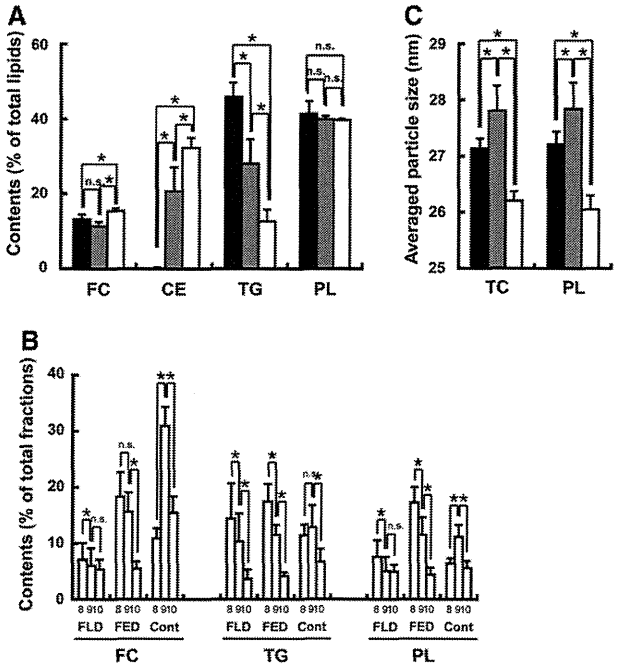

Figure 3. Characterization of lipid profiles in Lp8 of familial lecithin:cholesterol acyltransferase deficiency (FLD) and Fish-eye disease (FED). **A**, Lipid compositions of Fr. 7 to 10 fractions (Lp8) were compared among FLD (closed column), FED (gray column), and normal (open column). *P<0.05. **B**, Lipid concentrations of fractions 8, 9, and 10 were compared in FLD (n=5), FED (n=4), and controls (n=4). *P<0.05. Cholesteryl ester (CE) concentrations in FLD are not shown because levels were undetectable. **C**, Size distribution of lipoproteins in Lp8 (Fr. 7–10) was compared among FLD (closed column), FED (gray column), and normal (open column) based on total cholesterol (TC) and phospholipid (PL) concentrations. *P<0.05. FC indicates free cholesterol; and TG, triglyceride.

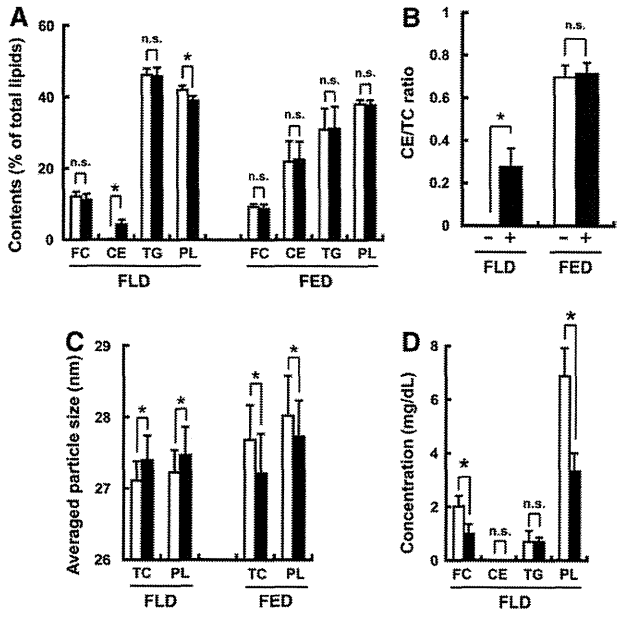

Figure 4. Effects of in vitro familial lecithin:cholesterol acyltransferase (LCAT) supplementation on the lipid profiles of abnormal lipoproteins in LCAT-deficiency syndrome. After analyses described in Figure II in the online-only Data Supplement, lipid composition (**A**), cholesteryl ester (CE)/TC ratio (**B**), averaged particle size based on total cholesterol (TC) and phospholipid (PL) concentrations (**C**), in Lp8, and lipid concentrations in Lp12 to 16 (**D**), were compared between culture media containing recombinant LCAT (rLCAT; closed column) and media without rLCAT (open column). *P<0.05. FC indicates free cholesterol; FED, Fish-eye disease; and TG, triglyceride.

deficiency (Figures 1 and 4B). Lp8 also differed in composition between FLD and FED: in FLD, it contained increased TG and decreased CE in comparison with FED (Figure 3A). Importantly, although the levels varied with the severity of renal damage as did those in Lp1, the buoyance of the peak at Fr. 8 did not vary with severity of renal damage (Figure 2), strongly suggesting that Lp8 directly results from a lack of LCAT and not from metabolic disturbances that occur during proteinuria and progressive renal failure.

In addition to the above-mentioned characteristics for Lp8 in LCAT-deficiency syndrome, HPLC-GFC analyses clarified novel unique lipid properties of Lp8 in FLD in comparison with that in FED; the averaged sizes of Lp8 are smaller in FLD than those in FED (Figure 3C). The lipid compositions of Lp8 in FLD were, in part, ameliorated by rLCAT incubation (Figure 4A). The averaged sizes of the Lp8 increased in FLD, whereas those in FED decreased (Figure 4C). rLCAT increased the CE formation in both LDL and HDL fractions in FLD sera. Thus, these findings indicated that the abnormal compositions were most likely caused primarily by the dysfunction of LCAT in the patients, and that the abnormal characteristics of Lp8 were not because of metabolic disturbances that occur during proteinuria and progressive loss of kidney function.

Previous extensive analyses using electron microscopy have identified 3 abnormal lipoproteins in the LDL fraction of FLD[12]: TG-rich and CE-poor particles of sizes similar to normal LDL (FLD-LDL); FC- and PL-containing particles of sizes distributing from 40 to 60 nm (LpX-like particle)[2]; particles with a diameter of 100 nm (designated as LM-LDL)[17,30] that were later reported to be identical to LpX.[15] LpX is FC- and PL-rich but TG-poor lipid particles (30%, 60%, and 2%, respectively)[22] without apolipoproteins, which range from very low density lipoprotein to large LDL fractions in fast performance liquid chromatography analysis.[31] The abnormal particles have been shown to be decreased by lipid-lowering therapy in a patient with FLD.[21] Lipoproteins in Lp8 were different from LpX in the lipid contents; the fractions were rich in FC and PL and also rich in TG (13.2±1.3%, 41.4±3.3%, and 45.8±3.8%, respectively). The composition analyses suggested that Lp8 corresponds to FLD-LDL, but the calculated sizes of Lp8 were larger than normal LDL using the data obtained by size fractionation with HPLC-GPC in the present study. Thus, the identified Lp8 in LCAT-deficiency syndrome was most likely not identical to LpX in the characteristics.

There is a limitation for the interpretation of the quantitative measurement of LpX in the frozen samples collected in our study because the abnormal lipoproteins were known to be labile to freezing-and-thawing treatment. In this context, fresh sera were collected from patients 2 and 4 and analyzed by agarose gel electrophoresis. The lipid staining of lipoproteins electrophoresed in agarose gel detected the abnormally slowly migrating TG-poor lipoproteins, LpX, at the expectedly migrating position, as well as TG-rich abnormal β-lipoproteins (LDL) in the once-frozen sample, as well as the fresh sample

in patient 4, although the staining intensity tended to decrease in comparison with the fresh counterpart. However, LpX was not detected in either sample with or without freeze-and-thaw treatment from patient 2. Thus, LpX was indeed labile to freeze/thawing, and the frozen samples were not adequate for the quantitative measurement. However, the presence was still able to be evaluated after once-freezing treatment. On the basis of background data, HPLC-GFC analysis showed that lipid contents in Lp8 were not largely affected by once-freezing treatment in both patients 2 and 4: in contrast, the contents of TG and PL were slightly decreased in lipoproteins with peak of Fr. 5 (data not shown). Additional studies using fresh samples of patients with distinct mutations and manifestations are needed to interpret the significance of novel lipoproteins in comparison with LpX for the development of renal insufficiency in LCAT0deficiency syndrome quantitatively.

In FLD but not in FED, Lp12 to 16 were heterogeneous in size and rich in PL. rLCAT decreased PL in these fractions specifically (Figure 5D; Figure II in the online-only Data Supplement). This may suggest that the heterogeneous-sized PL-rich particles in Fr. 12 to 16 converge to normal-sized HDL (Fr. 16–18) on incubation with rLCAT, with concomitant esterification of FC.

In conclusion, 4 lipoprotein fractions specific to LCAT-deficiency syndromes were identified by the HPLC-GFC analysis of samples from genetically diagnosed patients with different mutations and manifestations. The composition of 2 of these was unique to only FLD; these were not likely compatible with the previously reported LpX. These abnormal lipoproteins may be causal to the renal pathology in FLD, the main cause of increased morbidity and mortality in this condition. The regular evaluation of these specific lipid fractions during LCAT enzyme replacement therapy in patients with LCAT deficiency may provide guidance for success of the intervention. The value of these lipid fractions for risk of future renal disease needs to be addressed in prospective follow-up studies in patients with FLD with various mutations in the *LCAT* gene before the onset of proteinuria.

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Disclosures

None.

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Significance

Lecithin:cholesterol acyltransferase-deficiency syndromes are classified into 2 forms: familial lecithin:cholesterol acyltransferase deficiency and fish-eye disease. Patients with familial lecithin:cholesterol acyltransferase deficiency develop renal failure, whereas fish-eye disease patients do not. This study was performed to identify abnormal lipoproteins associated with the renal damage of patients with different mutations and manifestations. Size fractionation with gel filtration of patients' sera and in vitro incubation experiments with recombinant lecithin:cholesterol acyltransferase showed abnormal lipoproteins associated with the renal damage. Thus, our novel analytic approach identified large low-density lipoprotein and high-density lipoprotein with a composition specific to familial lecithin:cholesterol acyltransferase deficiency but not to fish-eye disease. The identification of abnormal lipoproteins may shed light on the clarification of renal pathology and the development of treatment for the patients with familial lecithin:cholesterol acyltransferase deficiency.

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Association of lifestyle-related factors with circadian onset patterns of acute myocardial infarction: a prospective observational study in Japan

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ABSTRACT

Objective: The onset of acute myocardial infarction (AMI) shows characteristic circadian variations involving a definite morning peak and a less-defined night-time peak. However, the factors influencing the circadian patterns of AMI onset and their influence on morning and night-time peaks have not been fully elucidated.

Design, setting and participants: An analysis of patients registered between 1998 and 2008 in the Osaka Acute Coronary Insufficiency Study, which is a prospective, multicentre observational study of patients with AMI in the Osaka region of Japan. The present study included 7755 consecutive patients with a known time of AMI onset.

