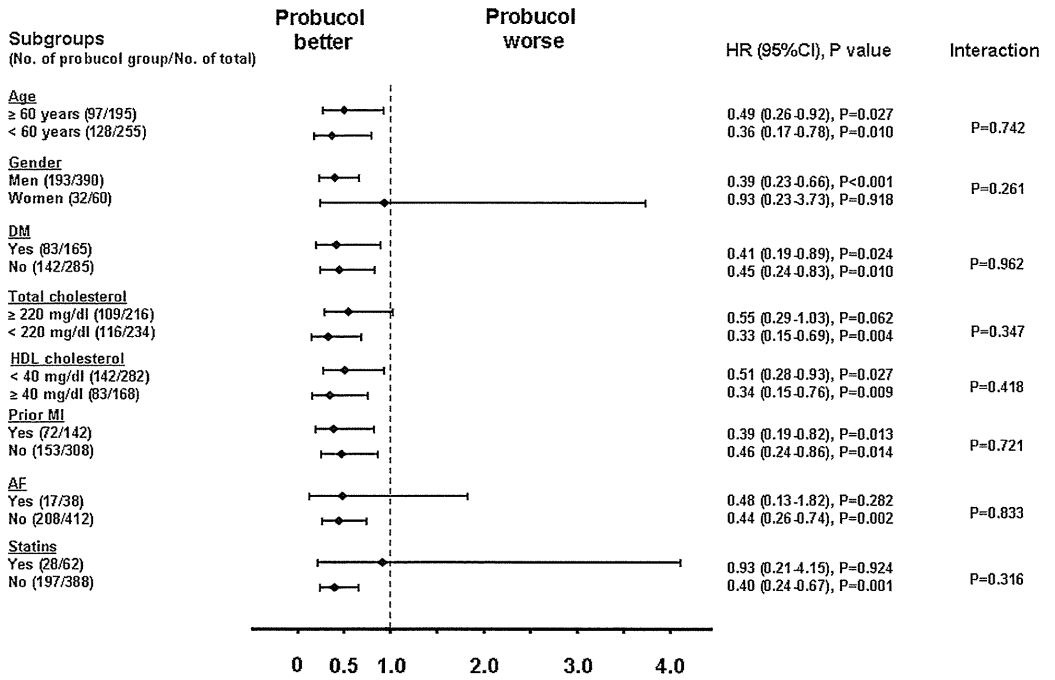

Fig. 2. Association between probucol use and all-cause mortality in subgroups of propensity score matched patients HR, hazard ratio; CI, confidence interval; HDL, high-density lipoprotein; MI, myocardial infarction; AF, atrial fibrillation.

large-scale randomized, double-blind, placebo-controlled trial to assess the effect of succinobucol, the monosuccinic acid ester of probucol, which has greater intracellular antioxidant efficacy *in vitro* than probucol without a QT interval prolongation effect [10,26], on the incidence of cardiovascular events after acute coronary syndrome. The ARISE trial showed that succinobucol significantly reduces the incidence of composites of cardiovascular death, resuscitated cardiac arrest, non-fatal MI, or stroke as pre-specified secondary endpoints. However, this study failed to show a benefit for primary endpoint, a composite of the abovementioned secondary endpoints as well as unstable angina or coronary revascularization. Although this is a large-scale randomized clinical trial using very similar advantageous drug based on QT interval prolongation as compared to probucol, specific beneficial effects of probucol on long-term morbidity and mortality in patients with severe CAD remains unclear. The Probucol Observational Study Illuminating Therapeutic Impact on Vascular Events (POSITIVE) study [21] assessed the efficacy of long-term probucol treatment on reduction of cardiovascular event risk in Japanese patients with heterozygous familial hypercholesterolemia. The POSITIVE study enrolled relatively large number of patients ($N=410$) from 15 institutions and showed that in a subset of patients with prior cardiovascular diseases ($N=88$), probucol therapy was associated with a reduced risk of long-term cardiovascular event incidence. This study indicates that probucol therapy may be beneficial in reducing cardiovascular events in patients with prior cardiovascular diseases. However, number of subjects in the secondary prevention cohort was very small and it is not clear whether patients enrolled in their study had undergone coronary revascularization. In the present study, we showed that probucol therapy at the time of complete coronary revascularization is associated with reduced all-cause mortality for a long-term follow-up period (>10 years). To the best of our knowledge, there have been no studies involving patients with significant CAD treated with coronary revascularization. Furthermore, it was important to assess data only from patients who had achieved complete revascularization because initial CAD events may be prevented or delayed by complete coronary revascularization, even in patients with severe coronary atherosclerosis. This minimizes the bias of treatment procedures for initial CAD events. Therefore, benefits of probucol use in long-term mortality among a secondary prevention cohort of patients with CAD were assessed in this study.

