)	0.001
TG[§]	120 (28-1289)	121 (34-1068)	120 (28-1289)	0.96
HDL-C[§]	49 (20-108)	47 (20-90)	52 (27-108)	<0.001
LDL-C[§]	244 (45-425)	253 (98-425)	223 (45-403)	<0.001
Blood Pressure, mmHg				
SBP[§]	129 (82-190)	128 (82-190)	131 (90-190)	0.57
DBP[§]	0 (48-120)	80 (48-120)	80 (56-120)	0.91
FBS (mg/dL)[§]	95 (63-276)	94 (63-140)	95 (81-276)	0.41
HbA_{1c} (%)[§]	5.7 (4.1-12.4)	5.8 (4.1-9.7)	5.3 (4.3-12.4)	0.03
Tendon xanthoma thickness (mm)[§]	12.1 (7.5-49.0)	12.5 (7.5-49.0)	10.5 (8.0-20.0)	<0.01
Treatment				
Cholesterol-lowering drugs (non-probucol)	302 (93.8%)	219 (94.0%)	83 (93.3%)	0.80
LDL-apheresis	7 (2.2%)	6 (2.6%)	1 (1.1%)	0.68
Anti-platelet drugs	49 (15.2%)	41 (17.6%)	8 (9.0%)	0.06
Anti-hypertensive drugs	69 (21.4%)	59 (25.3%)	10 (11.2%)	<0.01
Diabetic drugs	15 (4.7%)	12 (5.2%)	3 (3.4%)	0.37

[†]Continuous variables compared by Wilcoxon's rank sum test, distribution of categorical variables by Fisher's exact test. [§]Data are median (range). All data are number (%) unless otherwise indicated. Each percentage shown is related to the total number with measurement data. BMI, body mass index; PAD, peripheral artery disease; TC, total cholesterol; TG, triglyceride; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; SBP, systolic blood pressure; DBP, diastolic blood pressure; FBS, fasting blood sugar; HbA_{1c}, hemoglobin A_{1c}. LDL-C was calculated with the Friedewald formula.

nificance level as a result of multivariate Cox regression analysis using a backward variable selection. After adjustment for these two baseline variables, the hazard ratio of probucol use for CV events was estimated to be 0.13 (95% CI 0.05-0.34) and significant ($p<0.001$). In sensitivity analyses, we also obtained similar estimation results on probucol for various sets of baseline covariates for adjustment.

The lipid levels of TC, LDL-C and HDL-C were lowered after probucol treatment both in primary and secondary prevention. In the primary prevention group, the median (range) levels of TC, TG, LDL-C and HDL-C closest to before treatment were respectively 305 (165-493), 119 (35-1068), 228 (107-425) and 48 (25-96) mg/dL, and those at 10-year treatment were, respectively, 222 (141-371), 94 (43-335), 157 (91-311) and 39 (17-81) mg/dL. In the secondary prevention, the median levels of TC, TG, LDL-C and HDL-C closest to before atment were, respectively, 320 (191-469), 129 (37-636), 240 (117-381) and 44 (24-90) mg/dL, and those at 10-year treatment were, respectively, 211(135-305), 71 (48-475),

Table 2. Baseline characteristics of patients in secondary prevention group

Characteristics	Secondary prevention No. (%) of patients			*p*
	All *n*=88	Exposed *n*=74 (84.1)	Unexposed *n*=14 (15.9)	
Age, mean (range)	52 (23-71)	51 (29-70)	53 (23-71)	0.62
Men, No. (%)	53 (60.2%)	46 (62.2%)	7 (50.0%)	0.55
BMI ≥25	21 (25.3%)	17 (24.3%)	4 (30.8%)	0.73
Smoker	42 (50.0%)	38 (53.5%)	4 (30.8%)	0.23
Drinker	39 (46.4%)	33 (46.5%)	6 (46.2%)	1.00
Xanthoma	75 (85.2%)	63 (85.1%)	12 (85.7%)	1.00
Tendon xanthoma	71 (80.7%)	61 (82.4%)	10 (71.4%)	0.46
Nodular xanthoma	7 (8.0%)	6 (8.1%)	1 (7.1%)	1.00
Palpebral xanthoma	8 (9.1%)	5 (6.8%)	3 (21.4%)	0.11
PAD	2 (2.3%)	2 (2.7%)	0 (0.0%)	1.00
Hypertension	36 (40.9%)	30 (40.5%)	6 (42.9%)	1.00
Diabetes	14 (15.9%)	9 (12.2%)	5 (35.7%)	0.04
Lipid profile, (mg/dL)				
TC†	332 (191-469)	334 (191-469)	322 (229-444)	0.41
TG†	128 (37-636)	128 (37-636)	136 (63-318)	0.85
HDL-C†	42 (20-90)	42 (20-90)	39 (26-73)	0.91
LDL-C†	249 (117-381)	256 (117-381)	245 (138-354)	0.57
Blood Pressure, mmHg				
SBP†	129 (90-180)	128 (96-180)	136 (90-166)	0.97
DBP (mmHg)†	80 (52-114)	80 (52-114)	78 (60-104)	0.33
FBS (mg/dL)†	96 (72-252)	97 (72-197)	94 (79-252)	0.96
HbA1c (%)†	5.8 (4.1-10.6)	5.5 (4.1-8.1)	6.4 (5.3-10.6)	0.06
Tendon xanthoma thickness (mm)†	14.5 (5.8-25.0)	15.0 (5.8-25.0)	10.0 (8.5-18.8)	0.09
Prior CV events				
Angina Pectoris	45 (51.1%)	36 (48.6%)	9 (64.3%)	0.39
Myocardial Infarction	34 (38.6%)	33 (44.6%)	1 (7.1%)	<0.01
Stroke	7 (8.0%)	4 (5.4%)	3 (21.4%)	0.08
Heart failure	2 (2.3%)	2 (2.7%)	0 (0.0)	1.00
TIA	2 (2.3%)	1 (1.4%)	1 (7.1%)	0.29
Treatment				0.08
Cholesterol-lowering drugs (non-probucol)	81 (92.0%)	70 (94.6%)	11 (78.6%)	
LDL-apheresis	13 (14.8%)	11 (14.9%)	2 (14.3%)	1.00
Anti-platelet drugs	50 (56.8%)	44 (59.5%)	6 (42.9%)	0.38
Anti-hypertensive drugs	47 (53.4%)	42 (56.8%)	5 (35.7%)	0.24
Diabetic drugs	6 (6.8%)	3 (4.1%)	3 (21.4%)	0.05