Main outcomes and measures: A mixture of two von Mises distributions was used to examine whether a circadian pattern of AMI had uniform, unimodal or bimodal distribution, and the likelihood ratio test was then used to select the best circadian pattern among them. The hierarchical likelihood ratio test was used to identify factors affecting the circadian patterns of AMI onset. The Kaplan-Meier method was used to estimate survival curves of 1-year mortality according to AMI onset time.

Results: The overall population had a bimodal circadian pattern of AMI onset characterised by a high and sharp morning peak and a lower and less-defined night-time peak (bimodal $p<0.001$). Although several lifestyle-related factors had a statistically significant association with the circadian patterns of AMI onset, serum triglyceride levels had the most prominent association with the circadian patterns of AMI onset. Patients with triglyceride ≥150 mg/dL on admission had only one morning peak in the circadian pattern of AMI onset during weekdays, with no peaks detected on weekends, whereas all other subgroups had two peaks throughout the week.

Conclusions: The circadian pattern of AMI onset was characterised by bimodality. Notably, several lifestyle-related factors, particularly serum triglyceride levels, had a strong relation with the circadian pattern of AMI onset.

Strengths and limitations of this study

- We comprehensively analysed the circadian patterns of acute myocardial infarction (AMI) onset in a large, multicentre cohort of patients in relation to patient characteristics, lifestyle factors and the day of the week.
- A mixture of two von Mises distributions revealed that the circadian pattern of AMI onset exhibited bimodality.
- Several lifestyle-related factors were shown to be associated with the circadian patterns of AMI onset, depending on the day of the week. In particular, it was demonstrated that elevated serum triglyceride levels on admission accentuated morning peak of AMI onset during weekdays.
- Participants were limited to those who were hospitalised for AMI.
- Laboratory data were evaluated on admission.

Trial registration number: UMIN000004575.

INTRODUCTION

Onset patterns of acute myocardial infarction (AMI) exhibit circadian variation which is characterised by an increased frequency in the morning and a secondary peak incidence at night-time.[1] Several studies have confirmed that AMI onset exhibits a bimodal circadian pattern, with peaks occurring in the morning[2–4] and night-time hours.[1 4–7] However, it is not well understood what factors, particularly lifestyle-related factors, influence the circadian patterns of AMI. Moreover, although these patterns appear to vary according to the day of the week,[8] it is unclear how the circadian patterns

of AMI onset vary throughout the week, particularly, in association with socioeconomic factors.

As AMI and subsequent ischaemic heart failure is the leading cause of death in developed and developing countries, primary prevention of AMI is a major healthcare issue worldwide. Accordingly, identifying potential factors influencing the circadian pattern of AMI may help in the clinical management of patients to prevent the onset of AMI.

In the present study, we comprehensively analysed the circadian patterns of AMI onset in a large, multicentre cohort of patients in relation to patient characteristics, lifestyle factors and the day of the week.

METHODS

OACIS registry and study participants

The Organisation to Assess Strategies for Ischaemic Syndromes (OACIS) is a prospective, multicentre observational study collecting demographic, procedural, biological and outcome data as well as blood samples from patients with AMI hospitalised at 25 collaborating hospitals from the Osaka region of Japan (UMIN-Clinical Trial Registry ID: UMIN000004575; see online supplementary appendix).[9 10] A diagnosis of AMI was made if the patient fulfilled at least two of the following three criteria: (1) history of central chest pressure, pain or tightness lasting 30 min, (2) typical ECG changes (ie, ST-segment elevation ≥0.1 mV in one standard limb lead or two precordial leads, ST-segment depression ≥0.1 mV in two leads, abnormal Q-waves or T-wave inversion in two leads) and (3) an increase in serum creatine kinase levels two times the upper normal limit in each hospital. All the collaborating hospitals were encouraged to enrol consecutive patients with AMI.

We prospectively collected data with the help of research cardiologists and trained research nurses using a specific reporting form, and the following variables were extracted from the OACIS registry database: age, gender, working status, body mass index, coronary risk factors (diabetes, hypertension, dyslipidaemia, smoking, drinking, previous MI, multivessel disease and collateral circulation), clinical presentation on admission (KILLIP classification, initial TIMI flow and ST-elevation MI (STEMI)), coronary angiography data, reperfusion therapy, laboratory data on admission (glycated haemoglobin (HbA1c), total cholesterol, low-density (LDL) and high-density lipoprotein (HDL) cholesterol, triglyceride and estimated glomerular filtration rate) and medications at discharge (RAS inhibitors, β-blocker, calcium channel blocker, statin, antiplatelet agent and diuretics). Diabetes mellitus was defined as fasting plasma glucose ≥126 mg/dL, HbA1c ≥6.5% or a history of antidiabetic therapy. Hypertension was defined as a history of systolic blood pressure ≥140 mm Hg, diastolic blood pressure ≥90 mm Hg or antihypertensive therapy. Dyslipidaemia was defined as fasting total cholesterol ≥220 mg/dL, LDL cholesterol ≥140 mg/dL, HDL cholesterol ≤40 mg/dL, fasting triglycerides ≥150 mg/dL or lipid-lowering therapy.

The study protocol has been approved by the ethics committee of each participating hospital. All in-hospital data were obtained after written informed consent had been received and were then transmitted to the data collection centre at the Department of Cardiovascular Medicine, Osaka University Graduate School of Medicine, Suita, Japan, for processing and analysis. The corresponding authors had full access to and validated all data in the study.

In the present study, we analysed 7755 patients with AMI whose time of AMI onset was definitely known among the 8603 consecutive patients registered in the OACIS registry between 1998 and 2008. Patients' baseline characteristics are presented in table 1.

Statistical analysis

Continuous variables were summarised as quartiles and were compared using the Wilcoxon rank-sum test for two-group comparisons, and the Kruskal-Wallis test for four-group comparisons. Categorical variables were presented as numbers and percentages, and were compared using the χ^2 test. A mixture of two von Mises distributions was used to examine whether a circadian pattern of AMI onset had uniform (no peak), unimodal (one peak) or bimodal distribution (two peaks), and the likelihood ratio test was then used to select the best circadian pattern among them.[11] In addition, the hierarchical likelihood ratio test was used to identify factors affecting the circadian patterns of AMI onset. The Kaplan-Meier method was used to estimate survival curves of 1-year mortality according to AMI onset time (morning (6:00–11:59), afternoon (12:00–17:59), evening (18:00–23:59) and night-time (0:00–5:59)). The log-rank test was used to compare survival curves between the groups, and the Cox proportional hazards regression model was used to estimate HRs and 95% CIs. To reduce potential confounding effects due to patient background variability in the comparison between the afternoon-onset and other groups, a stratified Cox proportional hazards regression model was used, in which the potential confounding variables were included into the model as stratification factors. Cosinor analysis was used to estimate the amplitude of serum triglyceride (TG) levels on admission according to AMI onset time. Then, an F-test for the existence of a rhythm (amplitude) was used to examine whether the amplitude of serum TG levels on admission in patients with AMI had circadian variation or not. Statistical significance was set as $p<0.05$. All statistical analyses were performed using an in-house validated Fortran program or SAS V.9.3 (SAS Institute Inc, Cary, North Carolina, USA).

RESULTS

Bimodal circadian patterns of AMI onset in the overall population

The daily patterns of AMI onset in our cohort of 7755 patients were first analysed using the likelihood ratio test (figure 1). In the overall population, AMI onset clearly

Table 1 Demographics and clinical characteristics of the study population

	N=7755
Patients	
Age (years)	66 (57–74)
Male (%)	5872 (75.7)
Job (%)	3364 (48.2)
BMI (kg/m^2)	23.4 (21.4–25.7)
Cardiovascular risk factors	
Smoker (%)	4865 (63.9)
Drinker (%)	3321 (45.3)
Diabetes (%)	2586 (33.4)
Hypertension (%)	4424 (58.9)
Dyslipidaemia (%)	3259 (44.1)
Previous MI (%)	983 (13.0)
Angina pectoris (%)	1737 (23.4)
Multivessel disease (%)	2790 (38.4)
Collateral circulation (%)	2576 (35.7)
Clinical presentation	
Onset admission time <24 h (%)	6804 (89.1)
KILLIP ≥II (%)	1331 (18.0)
Initial TIMI ≤II (%)	4759 (68.4)
STEMI (%)	6567 (86.0)
Laboratory data on admission	
Blood glucose level (mg/dL)	152 (122–209)
HDL cholesterol (mg/dL)	44 (37–53)
LDL cholesterol (mg/dL)	121 (99–147)
Triglycerides (mg/dL)	92 (58–142)
HbA1c (%)	5.9 (5.5–6.9)
Peak CK (IU/L)	2147 (1069–4006)
eGFR (mL/min/1.73 m^2)	64.5 (49.2–80.9)
Localisation of MI	
LAD	3050 (41.7)
RCA	2447 (33.4)
LCX	998 (13.6)
LMT	164 (2.2)

Categorical variables are presented as number (%), and continuous variables are presented as quartile. Laboratory data were measured on admission. Smoker was defined as a patient with a smoking history, and drinker was defined as an active drinker. Number (%) of localisation of MI was calculated out of 7319 patients who underwent coronary angiography.
BMI, body mass index; CK, creatine kinase; eGFR, estimated glomerular filtration rate; HbA1c, glycated haemoglobin; HDL, high-density lipoprotein; LAD, left anterior descending artery; LCX, left circumflex artery; LDL, low-density lipoprotein; LMT, left main trunk; MI, myocardial infarction; RCA, right coronary artery; STEMI, ST-elevation myocardial infarction.