Mechanisms contributing to the association of probucol use and reduced all-cause mortality may include a combination of oxidative stress reduction [1–3] and inflammatory response [4,5] in addition to lowering LDL cholesterol. Anti-oxidant and anti-inflammatory effects may explain why all-cause mortality, not cardiac mortality, was reduced in the probucol group. These two effects may affect not only progression of cardiovascular disease but also development or progression of other diseases such as inflammatory diseases, cancer, and neurodegenerative disease [27]. More importantly, mechanisms promoting cholesterol efflux and enhancing RCT by activation of CETP [14,17] and SR-BI [19] may have contributed to lower all-cause mortality in the probucol group. In our pre-match patients, the probucol group showed a significantly lower baseline HDL level. Although we did not have data regarding changes in HDL cholesterol level before starting probucol, lower HDL cholesterol levels in the probucol group imply that reduced HDL cholesterol levels are caused by probucol through enhancing RCT by activation of CETP and SR-BI. However, this reduction in HDL cholesterol may be due to altered HDL function from increasing preβ1-HDL, which can promote cellular lipid efflux. Therefore, the observed lowered HDL cholesterol levels in the probucol group may not be harmful, but rather may be a reflection of increased cholesterol efflux, which can beneficially affect mortality. Furthermore, based on reductions in new-onset DM cases and glycated hemoglobin levels among patients with succinobucol in the ARISE trial [25], probucol treatment contributes to reduced mortality through the prevention of new-onset DM or through the improvement of its control level [28]. However, we did not study new-onset DM or glycemic control during follow-up in this study.

In the present study, we observed a significant relationship between probucol and all-cause mortality but did not observe a significant relationship between probucol and cardiac

mortality. In addition, PS adjusted and PS matched analyses indicate that patients using probucol generally died due to non-cardiac-related causes. Therefore, we expected to observe a significant relationship between probucol and major sub-specific causes of non-cardiac death. However, we did not observe a significant relationship between probucol and deaths associated with cancer, which were the most frequent sub-specific cause of non-cardiac death. However, the probucol group tended to have reduced cardiac mortality in all models, as well as non-cardiac mortality. This suggests that reduced all-cause mortality in the probucol group is consistent with combined risk reduction in both cardiac and some sub-specific causes of non-cardiac death other than cancer-related deaths, which may include deaths related to heart failure. Since it is difficult to distinguish deaths due to heart failure from other non-cardiac causes of death, we did not include heart failure in the definition of cardiac death, which may have affected the results. Nevertheless, all-cause mortality is an objective endpoint and should be used as a primary endpoint in this type of observational study.

Importantly, no significant subgroup-treatment effect interactions were observed in any subgroup analyses. This suggests that the efficacy of probucol may not be affected by age, gender, presence or absence of DM, total and HDL cholesterol levels, presence or absence of prior MI, AF, and statins use. However, the absence of a significant relationship between probucol use and all-cause mortality in women and patients with AF might suggest an incidence of QT interval prolongation and fatal ventricular arrhythmia. Women and patients with a high risk of drug interaction such as those with AF treated with anti-arrhythmic drugs were at risk to have torsades de pointes among patients treated with drugs that prolong the QT interval such as probucol [29]. Absence of a significant relationship between probucol use and mortality in patients who receive statins should be taken into account, although the probucol group showed no significant overall mortality risk and there were no significant subgroup treatment effect interactions. In addition, negative results can be ignored because of the small number of patients in those subgroups and the high rate of false-negative results in the analysis within individual subgroups. Thus, studies specifically evaluating the effects of probucol on mortality in women, patients with AF, and statin-treated patients as well as large-scale studies are required.

4.1. Study limitations

This was a single center observational study of daily clinical practice. Although propensity analyses are powerful, they are inherently limited by the number and accuracy of variables evaluated. Furthermore, recent evidence suggests that PS is not always an accurate indicator for adjusting confounding factors by indication [30] and that results from propensity analyses pertain to population-averaged effects rather than the effect of receiving or not receiving probucol therapy within an individual. In addition, even after adjusted analysis was performed, other unknown confounders may have affected the outcomes. Further studies are required to determine if there is a benefit in long-term outcomes for patients undergoing probucol treatment.