†Data are the median (range). All data are numbers (%) unless otherwise indicated. Each percentage is related to the total number with measurement data. TIA indicates transient ischemic attack.

147 (124-197) and 33 (17-70) mg/dL. Sub-analysis of changes in the lipid profile after probucol treatment detected significant three predictors of CV event risk: higher baseline TC (HR 2.74, 95% CI 1.05-7.16; p= 0.04) in the primary prevention group; reduction in TG (HR 0.22, 95% CI 0.06-0.86; p=0.03); and reduction in LDL-C (HR 0.17,95% CI 0.03-0.90; p=0.04) after treatment in the subset of the secondary prevention group on stable doses of probucol. Neither TC nor HDL-C after treatment was associated with CV event risk in the probucol-exposed group, which indicates that reduction of the HDL-C level after probucol treatment is not related to CV event risk for probucol-exposed patients.

We evaluated the safety of probucol for all collected data from 541 patients, and found 56 adverse

Table 3. Incidence of cardiovascular events

		Cardiovascular Event		No event	Total	*p*
Primary prevention (n=322)	Exposed (n=233)		27 (11.6%)	206	233	
		MI	4			
		AP	18			
		Str.	3			
		TIA	1			
		PAD	1			0.058
	Unexposed (n=89)		4 (4.5%)	85	89	
		AP	1			
		Str.	2			
		TIA	1			
Secondary prevention (n=88)	Exposed (n=74)		20 (27.0%)	54	74	
		MI	6			
		AP	12			
		HF	1			
		Str.	1			0.012
	Unexposed (n=14)		9 (64.3%)	5	14	
		MI	2			
		AP	6			
		Str.	1			

MI, myocardial infarction; AP, angina pectoris; HF, heart failure; Str., stroke; TIA, transient ischemic attack; PAD, peripheral artery disease.
[1]One of the 4 patients died after 12 months of probucol termination.

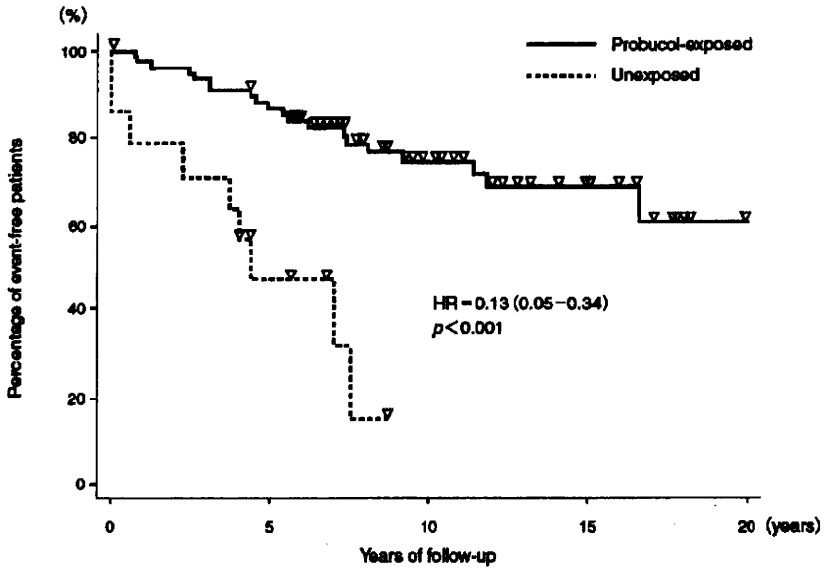
events in 18 patients. Malaise, pruritus, macrocytic anemia and pain in the extremities were recorded as adverse drug reactions associated with probucol. We noted and reported gastric cancer stage III immediately to the Ministry of Health and Welfare as an unexpected serious event, because of an unknown drug relation due to many concomitant drugs, although probucol was found to be non-carcinogenic alone[21]. Six deaths were observed in the population not taking probucol or stopping probucol. There was no other difference in the incidence of adverse events, including serious events, between probucol exposure and non-exposure.

Discussion

Many data from large-scale randomized controlled trials have overwhelmingly demonstrated the clinical benefits of lowering cholesterol with statins[22, 23], yet the rapid and extensive prophylactic use of cholesterol-lowering drugs remains controversial. Few studies have addressed the clinical risks and benefits of long-term treatment of hyperlipidemia among women[24] or elderly patients[25]. The safety of long-term cholesterol-lowering therapy, including the issue of associated cancer risk or benefit, remains inconclusive because of conflicting clinical evidence[26]. More importantly, conclusions from the results of randomized controlled trials are limited by their relatively short follow-up periods (generally less than 5 years) in the analyzed studies.