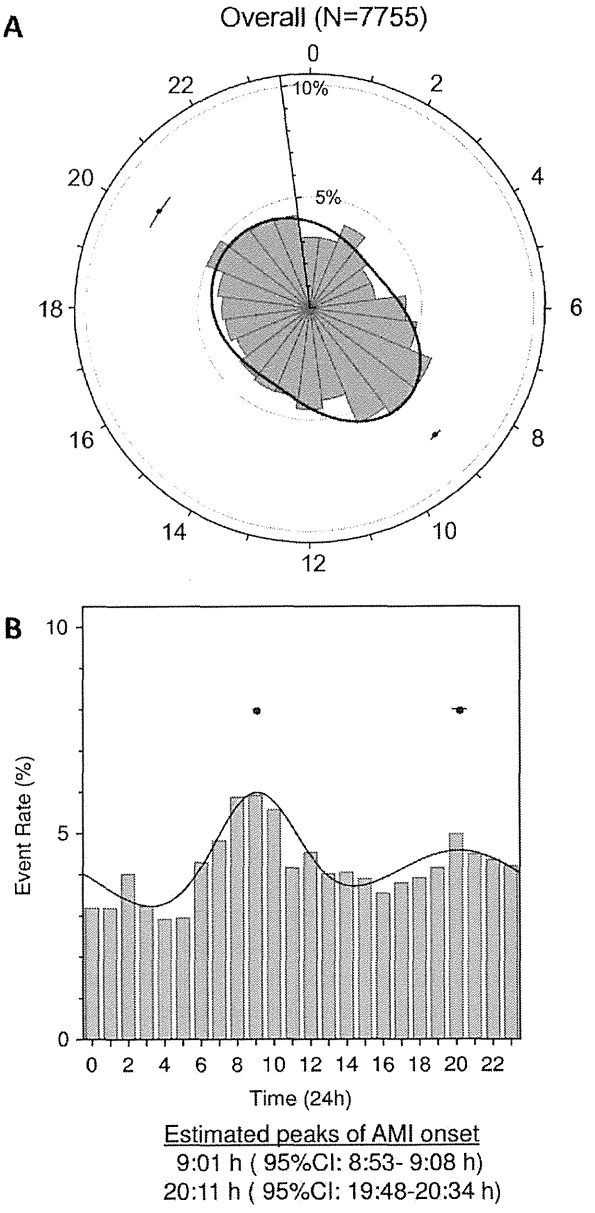


Figure 1 Circadian pattern of acute myocardial infarction (AMI) onset in the overall population. A circadian pattern of AMI onset in the overall population was clearly observed in a circular plot (A) and histogram (B). The solid line corresponds to the fitted von Mises distribution, and the dots with error bars are the estimated peak onset times and 95% CIs, respectively.

exhibited a circadian pattern consisting of two peaks (bimodal: p<0.001): a primary peak at 9:01 (95% CI 8:53 to 9:08) and a secondary peak at 20:11 (95% CI 19:48 to 20:34). The primary peak was more clearly defined than the secondary peak in the circular and columnar histograms (figure 1A, B, respectively).

The likelihood ratio test analysis revealed that the peak time of AMI onset varied according to the day of the week (figure 2). For example, the primary peak onset time was earliest on Monday (8:24 (95% CI 8:04 to 8:44)) and latest on Sunday (9:44 (95% CI 9:22 to 10:06)). On Tuesday, patients exhibited a circadian pattern of AMI onset characterised by late primary (9:28 (95% CI 9:06 to 9:51)) and secondary peak onset times (21:13 (95% CI 20:40 to 21:46)), whereas earlier peak onset times (8:43 (95% CI 8:15 to 9:10) and 19:09 (95% CI 18:23 to 19:55)) were detected on Thursday. Notably, the evening peak was higher and sharper than the morning peak on Saturday (figure 2).

Factors affecting the circadian patterns of AMI onset

The hierarchical likelihood ratio analysis revealed that serum TG levels on admission, smoking, age, drinking, blood glucose levels on admission, gender and working

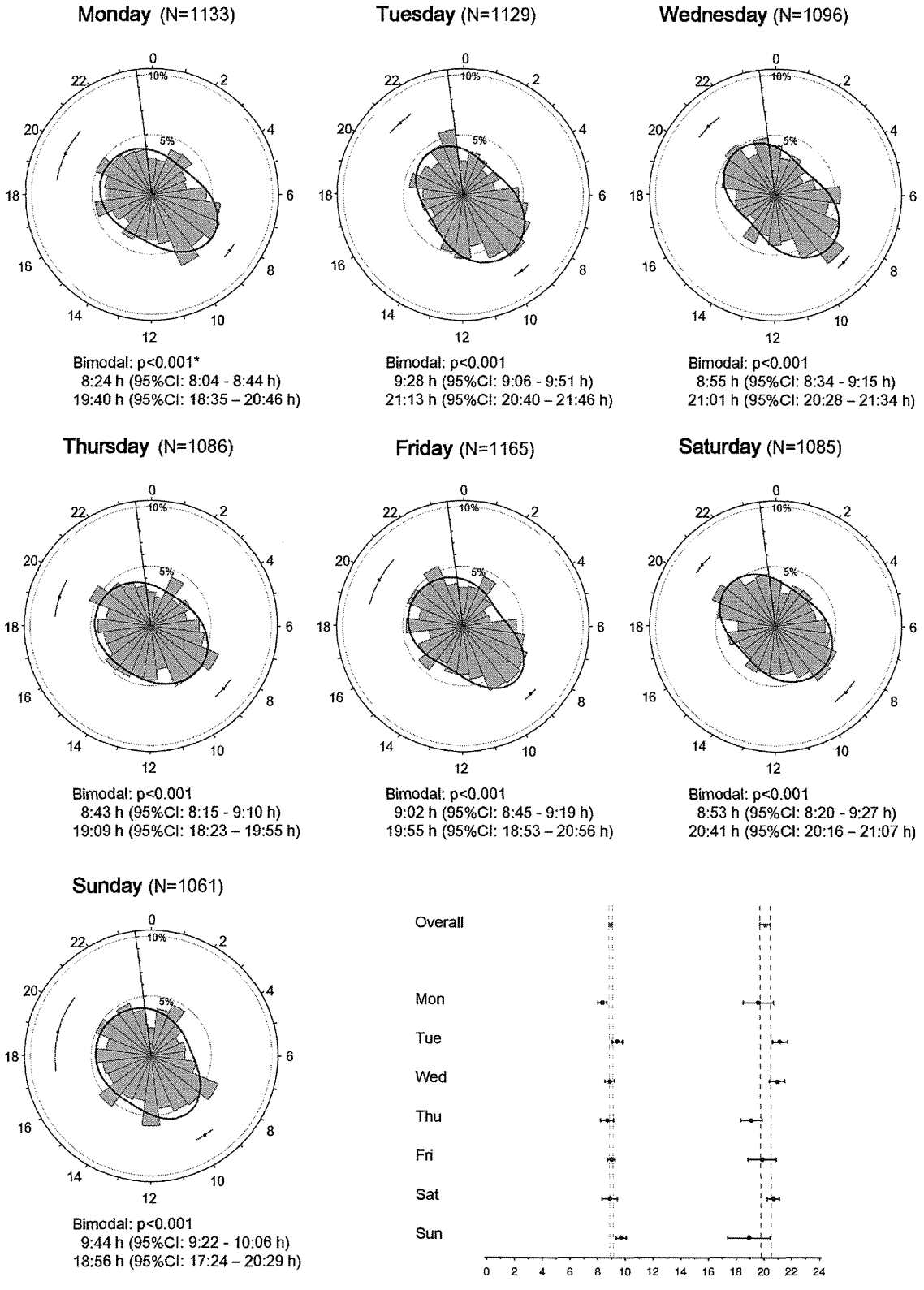


Figure 2 Circadian pattern of acute myocardial infarction (AMI) onset according to the day of the week. Circadian patterns of AMI onset based on the day of the week are shown. The estimated peak onset time and 95% CIs are shown below each circular plot. *p Values from the likelihood ratio test to examine whether the circadian pattern of AMI onset was uniform, unimodal or bimodal.

status had a statistically significant association with the circadian pattern of AMI onset, whereas several other known risk factors for AMI, including HDL and LDL cholesterol, HbA1c, hypertension, diabetes and dyslipidaemia were not related to the observed patterns (figure 3, supplementary table 1).

Among the positively associated factors, serum TG levels on admission had the greatest association with the

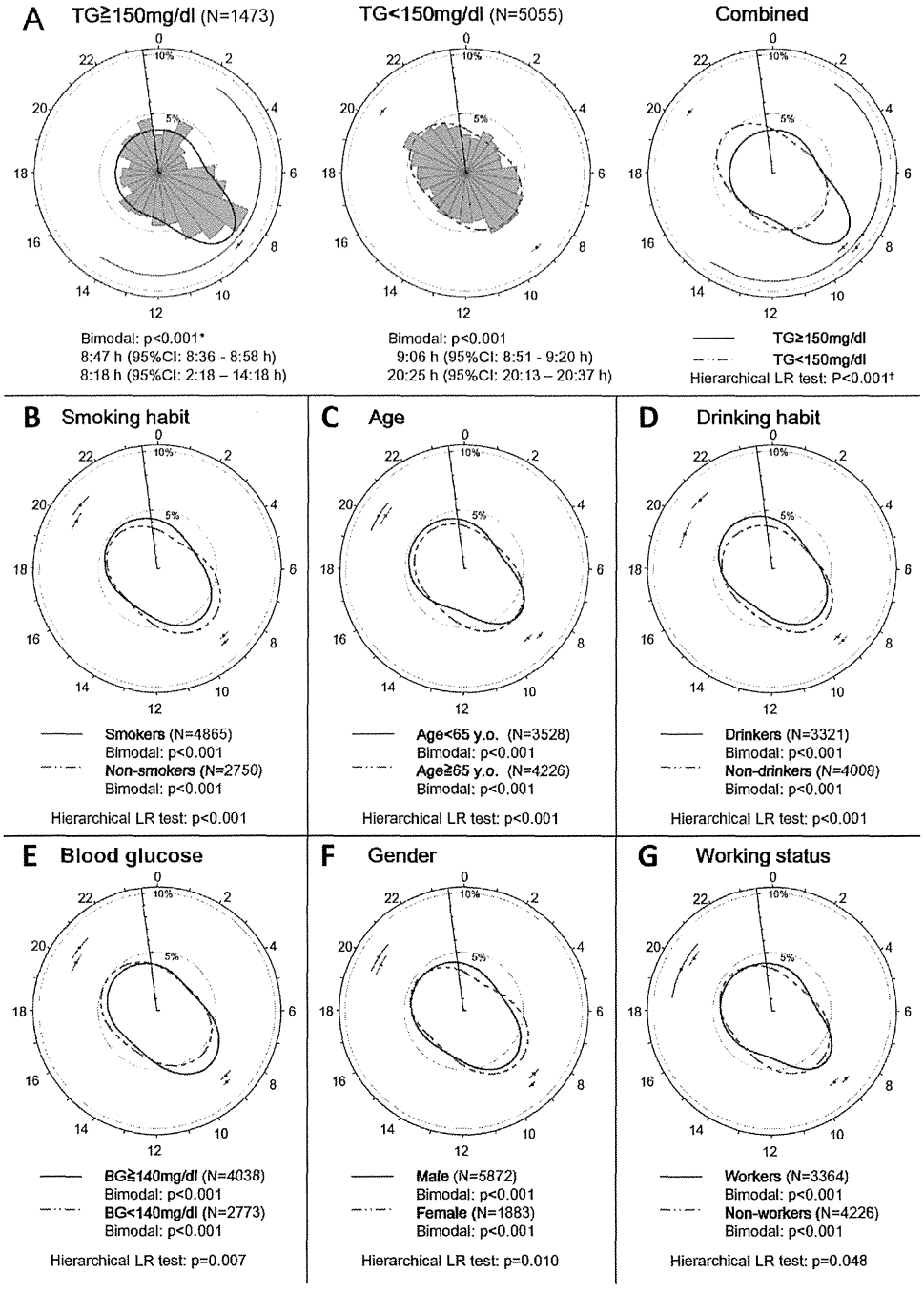


Figure 3 Circadian pattern of acute myocardial infarction (AMI) onset based on lifestyle-related factors. (A) Circular plots of the circadian pattern of AMI onset in the subpopulation with triglyceride (TG) levels ≥150 and <150 mg/dL, and the circular plot of the corresponding fitted von Mises distributions for each subgroup are shown. (B–M) Circular plots of the fitted von Mises distributions of each subgroup based on smoking habit, age, drinking habit, blood glucose levels, gender and working status, low-density lipoprotein (LDL) levels, high-density lipoprotein (HDL) levels, glycated haemoglobin (HbA1c) levels, hypertension, diabetes and dyslipidaemia. *p Values from the likelihood ratio (LR) test to examine whether the circadian pattern of AMI onset was uniform, unimodal or bimodal in each subgroup. †p Values from the hierarchical LR test to examine whether each factor affected the circadian pattern of AMI onset.