In addition, the total duration of probucol use and changes in dosage after complete revascularization was not examined and there was crossover between the two groups. However, if crossover bias was present, it would have led to underestimation of the association between probucol administration and survival. This further emphasizes that probucol therapy at the time of complete revascularization is associated with better long-term mortality. Although this may have affected the results of our study, the effect was very small.

5. Conclusion

In the present study involving consecutive revascularization patients, we showed that the use of probucol is significantly associated with reduced long-term all-cause mortality after complete revascularization. Although probucol should be used with caution in specific subgroups, findings of the present study may contribute to reappraisal of probucol as a therapeutic drug in patients with CAD.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.atherosclerosis.2011.09.051.

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Committee Report 1

Executive Summary of the Japan Atherosclerosis Society (JAS) Guidelines for the Diagnosis and Prevention of Atherosclerotic Cardiovascular Diseases in Japan — 2012 Version

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Committee for Epidemiology and Clinical Management of Atherosclerosis

Among the various atherosclerotic cardiovascular diseases (CVDs), these guidelines primarily deal with cerebrovascular disease, peripheral arterial disease (PAD) and coronary artery disease (CAD), which occur in association with atherosclerosis and is closely related to dyslipidemia.

1. Comprehensive Risk Management for the Prevention of Atherosclerotic CVD

To prevent CVD, it is important to manage dyslipidemia in addition to other risk factors. For this purpose, we propose comprehensive risk management for the prevention of CVD. Risk factors that should be considered include dyslipidemia, hypertension, diabetes mellitus, smoking, chronic kidney disease (CKD), a family history of premature CAD, a history of CAD, noncardiogenic cerebral infarction, PAD, age and sex. In this article, we describe the comprehensive management of CVD.

2. Diagnostic Criteria for Dyslipidemia

It has been shown in epidemiological studies conducted in Japan, as well as in Western countries, that the incidence of CAD increases in association with increases in the levels of LDL-cholesterol (LDL-C)[1] and triglycerides (TGs)[2, 3] and decreases in the level of HDL cholesterol (HDL-C)[4-7]. Currently in Japan, the incidence of CAD is much lower than that observed in Western countries[2, 3, 8, 9]; however, this incidence is anticipated to increase in the near future due to the recent Westernization of the Japanese lifestyle. Therefore, the current guidelines provide screening criteria for dyslipidemia to prevent CVD with a specific emphasis on the prevention of CAD, as shown in **Table 1**.

Received: November 2, 2012
Accepted for publication: December 17, 2012

Regarding the diagnosis of dyslipidemia, the total cholesterol (TC), TG and HDL-C levels should be measured after an overnight fast. The LDL-C level is then calculated using the Friedewald formula (LDL-C=TC−HDL-C−TG/5).

This formula cannot be used if blood is collected without fasting or if the TG is ≥400 mg/dL. In such cases, using the non HDL-C level is recommended, which is calculated by subtracting the HDL-C level from the TC level. Data obtained in Japan indicate that the non HDL-C level is approximately 30 mg/dL higher than the LDL-C level. This view is shared by the National Cholesterol Education Program (NCEP). When lipids are evaluated based on the non HDL-C level, the target value of non HDL-C is determined by adding 30 mg/dL to the value of LDL-C (**Table 2**).

The incidence and mortality of CAD increase continuously in association with increases in the LDL-C level. At present, the incidence of CAD is lower in Japanese individuals than in Westerners. To maintain this low rate, efforts directed toward early prevention are required. Therefore, from the perspective of the prevention and treatment of CAD, the current guidelines propose an LDL-C level of 140 mg/dL as the reference value when screening Japanese individuals for hyper-LDL cholesterolemia. This value was selected because it corresponds to a TC level of 220 mg/dL, at which point the relative risk is approximately 1.5-fold higher than that observed at a TC level of <180 mg/dL, according to the NIPPON DATA80[10]. Since the LDL-C goal may vary depending on concomitant risk factors, an LDL-C level between 120 and 139 mg/dL is defined as indicating borderline hyper-LDL cholesterolemia.