In long-term treatment for FH, probucol was used with other cholesterol lowering drugs in over 80% of the secondary prevention group–those with a more severe clinical outlook than the primary prevention group: a higher prevalence of hypertension and diabetes, significant thicker tendon xanthoma, more combined therapy with LDL-apheresis, anti-platelet drugs, and anti-hypertensive drugs. The high rate of probucol use in FH was surprising, different from expected. This might partly reflect the prescription behavior of experts with the result that intractable patients responded to the regimen.

In the secondary prevention, the higher-risk group, probucol exposure was associated with a reduction in the risk of cardiovascular events (HR 0.13; 95% CI 0.05–0.34) with high significance ($p<0.001$), while it was not significant in the primary prevention group. This result was also contrary to our expectation that probucol exposure would likely be associated with increased event risk due to a confounding indication–that patients considered more severe at diagnosis would receive more treatment, including probucol. We did not collect the details of non-probucol drugs



Number at risk

Years	0					5					10					15					20
Exposed	74	71	70	68	66	62	54	50	42	38	34	30	25	19	17	13	12	8	3	1	0
Unexposed	14	11	11	10	9	5	4	2	1	0	0	0	0	0	0	0	0	0	0	0	0

Estimates of event-free rates are according to whether patients received probucol. The cumulative probability of remaining without events was higher in patients treated with probucol ($p<0.001$; log-rank test).

Fig. 2. Kaplan-Meier Estimates of Event-free Rate.

For secondary prevention, the incidence of cardiovascular events was 27.0% in the exposed group and 64.3% in the unexposed group. An event-free survival curve for the secondary prevention group is given.

Table 4. The results of multivariate analysis using Cox regression procedure

Factor	Primary prevention			Secondary prevention		
	HR	95% CI	*p*	HR	95% CI	*p*
Baseline variables						
Total cholesterol	1.58	1.06–2.33	0.02	–	–	–
Drinking	2.43	1.09–5.44	0.03	–	–	–
Peripheral artery disease	5.27	0.51–54.63	0.16	–	–	–
Palpebral xanthoma	–	–	–	2.94	1.02–8.47	0.05
Diabetes	–	–	–	2.58	0.76–8.76	0.13
Treatment in follow-up						
Probucol use	1.50	0.48–4.67	0.49	0.13	0.05–0.34	<0.001
Anti-platelet drug use	–	–	–	2.48	1.00–6.17	0.05

to simplify the study procedure. However, we would likely exclude underused statins because of the reduced use of non-probucol drugs from the possible factors of the higher event rate in the unexposed group, because statins were available when all of the 9 recurrent patients (**Table 3**) started and the patients continued on cholesterol-lowering drugs. We suppose, therefore, that the reasons for this unanticipated great risk reduction include some antioxidant and anti-atherogenic actions[3, 4, 27] of probucol. The finding in second prevention may be suggested by the report[27] that probucol significantly decreased *in vitro* LDL oxidizability measured under typically strong oxidative conditions, and that long-term treatment with probucol had an

anti-atherogenic effect in Watanabe Heritable Hyperlipidemic rabbits. From the observation that the baseline lipid profile was not different between the two groups of exposure and non-exposure in secondary prevention, the drug might exhibit greates effectiveness in post-cardiovascular disease patients, in possibly advanced lipid accumulation and inflammation, which are associated with the circulation of oxidized LDL[28].

In primary prevention, we observed an almost significant increase of events in the exposed group (**Table 3**), and an apparently increased risk (HR 1.5), although not statistically significant after adjustment (**Table 4**). We suppose, however, that the ideal effects of probucol might be concealed by the following factors noted in primary prevention. The exposed group had a worse lipid profile (TC, LDL-C and HDL-C levels), higher HbA_{1c}, and thus definitely a higher risk than the unexposed group. Furthermore, 8 (nearly 30%) of the 27 patients experiencing cardiovascular events in the exposed group discontinued probucol when they had events. This was consistent with the different finding between primary and secondary preventions in the exposed group: less than half of the patients (113 of 233) in primary prevention continued on probucol, while 53 (72%) of 74 patients continued in secondary prevention. This estimation might be conservative.

The controversial and paradoxical action of probucol– lowering HDL-C– level was not associated with the risk of CV events in the cohort, therefore, the association between low levels of HDL-C and an increased risk for CV events or death indicated by the early Framingham Heart Study[29] may not be extrapolated to probucol-treated patients. This proposition is consistent with recent findings that a lowered HDL-C level is not always atherogenic, but that the quality or function of HDL-C is more important than the HDL-C levels[30]. In fact, increased levels of HDL-C with torcetrapib, a CETP inhibitor, were not associated with a significant clinical benefit in patients with coronary disease[31], FH[32] or mixed dyslipidemia[33].

We speculate that enhanced reverse cholesterol transport by CETP activation as a result of probucol treatment also contributed to the detected risk reduction in the cohort. The observed positive outcome of probucol, a CETP activator, might be a mirror image of the negative clinical trial results for the CETP inhibitor[34]. Reports[35,36] of increased coronary heart disease in CETP deficiency despite increased HDL-C levels, and the molecular approach to review CETP deficiency[37] support our hypothesis, at least in Japanese genealogy. Interestingly, a recent basic research reports that human CETP expression enhances the mouse survival rate in an experimental systemic inflammation model[38], indicating for the first time a role for CETP in the defense against the exacerbated production of proinflammatory mediators.