circadian pattern of AMI onset. Although the likelihood ratio test demonstrated that patients with admission serum TG levels of ≥150 mg/dL (N=1473) had two characteristic peaks during the day, the peak pattern clearly differed from the other subpopulation groups. In patients with admission serum TG levels of ≥150 mg/dL, both peaks occurred in the morning and nearly overlapped (8:18 and 8:47; figure 3A). Therefore, the subpopulation with admission TG levels ≥150 mg/dL was considered to have a high frequency of AMI onset only in the morning.

The baseline characteristics and laboratory data of patients with serum TG levels of ≥150 and <150 mg/dL

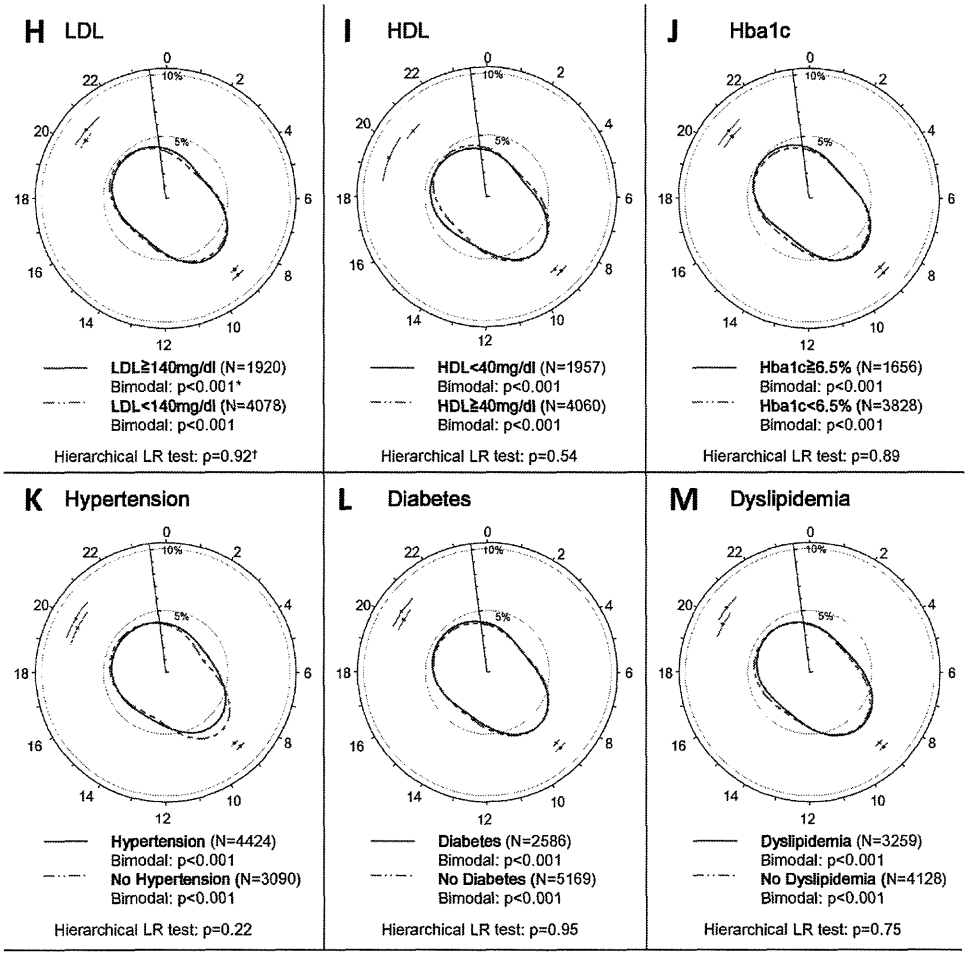


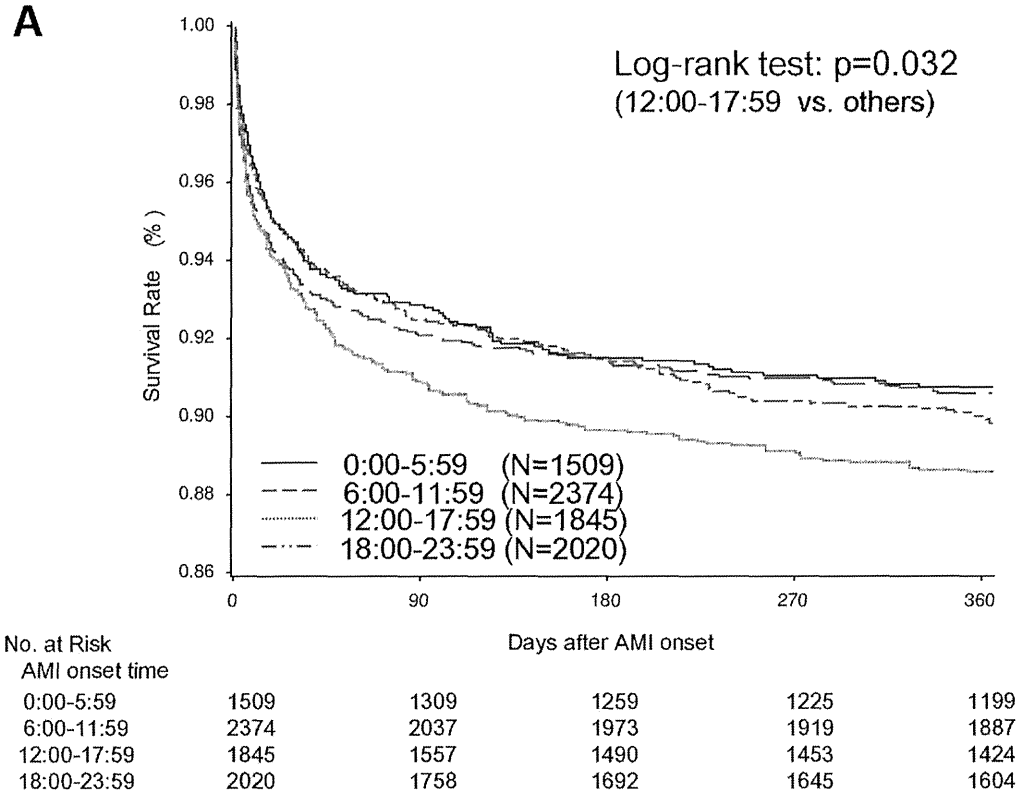
Figure 3 Continued.

on admission are shown in supplementary table 2. In the subpopulation with higher TG levels, the circadian patterns of AMI onset were characterised by a large, sharp peak in the morning from Monday to Friday, but no peaks were detected on Saturday and Sunday (bimodal: p=0.32 and 0.133, respectively; supplementary figure 1). In contrast, patients with admission serum TG levels of <150 mg/dL (N=5055) had onset peaks that occurred in the morning and evening consistently throughout the week (supplementary figure 1).

A likelihood ratio test demonstrated that all other subpopulations had two AMI onset peaks during the day: one in the morning and the other in the evening (figure 3, supplementary table 1). The subpopulations that were grouped according to smoking habit, age <65 years, male gender and active employment had a circadian pattern of AMI onset with a sharper primary peak and a less-defined sharp secondary peak compared with the other subpopulations (figure 3B, C, F, G, supplementary table 1), although the peak heights were similar between the subpopulations, with the exception of the smoker/non-smoker subpopulations. The primary AMI onset peak in the subpopulation of smokers was higher than that among non-smokers, whereas the secondary peaks were similar. Drinkers had a circadian pattern of AMI onset that was characterised by a lower and less sharp peak in the morning and a higher, sharper and later peak in the evening (9:00 (95% CI 8:48 to 9:13, 20:54 (95% CI 20:29 to 21:20)) compared with non-drinkers (9:03 (95% CI 8:53 to 9:14), 19:27 (95% CI 18:50 to 20:04); figure 3D, supplementary table 1). The subpopulation with admission blood glucose ≥140 mg/dL exhibited a circadian pattern of AMI onset with a higher and sharper primary peak and a less-defined secondary peak compared with the subpopulation of patients with AMI with blood glucose <140 mg/dL on admission (figure 3E).