Hypo-HDL cholesterolemia has also been established to be a risk factor for CVD. The current guidelines define an HDL-C level of <40 mg/dL as indicating hypo-HDL cholesterolemia, as determined in

Table 1. Dyslipidemia: Diagnostic Criteria for Screening (Fasting*)

Low-density lipoprotein cholesterol (LDL-C)	≥140 mg/dL	Hyper-LDL cholesterolemia
	120-139 mg/dL	Borderline hyper-LDL cholesterolemia**
High-density lipoprotein cholesterol (HDL-C)	<40 mg/dL	Hypo-HDL cholesterolemia
Triglycerides (TG)	≥150 mg/dL	Hypertriglyceridemia

- The LDL-C level is calculated using the Friedewald formula (TC − HDL-C − TG/5) (for TG <400 mg/dL).
- If the TG level is ≥400 mg/dL or non-fasting blood is used, the non HDL-C (TC − HDL-C) level should be used with a cutoff value of LDL-C + 30 mg/dL.

*Fasting is defined as deprivation of food for at least 10 to 12 hours; however, the ingestion of noncaloric beverages, such as water and tea, is allowed.

**If a patient is found to have borderline hyper-LDL cholesterolemia during screening, he/she should be examined for any high-risk conditions and the need for treatment should be considered.

Table 2. Lipid Management Targets for Patients with Different Risk Levels

Therapeutic principle	Management category	Lipid management target (mg/dL)			
		LDL-C	HDL-C	TG	Non HDL-C
Primary prevention Drug therapy should be considered after lifestyle modification	Category I	<160	≥40	<150	<190
	Category II	<140			<170
	Category III	<120			<150
Secondary prevention Drug therapy should be considered, together with lifestyle modification	History of CAD	<100			<130

- For patients at low absolute risk, such as the young, the relative risk chart (Supplementary Table) should be used and changes in the absolute risk should be monitored carefully while encouraging the patient to modify their lifestyle.
- These values should be considered general, not mandatory, goals.
- A 20%-30% reduction in the level of LDL-C is considered to be a prime target for pharmacological intervention.
- The management target for the non HDL-C level is the secondary target to be used after a patient with hypertriglyceridemia has achieved the management target for the LDL-C level. The non HDL-C level should be used if blood is collected after meals or if the TG level is ≥400 mg/dL.
- For patients in any category, the management goals should generally be achieved via lifestyle modification.
- For patients in category I, drug therapy should be considered if the LDL-C level is ≥180 mg/dL.

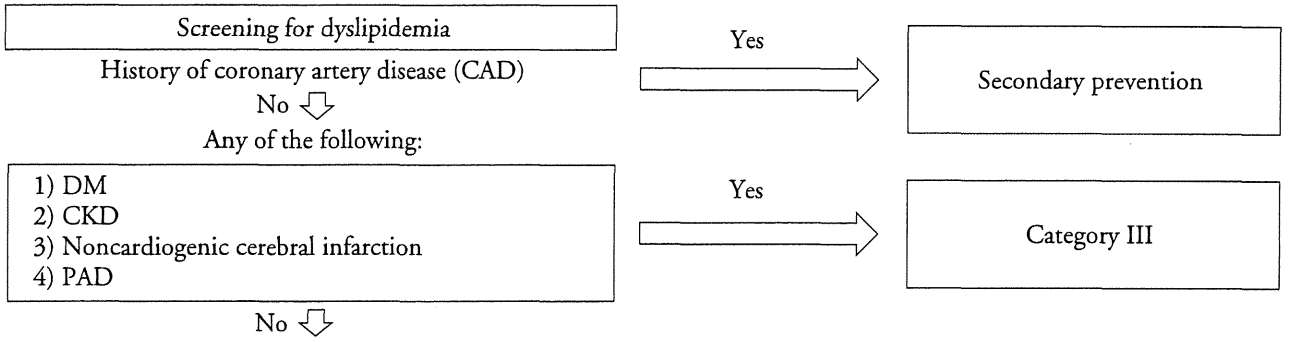
our previous guidelines. A number of studies have demonstrated sex differences in the HDL-C levels; however, it remains unclear whether these sex differences are reflected in the diagnosis of hypo-HDL cholesterolemia.

Hypertriglyceridemia has been found to occur in association with various conditions. Although some researchers insist that more intensive management is required in patients with certain diseases, such as diabetes mellitus, the current guidelines define a TG level of ≥150mg/dL as indicating hypertriglyceridemia, based on epidemiological data obtained during screenings of the general population.