For the safety evaluation, we found no cardiotoxic adverse drug reaction including QT/QTc prolongation or torsade de pointes, in this study, although probucol can cause them[16,39,40].

We obtained these results from an observational study with no control for inaccuracy, unexpected bias or confounding factors. We could not assure the precision of the baseline measurements due to unrecorded data. The participant centers were major hospitals for FH, but not all hospitals in Japan, because the study was conducted as part of a post-marketing study by a pharmaceutical manufacturer within the framework of the Japanese government regulations. Some restrictions on collecting data might have resulted in unexpected small numbers in the unexposed group in secondary prevention, although we think that the study cohort represents nearly a nationwide population of heterozygous FH in Japan. The results derived from patient data in Japan can not necessarily be generalized to patients in western countries.

Despite these limitations of the study, however, we could evaluate the outcome of long-term probucol treatment in the medical practice setting for FH, a high-risk population, for as long as 20 years in Japan. The significant risk reduction of CV events observed in the secondary prevention group holds clinical significance and suggests some beneficial therapeutic actions of this drug in arteriosclerotic diseases. The hypothesis from the findings warrants a randomized controlled trial for verification of the secondary prevention, and needs further research into the molecular mechanisms or roles of CETP in pathogenesis.

Author Contributions

Dr. Yamashita 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: Matsuzawa, Kita, Saito, Fukushima, Matsui. Acquisition of data: Yamashita, Bujo, Arai, Harada-Shiba, Saito, Kita, Matsuzawa. Analysis and interpretation of data: Yamashita, Bujo, Arai, Harada-Shiba, Matsui, Saito, Fukushima, Kita, Matsuzawa.

Drafting of the manuscript: Yamashita, Bujo, Arai, Harada-Shiba, Matsui, and Fukushima. Critical revision of the manuscript for important intellectual content: Yamashita, Matsui, Fukushima, Kita, Saito,

and Matsuzawa. Statistical analysis: Matsui and Fukushima. Administrative, technical, or material support: Fukushima, Matsui, Kita, Saito, and Matsuzawa. Study supervision: Yamashita, Fukushima, Matsui, Kita, Saito, and Matsuzawa.

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Disclosures

From the formerly Daiichi and Otsuka, Dr. Matsui, Dr. Fukushima, Dr. Matsuzawa, and Dr. Kita received fees and expenses for meetings related to protocol design, statistical and clinical interpretation of the data; Dr. Bujo, Dr. Arai, Dr. Harada-Shiba received honoraria and travel expenses for lectures, Dr.Yamashita, Dr. Bujo, Dr. Arai received fees and travel expenses for a meeting related to clinical interpretation of the data. Dr. Yamashita received consultancy fees from Otsuka. Dr. Matsuzawa is contracted as a short-term adviser to Otsuka in medical science. Dr. Saito received travel expenses only.

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Association of serum apolipoprotein B48 level with the presence of carotid plaque in type 2 diabetes mellitus

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ABSTRACT

Aims: The atherogenicity of chylomicron remnants has been discussed. We examined whether serum apoB48 level is associated with the presence of carotid plaque in type 2 diabetic patients.

Method: Forty type 2 diabetic patients (21 males and 19 females, 52.8 ± 11.8 years old; mean ± S.D.) were divided into two groups by the presence or absence of carotid plaque. The diurnal change of serum apoB48 level was measured by enzyme-linked immunosorbent assay.

Results: Fasting serum apoB48 level was higher in the subjects with carotid plaque than those without (6.5 ± 3.8 vs. 4.1 ± 1.9 μg/ml, $p = 0.01$). Age- and gender-adjusted analysis showed that the presence of carotid plaque was associated with fasting apoB48 (OR 1.43; 95% CI, 1.07–2.09, $p = 0.04$) and triglyceride (OR 1.14; 95% CI, 1.02–1.32, $p = 0.04$) levels. In normal LDL-cholesterol (<140 mg/dl) subjects, the presence of carotid plaque was associated with fasting apoB48 level (OR 2.16; 95% CI, 1.22–5.32, $p = 0.04$), but not associated with fasting triglyceride level (OR 1.11; 95% CI, 0.99–1.30, $p = 0.13$).

Conclusions: Serum apoB48 level was strongly associated with the presence of carotid plaque in type 2 diabetic patients.

1. Introduction

Dyslipidemia is related to the pathogenesis of atherosclerosis. The relationship between cardiovascular disease (CVD) and hypercholesterolemia, increased low-density lipoprotein cholesterol (LDL-C) level, or decreased high-density lipoprotein cholesterol (HDL-C), has been well documented. In contrast, the association between CVD and abnormality in triglyceride (TG) metabolism is still debated. Several prospective studies have shown positive association between serum TG level and CVD [1–3]; however, other studies have demonstrated that TG level was no longer associated with atherosclerosis after adjustment for HDL-C [4,5]. The heterogeneity of TG-rich lipoproteins (i.e., chylomicron, VLDL, and those remnant lipoproteins) would make it difficult to evaluate the atherogenicity of hypertriglyceridemia. Recently, it was reported that elevated nonfasting TG level was associated with increased risk of cardiovascular events [6]. It suggests an association between increased remnant lipoproteins and increased risk of CHD. However, it has not been clinically

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established which TG-rich lipoproteins play a crucial role in atherosclerosis.