One-year mortality according to onset time of AMI

One-year mortality was compared among four patient subpopulations that were grouped according to the time range of AMI onset. The baseline characteristics and laboratory data for the four groups are presented in supplementary table 3. A total of 753 deaths were recorded during a median follow-up period of 365 days. The Kaplan-Meier survival analysis demonstrated that the afternoon-onset (12:00–17:59) group had worse 1-year mortality than the other three groups (log-rank test, p=0.032; figure 4A). In the subgroup of patients with STEMI, the result was similar (log-rank test, p=0.007). The



No. at Risk AMI onset time					
0:00-5:59	1509	1309	1259	1225	1199
6:00-11:59	2374	2037	1973	1919	1887
12:00-17:59	1845	1557	1490	1453	1424
18:00-23:59	2020	1758	1692	1645	1604

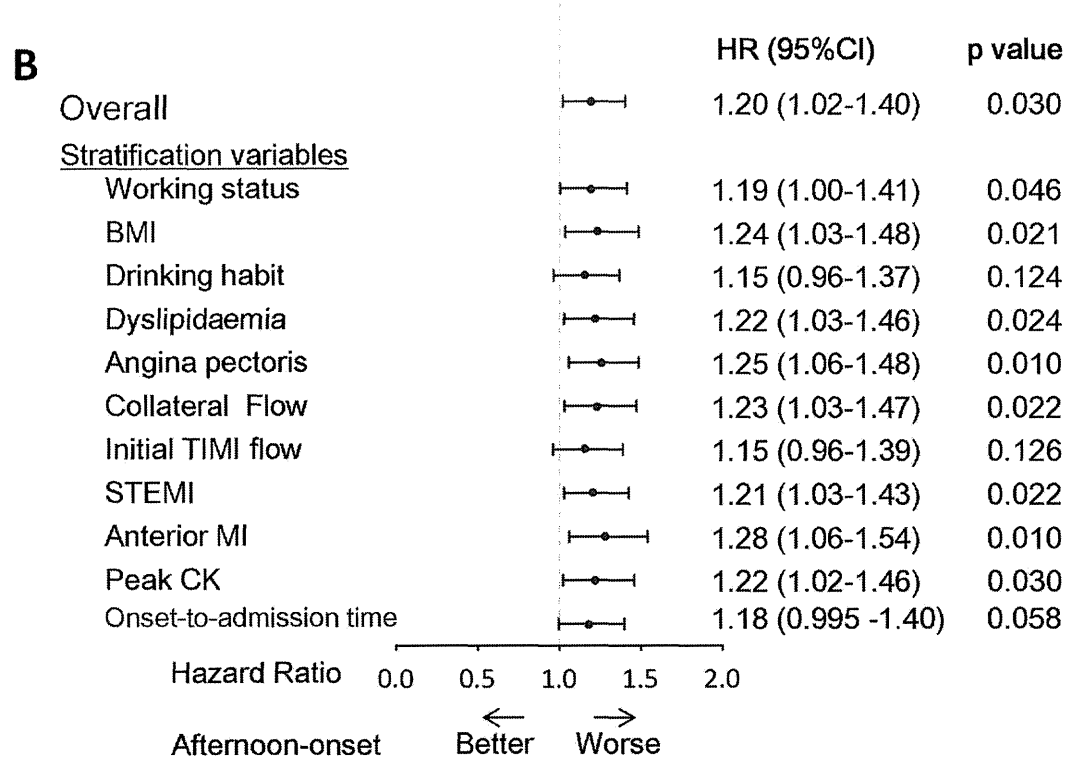


Figure 4 One-year mortality according to the onset time of AMI onset. (A) One-year mortality among the four subgroups based on AMI onset time. (B) HRs for 1-year mortality in the afternoon-onset group versus the other three onset time groups. The Kaplan-Meier survival curves of 1-year mortality among the four AMI onset time subgroups (A). A p value from the log-rank test was used to examine difference in the Kaplan-Meier curves. The HR and 95% CI, and p value for the overall population was calculated using univariable Cox regression analysis. The HRs and 95% CIs, and p values for the individual potential confounding variables were calculated using stratified Cox regression analysis, in which the variables were included into the model as stratification factors (B). AMI, acute myocardial infarction; BMI, body mass index; CK, creatine kinase; STEMI, ST-elevation MI.

univariable Cox regression analysis revealed that the HR of 1-year mortality in the afternoon-onset group as compared with the other three groups was 1.20 (95% CI 1.02 to 1.40, p=0.030, figure 4B). This result did not generally change after stratification with potential confounding factors that showed a different trend between the afternoon-onset group and the other three groups (figure 4B).

DISCUSSION

In the present study, we confirmed that AMI onset exhibits a circadian pattern characterised by bimodality, with a definite morning peak and a less-defined evening peak. Notably, several lifestyle-related factors were associated with variation in the circadian pattern of AMI onset. In particular, serum TG levels on admission for AMI were associated with a unique pattern of AMI onset that is characterised by augmented unimodal peaks on weekday mornings, suggesting that an individual's lifestyle may affect the onset pattern of AMI.

Bimodal pattern of AMI onset: morning and night-time peaks

AMI onset in our large patient cohort generally followed a circadian pattern that was characterised by a high and sharp morning peak and a lower and less-defined sharp night-time peak (figure 1), a finding that is consistent with the results of previous investigations.[1–7] Interestingly, the time of two peaks shifted in a synchronous fashion during weekdays; the secondary peaks generally occurred around 11–12 h after the morning peaks on Monday through Friday (figure 2). For example, AMI onset exhibited early morning and night-time peaks on Monday and Thursday, whereas that on Tuesday exhibited late morning and night-time peaks. Although this finding is partly consistent with the observation of Peters *et al*,[5] who reported that a secondary peak in AMI onset occurs 11–12 h after waking, the present study first demonstrated that this synchrony was present on weekdays, but absent on weekends.

Several physiological processes are considered to contribute to the bimodal pattern of AMI onset. For example, Stergiou *et al*[12] demonstrated that the two-peak diurnal variation in stroke onset occurs in parallel with variation in blood pressure, pulse rate and physical activity. Thus, the bimodality of blood pressure and heart rate[13 14] is the most likely explanation for the circadian patterns of AMI onset observed in the present study. A greater morning surge of blood pressure and heart rate[13] may explain why the night-time peak of AMI onset was lower and less-defined than the morning peak. In addition, increased blood viscosity[15] and thrombogenicity due to morning hypercoagulability[16] and hypofibrinolysis[17] also likely increased the frequency of AMI onset in the morning. It is also possible that external factors, such as physical exertion and mental stress, could be triggers for the morning onset of AMI.[18] In the present study, the younger (<65 years old), working, male and smoker subpopulations had a sharp morning peak of AMI onset compared with the elderly, non-working, female and non-smoking subpopulations (figure 3B, C, F, G). The sharpness of the morning peak might be related to increased susceptibility to physical and mental stress in these subpopulations, when they are more likely to start activities or go to work soon after waking up. Similarly, the sharp and early morning peak of AMI onset that was detected on Monday may be due to the increased physical and mental stress that is associated with the first morning of the week (figure 2). We also found that the morning peak occurred latest on Sunday (figure 2). Together, these findings strongly suggest that mental and physical activity and/or stress may act as a trigger for the morning onset of AMI.

Although many reports have examined the primary peak of AMI onset, relatively little attention has been paid to the secondary peak. We demonstrated that drinkers had a higher, sharper and later night-time peak of AMI onset than non-drinkers (figure 3D). Moreover, the night-time peak on Saturday was the highest and sharpest among the 7 days of the week (figure 2). This observation may be explained by the fact that people might likely consume alcohol and engage in social activities on Saturday night in Japan. Thus, these evening activities can result in increased sympathetic nerve activity and therefore may have contributed to the increased frequency of AMI onset at night. Taken together, our findings suggest that the morning and night-time peaks of AMI onset are influenced by physiological and socioeconomic factors.

Associations of lifestyle-related factors with the circadian patterns of AMI onset

Many previous studies on the circadian pattern of AMI onset considered gender, age, working status as potential factors affecting the circadian patterns of AMI onset.[1 4–6] We additionally incorporated laboratory data, disease and other socioeconomic factors into our analyses and found that several lifestyle-related factors, including admission serum TG and blood glucose levels, age, gender, working status and smoking and drinking habits had statistically significant associations with the circadian pattern of AMI onset. Among these factors, elevated serum TG levels (≥150 mg/dL) on admission had the largest associations with the circadian patterns of AMI onset, while the amplitude of serum TG levels on admission in patients with AMI did not have circadian variation (p=0.52; supplementary figure 2).

There are several evidences to support our findings. First, fasting hypertriglycaemia and postprandial hyperlipidaemia, which is characterised by postprandial accumulation of TG-rich lipoproteins and their partially hydrolysed products, are closely related to the development of atherosclerotic cardiovascular diseases.[19–21] Several studies have also reported that elevated serum TG levels are associated with an increased risk of MI.[22 23] Hypertriglycaemia is associated with increased

thrombogenicity,[24 25] which is reportedly associated with increased plasminogen activator inhibitor-1 (PAI-1)[26–28] and factor VII coagulant activities,[29 30] and viscosity.[31] These three factors have also been reported to affect the development of MI.[32–34] Moreover, hypertriglyceridaemia is also related to endothelium dysfunction,[35 36] which contributes to the pathogenesis of coronary artery disease.[37] In healthy participants, serum TG levels also exhibit circadian variation with a peak around 3:00.[38] Thus, it is conceivable that patients with hypertriglycaemia have further augmented TG levels and are therefore exposed to increased thrombogenicity and endothelium dysfunction in the early morning hours before dawn, which may explain the accentuated morning peak of AMI onset in patients with admission TG ≥150 mg/dL. Finally, it is reported that high plasma PAI-1 levels and excessive surges in morning blood pressure are independently and additively associated with increased risk of stroke in older patients with hypertension.[39] Thus, these lines of evidence strongly support our observation of a higher morning risk of AMI onset in the subpopulation with admission hypertriglyceridaemia.

Altered circadian patterns of AMI onset in patients with increased TG levels on admission

To the best of our knowledge, this is the first study to demonstrate an association between admission serum TG levels and the circadian patterns of AMI onset, as characterised by a lack of an evening peak in AMI onset in the subgroup of serum TG levels on admission ≥150 mg/dL compared with all other subgroups (figure 3). While LDL/HDL levels are considered to be closely associated with the development of atherosclerosis, LDL/HDL levels were not associated with onset patterns of AMI in the present study. Although the precise mechanisms for altered circadian patterns of AMI onset in patients with increased admission serum TG levels are unclear, increased serum TG might have influenced peripheral clocks residing in various tissues throughout the body, disrupting the circadian patterns of AMI onset. Indeed, recent studies have shown that energy metabolism is an important modulator of peripheral circadian clock in cardiovascular tissues.[40 41]

Our subpopulation analyses also revealed that the circadian patterns of AMI onset in patients with admission TG levels of ≥150 mg/dL had a sharp morning peak during weekdays, whereas no such peak was detected on Saturday or Sunday. This observation strongly suggests that increased thrombogenicity and endothelium dysfunction was a factor, but not the trigger, for the morning onset of AMI in our study cohort. Thus, it is conceivable that the accentuated morning peak of AMI onset in patients with admission TG ≥150 mg/dL may be due to the combination of the following three factors: (1) increased hypercoagulability, hypofibrinolysis, viscosity and endothelium dysfunction resulting from elevated serum TG levels, (2) increased risk of a morning surge of blood pressure and heart rate and (3) mental and physical stress.

One-year mortality according to AMI onset time

The association between AMI onset time and mortality is controversial. For example, Manfredini *et al*[42] reported that patients with a morning onset of AMI are characterised by higher fatal outcome, independent of site and size of infarction, while Bae *et al*[43] reported that patients with an evening-onset AMI had the worst 1-year mortality in association with poor baseline clinical characteristics. On the other hand, Holmes *et al*[44] observed no significant association between the circadian patterns of onset time and in-hospital mortality in patients with STEMI after adjusting for clinical risk factors.