3. Risk Stratification Based on Absolute Risk

The current guidelines stratify the risk of CVD for primary prevention according to the absolute risk calculated based on the results of the NIPPON DATA80[11]. This study identified age, sex, diabetes mellitus, current smoking, systolic blood pressure and the TC level as risk factors and determined the absolute risk of death from CAD depending on the degree or existence of these factors.

How absolute risk categories should be determined is based on clinical consensus and/or conventional wisdom. The U.S. NCEP Adult Treatment Panel III classifies a 10-year risk of death from CAD or the development of nonfatal myocardial infarction of ≥20% (based on the Framingham score) as high risk[12], whereas European guidelines classify a 10-year risk of death from CVD (including strokes and CAD) of ≥5% as high risk[13]. The current guidelines classify



Management categories based on absolute risk for the primary prevention of CAD

10-year probability (absolute risk) of CAD death derived from NIPPON DATA80	Additional risk factors	
	No additional risk factors	One or more of the following: (1) Hypo-HDL cholesterolemia (HDL-C <40 mg/dL) (2) Family history of premature CAD in first-degree relatives (a man aged <55 years or a women aged <65 years) (3) Impaired glucose tolerance
<0.5%	Category I	Category II
≥0.5%–<2.0%	Category II	Category III
≥2.0%	Category III	Category III

This flow chart is not applicable to patients with FH.

Fig. 1. Flow chart for setting management targets for LDL cholesterol

patients with a 10-year risk of death from CAD of ≥ 2% as belonging to the high-risk group (category Ⅲ), those with a risk of ≥0.5% to <2% as belonging to the intermediate-risk group (category Ⅱ) and those with a risk of <0.5% as belonging to the low-risk group (category Ⅰ), considering that there is little evidence of an association between hypercholesterolemia and cerebrovascular diseases in Japanese individuals. Since diabetes mellitus, CKD and a history of noncardiogenic cerebral infarction or PAD are considered to be important risk factors, patients with any of these conditions are classified as belonging to the high-risk group (**Fig. 1**).

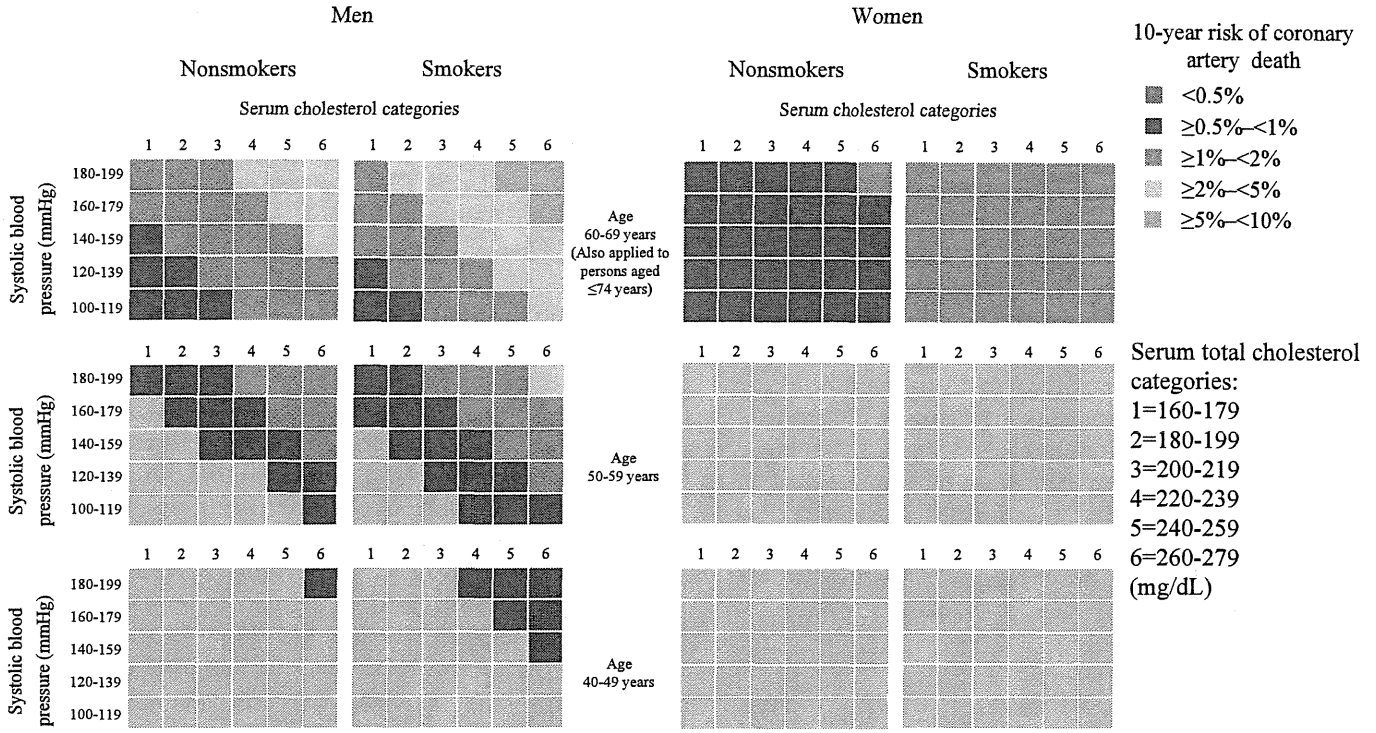
The 10-year absolute risk of CAD-related death should be determined based on the risk assessment chart provided in the NIPPON DATA80[11]. However, since this chart does not include hypo-HDL cholesterolemia, a family history of premature CAD or impaired glucose tolerance, the category should be raised if the patient meets one or more of these criteria (**Fig. 2**).