Chylomicron and its remnant have a characteristic apolipoprotein, apolipoprotein B48 (apoB48), each having one apoB48 molecule per particle. Hence, apoB48 could be a good marker for TG-rich lipoproteins derived from intestine. Several clinical studies indicated that apoB48 level was associated with atherosclerosis [7–10], but the association was not confirmed in other studies [11,12]. The inconsistent results may be due in part to differences in assay method for apoB48. To date, apoB48 level has been commonly evaluated by SDS-PAGE. However, the apoB48 value based on SDS-PAGE is essentially semi-quantitative [13]. Recently, an enzyme-linked immunosorbent assay (ELISA) method for apoB48 was newly established [14,15]. The ELISA method is simple and quantitative, and it has been used in some clinical studies [10,14].

Type 2 diabetes mellitus (DM) is one of the important risk factors for CVD. The mortality and morbidity of diabetic patients are markedly high compared to non-diabetic subjects [16]. In diabetic patients, dyslipidemia is recognized as an important CVD risk factor. A characteristic pattern of dyslipidemia in DM is high TG, low HDL-C, elevated small dense LDL-C, and postprandial lipemia. In type 2 DM, the production and clearance of TG-rich lipoproteins are disturbed [17]. Previous studies in type 2 DM demonstrated that apoB48 level was elevated [18], and apoB48-containing lipoproteins were increased in production and decreased in clearance [19]. Therefore, serum apoB48 level is expected to be increased throughout the day in type 2 DM; however, at present, little is known about the diurnal change. Furthermore, it remains uncertain whether serum apoB48 level is associated with atherosclerosis in type 2 DM. In the present study, we examined the diurnal change of serum apoB48 level and its association with atherosclerosis in type 2 DM.

2. Subjects and methods

2.1. Subjects

Basal data of 147 type 2 diabetic patients admitted to Nippon Medical School Hospital (Tokyo, Japan) for glycemic control were checked. Among these patients, 107 were excluded for the following reasons: treatment with steroid or lipid lowering drugs, complication with diabetic proliferative retinopathy or macroalbuminuria (>300 mg/day), primary hyperlipidemia, liver diseases, chronic renal diseases (>1.2 mg/dl of serum creatinine), infection, malignancy, endocrine diseases, recent major surgery or illness. After receipt of informed consent, 40 patients [21 males, 19 females; age 30–72 years (52.8 ± 11.8, mean ± S.D.)] were enrolled as the subjects of the present study. The subjects were divided into two groups by the presence or absence of carotid plaque (plaque (+) or (−), respectively). Standard lifestyle modifications (exercise and dietary changes) had been adopted for each subject before admission. Six subjects were treated with insulin (14.3% and 15.8% in plaque (+) and (−), respectively, $p = 0.89$) and 20 were treated with oral agents for diabetes (42.9% and 57.9%, $p = 0.34$), including sulphonylurea, glimepiride, metformin, thiazolidinedione, and alpha-glucosidase inhibitor. Fifteen subjects did not take any medication for diabetes (42.9% and 26.3%, $p = 0.30$). There was no bias in treatment for diabetes between the two groups. This study was approved by the Nippon Medical School Hospital Ethics Committee.

2.2. Blood specimen preparation

The study-related assessments were performed within 10 days after admission. All subjects were assessed after an overnight fast of 14 h. The total calories of daily diet (kcal/day) were calculated as 27.5 (kcal) × ideal body weight (IBW). IBW (kg) was calculated as 22 × [height (m)]^2 according to the recommendation of the Japan Society for the Study of Obesity. The nutritional composition of daily diet was 20% fat, 25% protein, and 55% carbohydrate. Each subject was provided three meals per day, at 8:00, 12:00, and 18:00. To assess the diurnal changes in serum apoB48, serum lipids, and plasma glucose (PG), blood specimens were obtained at 30 min before and 2 h after each meal (7:30, 10:00, 11:30, 14:00, 17:30, and 20:00), night (23:00), and early morning (3:00). Blood was drawn from the cubital vein and collected in test tubes. The blood samples collected from 7:30 to 14:00 were immediately centrifuged, and the samples from 17:00 to 3:00 were kept at 4 °C and centrifuged the next morning. To analyze PG, a portion of each blood sample was collected into a tube with NaF and anticoagulant, and centrifuged immediately after blood collection. Serum and plasma were immediately collected after the centrifugations, and stored at −80 °C until assayed.

2.3. Clinical and biochemical assessment

Subjects underwent a physical examination (height, weight, blood pressure, and waist circumference). Blood pressure was measured with a standard mercury sphygmomanometer in a sitting position in the morning. Smoking habits, a familial history of CVD, and duration of DM were assessed by interview. Smoking was defined as current or past smoking. Serum total cholesterol (TC), HDL-C, and TG were measured by enzymatic methods (LABOSPECT 008, Hitachi, Tokyo, Japan). Glycated albumin (GA) was also measured enzymatically (JCA-BM12, Japan Electron Optics Laboratory, Tokyo, Japan). LDL-C level was calculated by the Friedewald formula [20]. Plasma glucose (PG) was measured by a glucose oxidase method (ADAMS Glucose GA-1170, Arkray, Kyoto, Japan), and hemoglobin A_{1c} (HbA_{1c}) was measured by high performance liquid chromatography (ADAMS A1c HA-8160, Arkray). The plasma levels of lipoprotein lipase mass and adiponectin were measured by ELISA (Daiichi Pure Chemicals and Otsuka pharmaceutical, Tokyo, Japan, respectively). Serum apoB48 level was measured by ELISA using an anti-human apoB48 monoclonal antibody (B-48-151) as previously described (Fujirebio, Tokyo, Japan) [14,15]. The apoB48 assay was highly reproducible, with coefficients of variation of 1.9–3.1% and 2.2–4.4% for intra- and inter-assay, respectively.