In the present study, patients with an afternoon onset of AMI had the worst 1-year mortality (figure 4A). However, the baseline clinical characteristics were comparable among the four onset time groups in our study cohort. Indeed, stratification for potential confounding variables did not generally change the results, suggesting that the increased prognostic risk of AMI in the afternoon-onset group was not simply explained by differences in baseline characteristics in the present study (figure 4B). Anyway, a patient's background and physiological circadian rhythms might complexly interact with each other and affect mortality after AMI, which could lead to these different results among the studies and difficulty in interpreting the results. Further investigations are required to clarify the association of mortality after AMI and onset time.

Limitations

A few limitations of the present study warrant mention. First, this was an analysis of a prospective observational study and the results may have therefore been influenced by potential confounding factors, even after adjustment for baseline clinical and angiographic characteristics. Thus, caution is needed when interpreting the data and making generalisations to other cohorts. Second, the laboratory findings, including serum TG levels, were evaluated on admission. Therefore, we could not exclude the influence of food consumption and circadian variation of several factors, particularly serum TG levels, making interpretation of the data difficult. However, our results also demonstrated that serum TG levels were not likely the final trigger for AMI onset, as patients with TG ≥150 mg/dL on admission did not exhibit a morning peak of AMI onset on the weekend. In patients with hypertriglycaemia, hypercoagulability, hypofibrinolysis, viscosity and endothelium dysfunction are generally increased during the early morning hours before dawn,[26–31 35 36 38] resulting in enhanced susceptibility to AMI onset. Thus, under such conditions, it is conceivable that increased sympathetic activity, which was further enhanced in association with mental, physical and/or other factors, could be the final trigger for AMI onset on weekday mornings in patients with TG ≥150 mg/dL on admission. Based

on these findings, the influence of meal intake and circadian variation of serum TG levels on the morning peak of AMI onset in the population with TG ≥150 mg/dL may be minimal, if not negligible.

CONCLUSIONS

In our large cohort of consecutive patients with AMI, the circadian pattern of AMI onset exhibited bimodality and was shown to be associated with several lifestyle-related factors. Among these factors, increased serum TG levels on admission had the most marked association with circadian variation, which was characterised by an increased morning risk of AMI onset during weekdays in this subpopulation. Our findings may help to identify the underlying triggers and substrates of AMI onset and help suggest preventive measures of AMI. However, caution is warranted to interpret our results and confirmation in other cohorts is required.

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Patient consent Obtained.

Ethics approval The study protocol has been approved by the ethics committee of each participating hospital.

Provenance and peer review Not commissioned; externally peer reviewed.

Data sharing statement No additional data are available.

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Association of lifestyle-related factors with circadian onset patterns of acute myocardial infarction: a prospective observational study in Japan

Ryuya Edahiro, Yasuhiko Sakata, Daisaku Nakatani, Shinichiro Suna, Masaya Usami, Sen Matsumoto, Masahiko Hara, Tetsuhisa Kitamura, Hiroshi Sato, Shizuya Yamashita, Shinsuke Nanto, Shungo Hikoso, Yasushi Sakata, Masatsugu Hori, Toshimitsu Hamasaki, Issei Komuro and on behalf of the OACIS Investigators

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LDL cholesterol performance of beta quantification reference measurement procedure☆

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ABSTRACT

Background: Accurate measurement of blood lipids is crucial in cardiovascular disease risk management. The Centers for Disease Control and Prevention (CDC) Cholesterol Reference Method Laboratory Network (CRMLN) has assured the accuracy of these measurements for over 20 years using beta quantification (BQ) method as reference measurement procedure (RMP) for high- and low-density lipoprotein cholesterol (HDL-C, LDL-C). Only limited data exist about the performance of the BQ RMP.
Methods: Bottom fraction cholesterol (BFC), HDL-C, and LDL-C results after ultracentrifugation from the CDC lipid reference laboratory and the Japanese CRMLN laboratory were compared using 280 serum samples measured over the past 15 years. Data were compared statistically using method comparison and bias estimation analysis.
Results: Regression analysis between CDC (x) and Osaka (y) for BFC, HDL-C, and LDL-C were $y = 0.988x + 1.794$ ($R^2 = 0.997$), $y = 0.980x + 1.118$ ($R^2 = 0.994$), and $y = 0.987x + 1.200$ ($R^2 = 0.997$), respectively. The Osaka laboratory met performance goals for 90% to 95% of the CDC reference values.
Conclusions: The BQ method by the Osaka CRMLN laboratory is highly accurate and has been stable for over 15 years. Accurate measurement of BFC is critical for the determination of LDL-C.

1. Introduction

Increased concentrations of low-density lipoprotein cholesterol (LDL-C) are associated with an increased risk for the development of cardiovascular diseases (CVDs), especially coronary heart disease (CHD) [1,2]. Other major risk factors include hypertension, diabetes mellitus, smoking, and chronic kidney diseases [3,4]. Interventions to decrease LDL-C levels can improve the risk of CVD and result in reductions in atherosclerotic lesions [5–8]. Because of the strong and positive association between LDL-C and CVD, 2013 ACC/AHA Guideline on the Treatment of Blood Cholesterol to Reduce Atherosclerotic Cardiovascular Risk in Adults [9], the Third Report of the U.S. National Cholesterol Education Program (NCEP) [10,11], the European Atherosclerosis Society [12], and Japan Atherosclerosis Society Guidelines for the Prevention of Atherosclerotic Cardiovascular Diseases 2012 [13] focused primarily on LDL-C for the categorization and treatment of dyslipidemia. Thus, measuring LDL-C has been the cornerstone of cardiovascular risk assessment and prevention for the past decades.

The precise and accurate measurement of LDL-C is of particular importance for correctly and consistently classifying individuals at risk for CVD as outlined in clinical guidelines for subsequent treatment of patients. The precision and accuracy of LDL-C measurements needed to assure that appropriate patient care was established by the NCEP [14]. The beta quantification (BQ) procedure, which relies on ultracentrifugation (UC) to separate apo B lipoprotein (apo B) particles

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Table 1
Performance criteria applied to CRMLN lipid reference laboratory using BQ RMP.

Lipid	Precision	Accuracy
BFC	CV ≤ 1.5%	±(CDC LDL-C reference value × 0.02 + HDL-C bias vs. CDC) [max = ±2 mg/dL or 0.04 (HDL-C reference value) if smaller]
HDL-C	SD ≤ 1 mg/dL	±CDC HDL-C reference value × 0.04
LDL-C	CV ≤ 1.5%	±CDC LDL-C reference value × 0.02

CRMLN: Cholesterol Reference Method Laboratory Network. BQ RMP: Beta quantification reference measurement procedure.
CDC: US Centers for Disease Control and Prevention.
BFC: Bottom fraction cholesterol.

according to the hydrated density at d = 1.006, has been the established reference measurement procedure (RMP) for HDL-C and LDL-C [15,16]. BQ RMP performed at the U.S. Centers for Disease Control and Prevention (CDC) and Cholesterol Reference Method Laboratory Network (CRMLN) is considered the highest order RMP for this analyte. For over 15 years, the National Cerebral and Cardiovascular Center at Osaka, Japan has standardized their LDL-C BQ RMP through participation in the CRMLN. Members of the CRMLN are required to meet stringent performance criteria for precision and accuracy to allow both calibration and calibration verification of routine assays. Few reports are available on the performance of BQ RMP.

Using data obtained between May 1997 and October 2012, the precision and accuracy for HDL-C and LDL-C as measured at the Osaka laboratory were determined. We determined the fixed and/or proportional bias and correlations between the CDC and Osaka laboratories, and assessed factors that may affect results obtained with the BQ method by verifying relationships among bottom fraction cholesterol (BFC) — one major component of the BQ procedure, HDL-C, and LDL-C.

2. Material and methods

2.1. Materials

All materials were prepared according to Clinical Laboratory Standards Institute (CLSI) document C37-A. This implies that no preservatives or no additives were added. In this study, 67 different pool concentrations (lots) were used among the 280 survey samples provided by the CDC as part of the CRMLN monitoring surveys. One lot (bq47) was used 8 times over 2.5 years, which represented the longest period any lot was used. All CDC survey pools were blinded to the CRMLN participants. The pools were shipped frozen and stored at −70 °C before BQ analysis, and they were analyzed between May 1997 and October 2012 in 70 survey runs, with each survey run consisting of 3 to 5 different pools.

Measurements were conducted in the Osaka Medical Center for Cancer and Cardiovascular Diseases between July 1997 and June 2001, in the Osaka Medical Center for Health Science and Promotion between July 2001 and March 2012, and in the National Cerebral and Cardiovascular Center at Osaka continuously since April 2012 (all laboratories are referred to as 'Osaka laboratory').

2.2. Ultracentrifugation

BQ employs preparative ultracentrifuge (Beckman Coulter, Optima L-70K) to remove the chylomicrons and very-low-density lipoproteins (VLDL) of apo B-containing lipoproteins [17]. The methods at CDC and Osaka used 5 ml of serum per sample at a density of d = 1.006 kg/L (0.195 mol/L NaCL solution) and a 50.4 Ti rotor (Beckman Coulter) for UC. UC was carried out at CDC for 16.2 hours at 120,000 ×g, and 18 °C, and at Osaka for 18.5 hours, 105,000 ×g, and 18 °C. After UC, chylomicrons and VLDL in the top fraction (d < 1.006 kg/L) were removed and the remaining bottom fraction (d > 1.006 kg/L) including high-density lipoprotein (HDL), low-density lipoprotein (LDL), intermediate-density lipoprotein (IDL), and lipoprotein(a) (Lp(a)) was quantitatively transferred to a 5.00 mL volumetric flask and adjusted for volume with 0.15 mol/L NaCL solution [14,15]. The total cholesterol in this bottom fraction (BFC) was determined from one aliquot.

2.3. HDL-C precipitation

One mL aliquots of the apo B-containing lipoproteins in the bottom fraction were precipitated with 40 μL heparin (sodium injection, 5000 USP units/mL, Baxter Healthcare Corp.) and 50 μL manganese reagents (manganese(II) chloride solution, 1.00 mol/L ± 0.01 mol/L, SIGMA). The precipitate was removed by centrifugation for 30 min at 1500 ×g, 4 °C [18]. HDL-C was determined in the supernatant in duplicate measurements by the Abell–Kendall RMP [19]. LDL-C was calculated as the difference between BFC and HDL-C. A total of 8 replicate values per sample were obtained, and the mean of these replicates is used for comparison of assay performance.