The chart obtained from the NIPPON DATA80 addresses the risk of CAD-related death in individuals between 40 and 79 years of age. While the current guidelines are intended for adults younger than 65 years of age, they can also be applied to persons between 65 and 74 years of age. To calculate the absolute risk for individuals ≥70 and <75 years of age, the table for individuals between 60 and 69 years of age should be used. For adults <40 years of age, the table for individuals between 40 and 49 years of age should be used.

When assessing the absolute risk, it should be noted that the absolute risk greatly depends on age. If a low absolute risk is obtained for a young individual with a risk factor, such as hypertension or smoking, the risk factors should be managed appropriately. When secondary prevention is required, each risk factor should be dealt with separately, as outlined in the previous guidelines.

4. Management Targets for Dyslipidemic Patients

The management targets for dyslipidemic patients are presented by category in **Table 2**. For primary prevention, drug therapy should be considered after lifestyle factors have been improved for a certain



The section of hyperglycemia from the NIPPON DATA80 risk assessment chart is omitted here. These charts cannot be applied to high-risk patients, such as those with DM or CKD.

Fig. 2. Absolute risk assessment charts for death from coronary artery disease (primary prevention).

Absolute risk should be reassessed at least once a year since it may be affected by either risk factors or aging.

Step 1: The applicable portion of the above figures should be assessed based on gender, age, the present smoking status, systolic blood pressure (mmHg) and the TC level (mg/dL).
Absolute risk ≥ 2% → Category Ⅲ
Absolute risk < 2% → To Step 2
Step 2: Any of the following conditions: hypo-HDL-cholesterolemia (< 40 mg/dL), a family history of CAD and/or impaired glucose tolerance
Absolute risk ≥ 0.5% < 2% + Yes → Category Ⅲ
Absolute risk ≥ 0.5% < 2% + No → Category Ⅱ
Absolute risk < 0.5% + Yes → Category Ⅱ
Absolute risk < 0.5% + No → Category Ⅰ
Supplementary notes
(1) The TC category 160-179 mg/dL should be used in patients with a TC level of < 160.
(2) The TC category 260-279 mg/dL should be used in patients with a TC level of ≥ 280 mg/dL.
(3) The systolic blood pressure category of 100-119 mmHg should be used in patients with a systolic blood pressure of < 100 mmHg, while the systolic blood pressure category of 180-199 mmHg should be used in patients with a systolic blood pressure of ≥ 200 mmHg.
(4) The guidelines cannot be applied to persons 75 years of age or older. "The Elderly." For patients < 40 years of age, the relative risk chart (Supplementary Table) should be used.
(5) Blood pressure should be managed according to the guidelines established by the Japanese Society of Hypertension, while diabetes mellitus should be managed according to the guidelines established by the Japan Diabetes Society.
(6) It is desirable to encourage smokers to stop smoking irrespective of the level of absolute risk.