Carotid artery status was examined by high-resolution B-mode ultrasonography (SDU-2000, Shimadzu, Kyoto, Japan; iU22 and EnVisor, Philips Medical Systems, Andover, MA, USA; LOGIQ 7, GE Healthcare, Tokyo, Japan). The ultrasound devices

were used with electrical linear transducers (3–12 MHz). Subjects were placed in a supine position with the neck hyper-extended. Carotid plaque was assessed in common carotid artery, the carotid artery bifurcation, and internal carotid artery bilaterally. Plaque was defined as a localized intima-media thickness (IMT) ≥1.0 mm with marked protuberance. All measurements were performed by well-trained doctors.

2.5. Statistical analysis

Values are presented as means ± S.D. Statistical analysis was performed by χ^2 test or Fisher's exact test, as appropriate, and Student's t-test or Welch's t-test, as appropriate. Logistic regression analysis was used to calculate odds ratios of each variable for carotid plaque. Because TG values were highly dispersed, the value TG/10 was used for the calculation of odds ratio. Data were analyzed with JMP 6 software (SAS Institute, Cary, NC). All statistical tests were two-sided, and $p < 0.05$ were considered significant.

3. Results

3.1. Clinical and laboratory data

The clinical and laboratory data of all subjects are presented in Table 1. There were no differences in gender distribution, age, duration of DM, smoking habit, body mass index, or blood pressure between the plaque (+) and (−) groups. The two groups were similar in fasting TC, LDL-C, PG, and HbA_{1c} levels. The fasting serum apoB48 and TG levels were significantly higher in plaque (+) than in plaque (−).

3.2. Diurnal changes in apoB48, lipids, and glucose

Diurnal changes in serum apoB48 and TG levels are shown in Fig. 1A and B. Serum apoB48 level was sharply increased after breakfast and reached peaks after lunch and dinner, then returned toward the fasting levels at 3:00 in both plaque (+) and plaque (−). ApoB48 and TG levels were significantly higher in plaque (+) than in plaque (−) throughout the day, with the exception of a few time points. PG level was elevated after each meal in a similar pattern for both groups (Supplementary Fig. 1A). The diurnal fluctuation of TC and HDL-C levels was small and similar in both groups (Supplementary Fig. 1B and C).

3.3. Association of apoB48 with plaque

The results of logistic regression analysis showed that fasting serum apoB48 and TG levels were significantly associated with the presence of carotid plaque, regardless of adjustment for age and gender (Table 2). Other variables were not associated with the presence of carotid plaque.

3.4. Analyses of subjects with normal LDL-C level (<140 mg/dl)

To exclude the involvement of LDL-C level on the association between apoB48 and carotid plaque, we examined this factor in normal LDL-C (<140 mg/dl) subjects ($n = 25$). The normal LDL-C level of < 140 mg/dl was defined according to the recommendation of Japan Atherosclerosis Society [21]. Among the normal LDL-C subjects, 12 subjects were with carotid plaque [plaque (+)] and 13 were without the plaque [plaque (−)].

Table 1 – Clinical and laboratory data for all subjects

	Total ($n = 40$)	Plaque (+) ($n = 21$)	Plaque (−) ($n = 19$)	p value (+) vs. (−)
Male/female	21/19	13/8	8/11	0.21
Age (year)	52.8 ± 11.8	53.4 ± 9.9	52.1 ± 13.9	0.72
Duration of DM (year)	5.9 ± 6.3	5.9 ± 7.0	5.9 ± 5.7	0.99
Familial history of CVD (%)	10.0	14.3	5.3	0.37
Smoking (%)	57.5	66.7	47.4	0.22
BMI (kg/m^2)	24.1 ± 3.9	23.9 ± 3.1	24.4 ± 4.7	0.68
Waist circumference (cm)	83.8 ± 12.8	82.5 ± 13.3	85.5 ± 12.5	0.48
Blood pressure (mmHg)				
Systolic	121 ± 12	122 ± 11	120 ± 13	0.59
Diastolic	73 ± 10	73 ± 9	73 ± 11	0.80
TC (mg/dl)	210 ± 43	217 ± 38	201 ± 47	0.23
HDL-C (mg/dl)	52 ± 17	49 ± 14	55 ± 19	0.21
LDL-C (mg/dl)	130 ± 32	136 ± 30	123 ± 32	0.19
TG (mg/dl)	140 ± 73	163 ± 84	115 ± 48	0.03
Glucose (mg/dl)	177 ± 59	181 ± 67	172 ± 50	0.64
HbA_{1c} (%)	9.8 ± 2.1	10.1 ± 2.2	9.5 ± 1.9	0.40
GA (%)	28.0 ± 19.1	27.8 ± 8.5	28.1 ± 10.0	0.92
Adiponectin (μg/ml)	7.1 ± 4.4	6.3 ± 3.8	8.0 ± 4.9	0.25
LPL (ng/ml)	35.9 ± 14.4	34.4 ± 13.2	37.7 ± 15.7	0.49
ApoB48 (μg/ml)	5.4 ± 3.3	6.5 ± 3.8	4.1 ± 1.9	0.01

Values are means ± S.D. Plaque (+), subjects with carotid plaque; plaque (−), subjects without carotid plaque; DM, diabetes mellitus; CVD, cardiovascular disease; BMI, body mass index; TC, total cholesterol; HDL-C, HDL cholesterol; TG, triglyceride; LDL-C, LDL cholesterol; HbA_{1c}, hemoglobin A_{1c}; GA, glycated albumin; LPL, lipoprotein lipase; apoB48, apolipoproteinB48.