2.4. Performance criteria

Performance criteria applied to the CRMLN lipid reference laboratories are summarized in Table 1. Because the LDL-C is the difference between BFC and HDL-C, the bias criterion for BFC was determined by the allowable bias for LDL-C and HDL-C and was considered to be ± the sum of the allowable HDL-C and LDL-C bias.

Table 2
Measurement performance of the CRMLN laboratory at Osaka determined with 280 pooled sera measured between May 1997 and October 2012 in 70 survey runs.

Statistical item	BFC	HDL-C	LDL-C
Mean precision as %CV (SD)	0.60 (0.342)	1.01 (0.605)	0.85 (0.461)
Mean bias as % (SD)	−0.12 (0.853)	0.45 (1.708)	−0.34 (1.148)
Pass rate for imprecision (N)	95.4% (267)	95.4% (267)	91.8% (257)
Pass rate for bias (N)	91.4% (256)	94.6% (256)	89.6% (251)
Absolute bias (%)	0.63 ± 0.589	1.23 ± 1.270	0.86 ± 0.830
Bias in mg/dL (95% CI)	0.34 (0.14, 0.53)	−0.16 (−0.26, −0.07)	0.49 (0.32, 0.66)
Limits of agreement in mg/dL	−2.87 to 3.54	−1.76 to 1.43	0.31 to 0.66
Slope (95% CI)	0.988 (0.981, 0.995)	0.980 (0.971, 0.989)	0.987 (0.980, 0.993)
Intercept (95% CI)	1.794 (0.581, 3.006)	1.118 (0.676, 1.560)	1.200 (0.388, 2.011)
Correlation coefficient as R^2	0.997	0.994	0.997

CRMLN: Cholesterol Reference Method Laboratory Network.
BFC: Bottom fraction cholesterol.

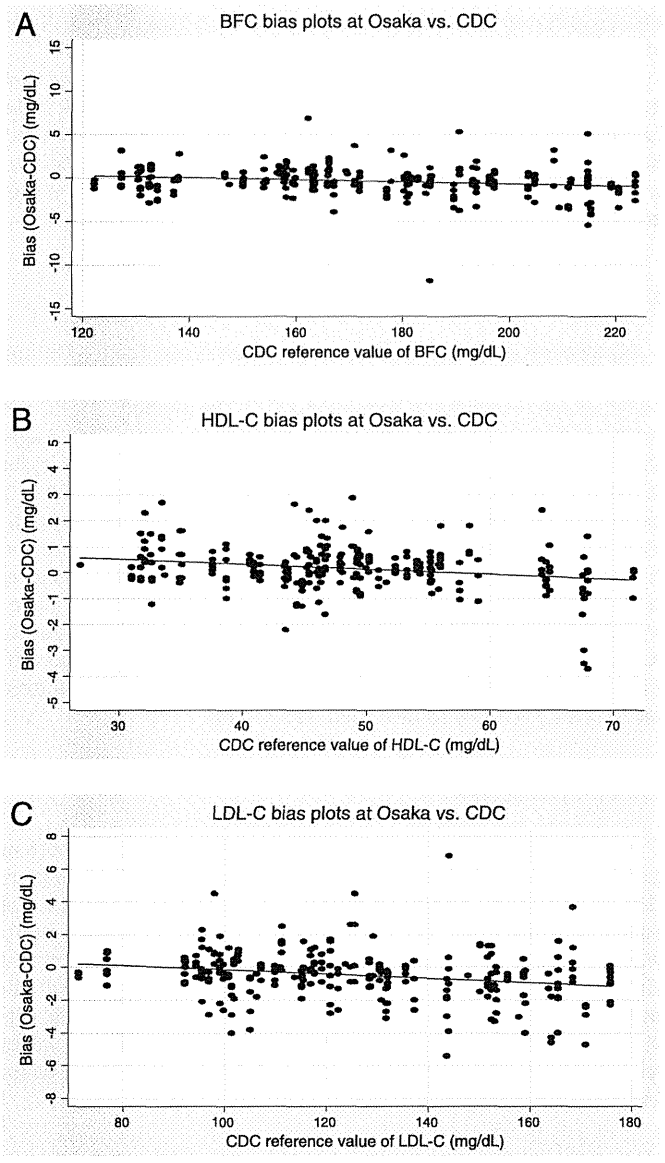

Fig. 1. Scatter plots of bias at Osaka vs. CDC for BFC (A), HDL-C (B) and LDL-C (C). (A) CDC: US Centers for Disease Control and Prevention. BFC: Bottom fraction cholesterol. x-axis indicates CDC reference value of BFC (unit: mg/dL) in the concentration range from 122.3 to 223.7 mg/dL and y-axis indicates the BFC bias between Osaka and CDC (unit: mg/dL). y (bias (Osaka–CDC)) = −0.012 × (CDC reference value) + 1.759 [n: 280, R^2 = 0.042 (p-value: 0.001)]. p-value and 95% CI are 0.001 and (−0.019, −0.005) for slope, respectively. p-value and 95% CI are 0.004 and (0.551, 2.968) for intercept, respectively. (B) CDC: US Centers for Disease Control and Prevention. HDL-C: High-density lipoprotein cholesterol. x-axis indicates CDC reference value of HDL-C (unit: mg/dL) in the concentration range from 27.0 to 72.4 mg/dL and y-axis indicates the HDL-C bias between Osaka and CDC (unit: mg/dL). y (bias (Osaka–CDC)) = −0.020 × (CDC reference value) + 1.112 [n: 280, R^2 = 0.063 (p-value: <0.001)]. p-value and 95% CI are <0.001 and (−0.029, −0.011) for slope, respectively. p-value and 95% CI are <0.001 and (0.671, 1.553) for intercept, respectively. (C) CDC: US Centers for Disease Control and Prevention. LDL-C: Low-density lipoprotein cholesterol. x-axis indicates CDC reference value of LDL-C (unit: mg/dL) in the concentration range from 71.5 to 173.3 mg/dL and y-axis indicates the LDL-C bias between Osaka and CDC (unit: mg/dL). y (bias (Osaka–CDC)) = −0.013 × (CDC reference value) + 1.186 [n: 280, R^2 = 0.059 (p-value: <0.001)]. p-value and 95% CI are <0.001 and (−0.020, −0.007) for slope, respectively. p-value and 95% CI are 0.004 and (0.376, 1.996) for intercept, respectively.

2.5. Statistical analysis

We used protocol EP9-A from the Clinical and Laboratory Standards Institute [20–22] for bias estimation and STATA12 analysis program for all other calculations.

3. Results

The concentration ranges of the 67 lots used in the CRMLN surveys were 122.3–223.7 mg/dL, 27.0–72.4 mg/dL, and 71.5–173.3 mg/dL for BFC, HDL-C, and LDL-C, respectively. For 15 years, the reference laboratory at Osaka meets CRMLN accuracy and precision performance goals for BFC, HDL-C and LDL-C (Table 2).

The mean percent bias between the Osaka laboratory and the CDC reference laboratory was <0.5% for all analytes, with limits of agreement being very narrow. Bias and regression analyses show that the bias, though small, is significant. The observed bias is well-below the allowable bias for CRMLN laboratories. The individual sample biases at low analyte concentrations tend to be positive, and at high concentration the biases are negative for all analytes (Fig. 1A–C).

From the estimation by regression line, the absolute bias between CDC and Osaka in the clinical decision levels was estimated as 0.40 mg/dL for BFC at 180 mg/dL, 0.32 mg/dL for HDL-C at 40 mg/dL and 0.62 mg/dL for LDL-C at 140 mg/dL. The bias was small, but the mean value of absolute bias in upper 10% and lower 10% concentration of reference value was larger than that in middle 80% for BFC (1.45 mg/dL vs. 0.98 mg/dL: p = 0.01). There was no difference of bias related to concentration for HDL-C (0.69 mg/dL vs. 0.54 mg/dL: p = 0.19) and LDL-C (1.04 mg/dL vs. 1.10 mg/dL: p = 0.70) (Table 3).

Assessing measurement bias over time showed no significant trend from May 1997 to October 2012. This is indicated in no significant bias observed with lot bq47, which was analyzed quarterly over 2.5 years. Furthermore, no significant trend in measurement bias was observed for this period (Fig. 2).

Correlation plots between BFC (x-axis, unit: %bias vs. CDC) and LDL-C (y-axis, unit: %bias vs. CDC) of the Osaka laboratory are positively correlated (y = 1.088x − 0.208, n = 280, R^2 = 0.652 (p-value < 0.001), p-value and 95% CI for slope are <0.001 and (0.994, 1.182), respectively, p-value and 95% CI for intercept are <0.001 and (−0.289, −0.128), respectively) (Fig. 3D). In contrast, only weak correlations are observed between the biases from BFC (x-axis, unit: %bias vs. CDC) and HDL-C (y-axis, unit: %bias vs. CDC). (y = 0.480x + 0.513, n = 280, R^2 = 0.057 (p-value < 0.001)) (Fig. 3E). Similarly, only weak correlations existed between the biases from LDL-C (x-axis, unit: %bias vs. CDC) and HDL-C (y-axis, unit: %bias vs. CDC). (y = −0.441x + 0.299, n = 280, R^2 = 0.087 (p-value < 0.001)) (Fig. 3F).

4. Discussion

LDL-C is a key biomarker for cardiovascular disease risk assessment, and it is the primary target for treatment. No RMP currently exists for direct measurement of LDL-C. Therefore, the BQ approach was established to assign LDL-C reference values to serum materials. Like all RMPs, it is not intended for use in patient care because of its technical demands (e.g. overnight UC, manual volumetric sampling, and reconstitution of the bottom fractions) [23,24]. However, the technical limitations of this method such as sample throughput or complexity are similar to those of other RMPs [25]. Because measurement results are traceable to an RMP and the International System of Units, it is important to assure that this method is highly reproducible and accurate over time. Efforts by CDC and its partners to assure the accuracy of LDL-C measurements have been ongoing for over 15 years. The CRMLN assures the accuracy of LDL-C measurements by providing reference measurement service to the clinical laboratory community to establish metrological traceability to the CDC RMP. Only a few studies have examined the performance of BQ RMP [26–28]. This study describes the performance of LDL-C value-assignment performed in one CRMLN laboratory over 15 years.

The actual cholesterol measurements are traceable to pure compound certified reference materials and thus are traceable to SI as outlined in ISO 17511. The isolation of the lipid fractions is traceable to a RMP, which is also outlined in ISO 17511. To our knowledge, ISO 17511 does not define nor require a so called "gold standard". Because

Table 3
Comparison of absolute bias between middle 80% and upper/lower 10% of reference values.