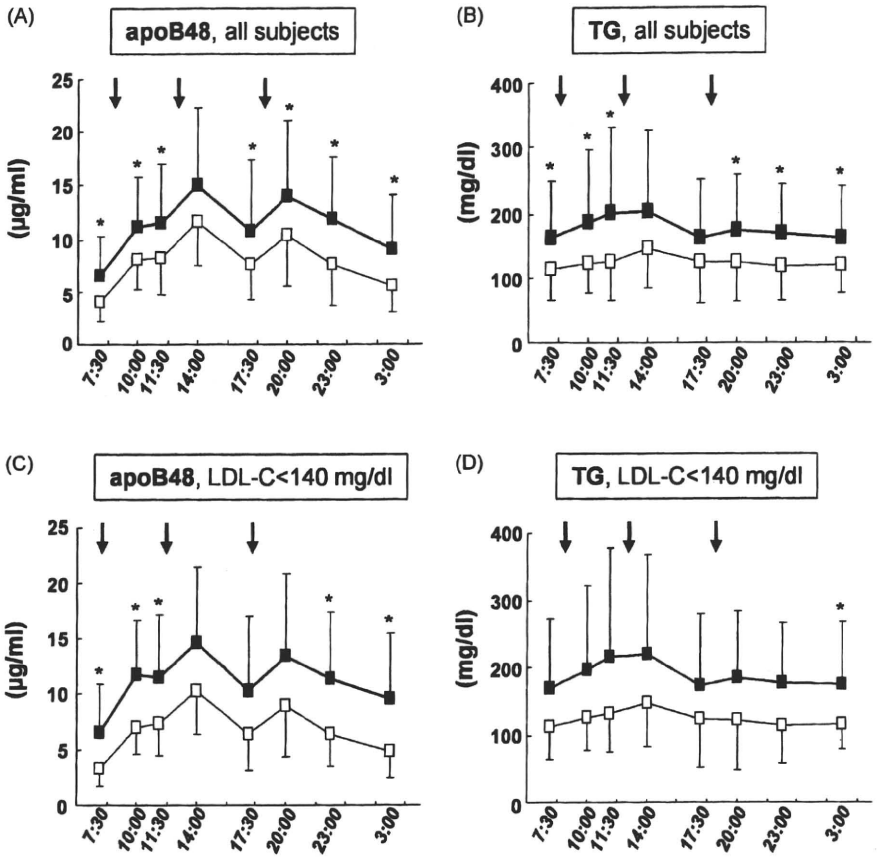

Fig. 1 – Diurnal changes of serum apolipoprotein B48 (apoB48, A and C) and triglyceride (TG, B and D) levels in the subjects with (■) and without (□) carotid plaque. A and B, all subjects (19 and 21 subjects with and without carotid plaque, respectively). C and D, normal LDL-cholesterol (LDL-C <140 mg/dl) subjects (13 and 12 subjects with and without carotid plaque, respectively). Values are means ± SD. *p < 0.05 vs. subjects without plaque. Arrows indicate breakfast, lunch, and dinner at 8:00, 12:00, and 18:00, respectively.

In the normal LDL-C subjects, fasting apoB48 level was significantly higher in plaque (+) than in plaque (–); however, the fasting TG level and other parameters were not different between the two groups (Table 3). The diurnal pattern of apoB48 level in the normal LDL-C subjects (Fig. 1C) was similar to that in all subjects (Fig. 1A). ApoB48 level was significantly higher in plaque (+) than in plaque (–) throughout the day, with the exception of a few time points (Fig. 1C). In contrast, TG level was not significantly different between the two groups, except at 3:00 (Fig. 1D). The diurnal changes of PG, TC, and HDL-C are similar in both plaque (+) and (–) (data not shown). Table 4 shows the results of logistic regression analysis in the normal LDL-C subjects. Regardless of gender and age adjustment, the fasting apoB48 level was significantly

Table 2 – Unadjusted, and age- and gender-adjusted odds ratios for the presence of carotid plaque in all subjects (n = 40)

Variable	Unadjusted		Adjusted	
	Odds ratio (95% CI)	p value	Odds ratio (95% CI)	p value
Age	1.01 (0.96–1.07)	0.71		
Gender (male)	1.50 (0.80–2.87)	0.21		
Smoking	1.49 (0.79–2.88)	0.22	1.33 (0.63–2.82)	0.45
HDL-C	0.97 (0.93–1.01)	0.21	0.98 (0.92–1.02)	0.34
LDL-C	1.01 (0.99–1.04)	0.19	1.02 (0.10–1.05)	0.12
TG	1.13 (1.02–1.28)	0.04	1.14 (1.02–1.32)	0.04
HbA_{1c}	1.15 (0.84–1.60)	0.39	1.16 (0.84–1.66)	0.39
ApoB48	1.42 (1.08–2.03)	0.03	1.43 (1.07–2.09)	0.04

CI, confidence interval; HbA_{1c}, hemoglobin A_{1c}; LDL-C, LDL cholesterol; HDL-C, HDL cholesterol; TG, triglycerides; ApoB48, apolipoprotein B48.

Table 3 – Clinical and laboratory data in normal LDL-C (<140 mg/dl) subjects

	Total (n = 25)	Plaque (+) (n = 12)	Plaque (–) (n = 13)	p value (+) vs. (–)
Male/female	14/11	7/5	7/6	0.82
Age (year)	51.1 ± 12.5	50.0 ± 3.7	52.0 ± 3.5	0.71
Duration of DM (year)	4.8 ± 5.3	3.7 ± 1.5	5.8 ± 1.5	0.32
Familial history of CVD (%)	8.0	8.3	7.6	0.43
Smoking (%)	60.0	66.7	53.8	0.76
BMI (kg/m^2)	24.0 ± 3.4	23.5 ± 1.0	24.5 ± 3.7	0.50
Waist circumference (cm)	83.2 ± 13.3	79.5 ± 3.8	86.9 ± 3.8	0.18
Blood pressure (mmHg)				
Systolic	120 ± 13	122 ± 4	118 ± 4	0.37
Diastolic	71 ± 11	71 ± 11	71 ± 12	0.89
TC (mg/dl)	189 ± 34	199 ± 31	179 ± 35	0.15
HDL-C (mg/dl)	49 ± 14	49 ± 14	49 ± 14	0.89
LDL-C (mg/dl)	111 ± 22	116 ± 6	107 ± 6	0.32
TG (mg/dl)	140 ± 83	169 ± 23	113 ± 22	0.09
Glucose (mg/dl)	183 ± 65	196 ± 72	172 ± 59	0.37
HbA_{1c} (%)	9.7 ± 2.1	10.0 ± 0.6	9.3 ± 0.6	0.45
GA (%)	28.0 ± 9.9	28.1 ± 2.9	27.9 ± 2.8	0.96
Adiponectin (μg/ml)	6.9 ± 4.3	6.5 ± 1.3	7.3 ± 1.2	0.61
LPL (ng/ml)	35.9 ± 15.0	37.1 ± 4.6	34.8 ± 4.4	0.73
ApoB48 (μg/ml)	4.8 ± 3.6	6.5 ± 4.4	3.2 ± 1.5	0.03

Values are means ± S.D. Plaque (+), subjects with carotid plaque; plaque (–), subjects without carotid plaque; DM, diabetes mellitus; CVD, cardiovascular disease; BMI, body mass index; TC, total cholesterol; HDL-C, HDL cholesterol; TG, triglyceride; LDL-C, LDL cholesterol; HbA_{1c}, hemoglobin A_{1c}; GA, glycated albumin; LPL, lipoprotein lipase; apoB48, apolipoproteinB48.

associated with the presence of carotid plaque. The fasting TG level and other variables were not associated with the presence of carotid plaque.

4. Discussion

The present study demonstrated that fasting serum apoB48 level was associated with the presence of carotid plaque in type 2 DM and that diurnal apoB48 level was significantly higher in the plaque (+) group than in the plaque (–) group, throughout most of the day. The present result that apoB48 is associated with atherosclerosis is consistent with several previous studies [7–10]. To date, possible underlying mechanisms for the association have been suggested in several in vitro experiments. Flood et al. [22] indicated that apoB48 possesses a binding site to arterial wall proteoglycans, and the interaction will induce the retention of apoB48-containing lipoproteins. It is also reported that chylomicron remnants were taken up by macrophages via LDL receptor-related protein [23] and apoB48 receptor [24,25]. These results suggest that apoB48-containing lipoproteins could penetrate into vascular subendothelial space, and where it could be involved in macrophage foam cell formation. In fact, apoB48-containing lipoproteins penetrated into the artery wall and were retained within the subendothelial space of the carotid artery in Watanabe heritable hyperlipidemic rabbits [26]. Furthermore, ApoB48 was also identified in human atherosclerotic plaque [27].

It is well established that high LDL-C level is a strong determinant of atherosclerosis; however, high LDL-C level was not significantly associated with the presence of carotid plaque in the present study. This result might be due to the range and the mean value of LDL-C in the subjects. In the present study, the range was not particularly wide and the mean value was not high (130 ± 32 mg/dl), which might be the

Table 4 – Unadjusted, and age- and gender-adjusted odds ratios for the presence of carotid plaque in normal LDL-cholesterol (<140 mg/dl) subjects (n = 25)

Variable	Unadjusted		Adjusted	
	Odds ratio (95% CI)	p value	Odds ratio (95% CI)	p value
Age	0.99 (0.92–1.05)	0.70		
Gender (male)	1.10 (0.49–2.46)	0.82		
Smoking	1.31(0.59–3.04)	0.51	1.38 (0.50–4.21)	0.54
HDL-C	1.00 (0.94–1.06)	0.88	1.00 (0.93–1.08)	0.90
LDL-C	1.02 (0.98–1.07)	0.31	1.03 (0.99–1.08)	0.21
TG	1.11 (0.99–1.29)	0.11	1.11 (0.99–1.30)	0.13
HbA_{1c}	1.17 (0.80–1.73)	0.43	1.17 (0.80–1.78)	0.43
ApoB48	1.91 (1.17–4.03)	0.04	2.16 (1.22–5.32)	0.04

CI, confidence interval; HbA_{1c}, hemoglobin A_{1c}; LDL-C, LDL cholesterol; HDL-C, HDL cholesterol; TG, triglycerides; ApoB48, apolipoprotein B48.