Lipid	Range of middle 80% of reference (CDC) value	Mean of absolute bias in middle 80% of reference (CDC) value	Mean of absolute bias in upper 10% and lower 10%	p-value
BFC	132.80–214.79 mg/dL	0.98 mg/dL	1.45 mg/dL	0.01
HDL-C	33.50–64.50 mg/dL	0.54 mg/dL	0.69 mg/dL	0.19
LDL-C	95.50–165.39 mg/dL	1.10 mg/dL	1.04 mg/dL	0.70

BFC: Bottom fraction cholesterol.

cholesterol measurements are traceable to SI, we prefer to use the term "accuracy" in the manuscript. The CDC BQ RMP is classified as a higher order reference measurement procedure used to assign reference values on frozen reference materials. The CDC LDL-C RMP is the reference point for LDL-C recommended by the NCEP Lipoprotein Measurement Working Group. The accuracy reported in the paper refers to the accuracy compared to the CDC LDL reference values. The CRMLN laboratories achieve traceability to CDC RMP through monitoring.

The BQ method combines the removal of triglyceride (TG)-rich VLDL by UC, isolation of HDL from the UC bottom fraction, and cholesterol analysis of the bottom fraction and HDL supernatant. Therefore, the performance of HDL-C and BFC measurements needs to be considered when assessing factors affecting LDL-C target value assignments.

Over 15 years, the BQ RMP operated at the Osaka laboratory provided highly accurate and precise measurements of HDL-C and LDL-C, as indicated in the high agreement with the CDC reference laboratory. The observed mean bias is well within the allowable bias for CRMLN laboratories. The CRMLN focuses mainly on assuring accuracy of measurements around the clinical decision levels, which would be 40–60 mg/dL for HDL-C and 100–160 mg/dL for LDL-C (Fig. 1B,C); most of the serum pools used in CRMLN cover these ranges. Within these ranges, no significant mean bias and no proportional bias between the 2 methods were observed (Table 3). Considering that the LDL-C value assignments are derived from two separate measurements and that this RMP is technically very demanding, the overall performance and performance over time is remarkable. The data demonstrate that this method can be operated in a highly precise manner over long periods of time.

The CDC BQ method has been accepted as the most reliable RMP for HDL-C and LDL-C measurements, and it was recommended by the NCEP as the RMP method for HDL-C and LDL-C. The BQ method was used to establish the concentrations of the major lipoprotein classes in almost all epidemiological studies and clinical trials on which current guidelines for CVD risk assessment are based. It is used in the assignment of LDL-C reference values to calibrators or standards, patient specimens or bench-level quality control materials, and in the evaluation of direct [29,30] and homogeneous methods [31–33]. In the "Program Recommendations for the Measurement of Low-Density Lipoprotein Cholesterol: Executive Summary" [16], Bachorik et al. encouraged the early development of homogeneous methods and suggested that new methods for measuring LDL-C should be developed that are capable of directly quantifying LDL-C, and which should not be based on calculations of the difference between two or more measured values. The developed homogeneous methods have some advantages, such as the direct measurement of LDL-C by automated analytical instruments and possible use of non-fasting samples. However, they do have limitations [31–33]. Therefore, the BQ method is needed to assure accurate patient data that can be compared to current clinical decision points.

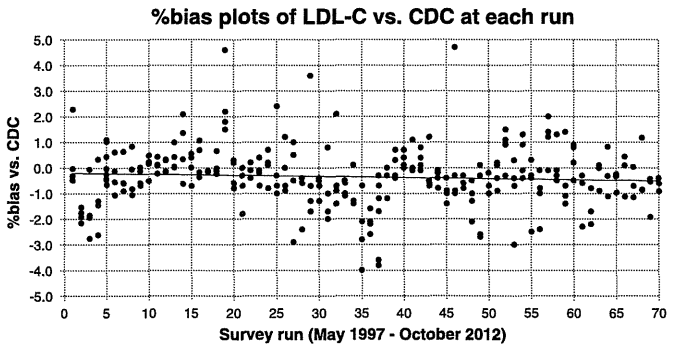

Fig. 2. %Bias plots of LDL-C vs. CDC at each survey run. CDC: US Centers for Disease Control and Prevention. LDL-C: Low-density lipoprotein cholesterol. x-axis indicates survey run number during May 1997 and October 2012 with 70 runs and y-axis indicates %bias of LDL-C vs. CDC. The accuracy criteria of %bias plots of LDL-C is ±2% of CDC reference value. Each survey run consists of 3 to 5 CDC pools for beta quantification analysis.

The reference values obtained with the BQ approach are based on the density of lipoprotein particles and their separation using specific UC conditions. LDL is not a unique molecular species; it consists of a group of similar, mixed, and atherogenic lipoproteins that vary to some degree in their chemical composition and physico-chemical particles [34]. The bottom fraction contains minor, but atherogenic lipoprotein classes such as IDL and Lp(a) [17,35,36]. In normal individuals, both lipoprotein classes can be expected to contribute 2–4 mg/dL, on average, to the total cholesterol measurement; however, their concentrations may be higher in patients with CHD and in patients at risk of developing CHD by virtue of dyslipidemia. The alterations of these lipid classes can affect cardiovascular disease risk, which may not be adequately detected by the BQ approach. Therefore, new approaches, such as measurement of LDL particle numbers, have been suggested to better assess cardiovascular risk in patients with such conditions [37]. The limitation of the BQ approach needs to be considered when using this RMP for reference value assignments.

The strong correlation between the BFC bias and the LDL-C bias, as well as the weak correlation between the LDL-C bias and HDL-C bias, suggests that the accuracy of LDL-C performed is directly affected by the accuracy of the BFC measurement and, to a much lesser extent, by the HDL-C measurement. This is expected because the LDL-C is calculated from the BFC, while HDL-C is an independent measurement. Because of the good agreement between CDC RMP and Osaka RMP, the different UC conditions used by these laboratories do not appear to have a profound effect on the mean bias or individual sample biases.

In conclusion, this study demonstrates that accurate measurement of BFC is critical for LDL-C value assignment. The BQ RMP performed at the Osaka laboratory is accurate and consistent over time. This assures that calibrations of assays used in patient care are accurate, and that measurements performed in patient care meet established performance criteria. Thus, the BQ RMP ensures that current guidelines using LDL-C levels for CVD risk assessments can be applied correctly and consistently.

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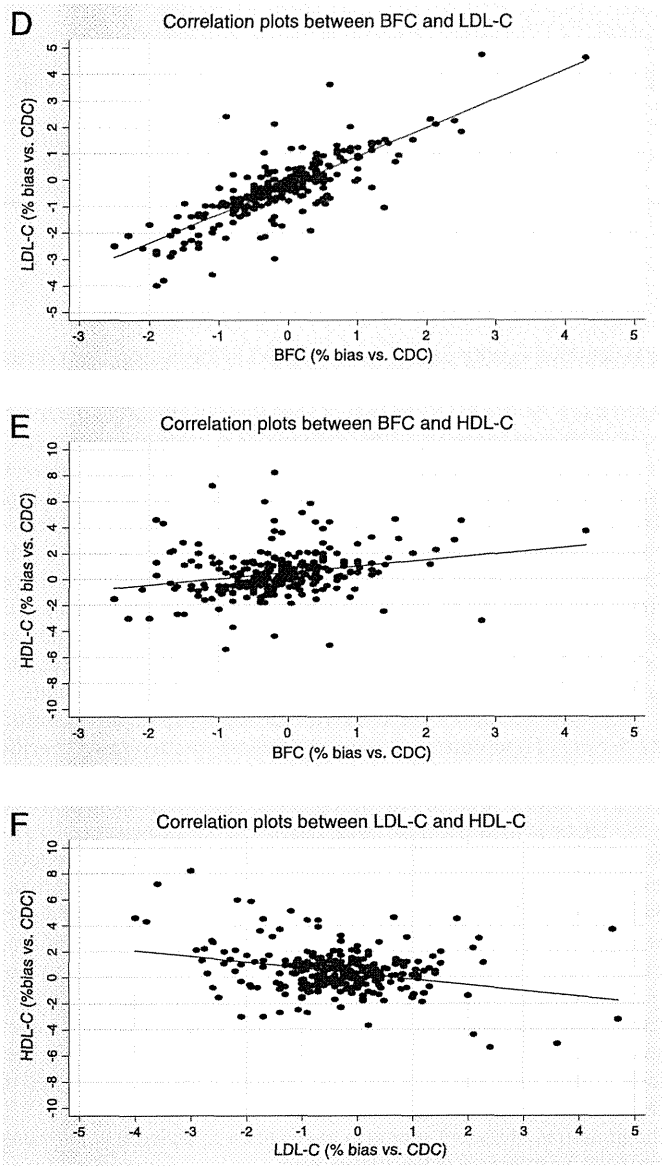

Fig. 3. Scatter plots of correlation and regression at Osaka between BFC and LDL-C (D), BFC and HDL-C (E), and LDL-C and HDL-C (F). (D) CDC: US Centers for Disease Control and Prevention. BFC: Bottom fraction cholesterol. LDL-C: Low-density lipoprotein cholesterol. CI: Confidence interval. x-axis indicates Osaka BFC (unit: %bias vs. CDC) and y-axis indicates Osaka LDL-C (unit: %bias vs. CDC). y (Osaka LDL-C) = 1.088 × (Osaka BFC) − 0.208 [n: 280, R^2 = 0.652 (p-value: <0.001)]. p-value and 95% CI are <0.001 and (0.994, 1.182) for slope, respectively. p-value and 95% CI are <0.001 and (−0.289, −0.128) for intercept, respectively. (E) CDC: US Centers for Disease Control and Prevention. BFC: Bottom fraction cholesterol. HDL-C: High-density lipoprotein cholesterol. CI: Confidence interval. x-axis indicates Osaka BFC (unit: %bias vs. CDC) and y-axis indicates Osaka HDL-C (unit: %bias vs. CDC). y (Osaka HDL-C) = 0.480 × (Osaka BFC) + 0.513 [n: 280, R^2 = 0.057 (p-value: <0.001)]. p-value and 95% CI are <0.001 and (0.250, 0.711) for slope, respectively. p-value and 95% CI are <0.001 and (0.316, 0.710) for intercept, respectively. (F) CDC: US Centers for Disease Control and Prevention. HDL-C: High-density lipoprotein cholesterol. LDL-C: Low-density lipoprotein cholesterol. CI: Confidence interval. x-axis indicates Osaka LDL-C (unit: %bias vs. CDC) and y-axis indicates Osaka HDL-C (unit: %bias vs. CDC). y (Osaka HDL-C) = −0.441 × (Osaka LDL-C) + 0.299 [n: 280, R^2 = 0.087 (p-value: <0.001)]. p-value and 95% CI are <0.001 and (−0.609, −0.273) for slope, respectively. p-value and 95% CI are 0.004 and (0.098, 0.499) for intercept, respectively.

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