period and the response has been evaluated. For individuals in category Ⅰ low absolute risk group), the management target for the LDL-C level is set at < 160 mg/dL. The target for individuals in category Ⅱ is set at < 140 mg/dL, while that for individuals in category Ⅲ (high absolute risk group) is set at < 120 mg/dL. It should be noted that achieving these targets is recommended but not obligatory. A meta-analysis of preventive clinical trials demonstrated that a 20%-30% reduction in the LDL-C level results in a decrease in the incidence of CAD of approximately 30%. Based on this finding, a 20%-30% decrease in

the LDL-C level can be considered a target. For secondary prevention, since the patient has already been diagnosed with CAD, the administration of drug therapy targeting an LDL-C level of <100 mg/dL is recommended in addition to lifestyle modification.

For the management of hypertriglyceridemia and hypo-HDL cholesterolemia, targeting a TG level of < 150 mg/dL and an HDL-C level of ≥40 mg/dL is recommended, as in the previous guidelines.

Some researchers have the opinion that stricter targets should be established for high-risk patients (such as those with diabetes mellitus or CKD) or those who require secondary prevention, depending on the patient's condition and severity of disease; however, there is insufficient evidence to support setting such goals. Nevertheless, the current guidelines also suggest that high-risk patients be stratified according to risk factors and that lower targets be established for such patients.

5. Treatment

Dyslipidemia should be treated with lifestyle modification, including smoking cessation and the administration of diet and/or exercise therapy. In primary prevention patients, drug therapy should only be considered when the lipid management targets are not achieved after sufficient effort has been made to improve lifestyle factors. In patients with a history of CAD, the use of drug therapy should be considered simultaneously with lifestyle modification.

When drug therapy is provided for patients with hyper-LDL cholesterolemia, statins are the first drug of choice. Resin, probucol and/or ezetimibe are used in combination with statins or selected when statins cannot be administered. The combination of statins and EPA is useful for treating high-risk patients with hyper-LDL cholesterolemia. For treating hypertriglyceridemia accompanied by hypo-HDL cholesterolemia, drugs such as fibrates and nicotinic acid derivatives should be considered.

6. High-Risk Conditions for CVD

The current guidelines include CKD in addition to a history of CAD (secondary prevention), diabetes mellitus, noncardiogenic cerebral infarction and PAD as high-risk conditions based on the findings of epidemiological studies, including evidence showing that the presence of CKD increases the incidence of CAD by at least two-fold. The previous guidelines classified a history of cerebral infarction as a high-risk condition, while the current guidelines classify a history of noncardiogenic cerebral infarction as a high-risk condition because cardiogenic cerebral infarctions are not caused by atherosclerotic disease.

7. Familial Hypercholesterolemia

Familial hypercholesterolemia occurs in approximately one in 500 individuals and is associated with a high risk of CAD. The current guidelines reference the diagnostic criteria for FH reported by the 2011 Primary Hyperlipidemia Research Group and set a target of an LDL-C level of <100 mg/dL or a decrease in the LDL-C level of at least 50%.

8. Evaluation of CVD

To prevent CVD, the presence or absence and severity of atherosclerosis must be evaluated before symptoms occur and risk factors must be managed or treated with the objective of preventing progression or possibly achieving regression. For this purpose, correctly staging CVD is important. At present, the degree of atherosclerosis is primarily evaluated using imaging techniques. Invasive techniques include angiography (to assess the severity of stenosis) as well as angioscopy and intravascular ultrasonography (to qualitatively assess the vessel walls). Noninvasive techniques include transcutaneous ultrasonography of the arteries, such as the carotid artery, to qualitatively and quantitatively evaluate the degree of atherosclerosis. Carotid artery ultrasonography is often used in general practice because the extent of carotid sclerosis has been shown to be correlated with the risk of cerebrovascular disease and/or CAD. The development of multidetector CT (MDCT) has allowed for easier detection of coronary artery lesions. At present, carotid artery ultrasonography and MDCT are less invasive and easier to perform than other imaging modalities. In the near future, developing guidelines for the assessment of atherosclerosis that can be employed before the onset of symptoms is necessary. At present, however, assessing the degree of atherosclerotic lesions using the above-mentioned imaging techniques is associated with some limitations. CVD should be diagnosed based on a clear understanding of these limitations.

Footnotes

This is an English version of the guidelines of the Japan Atherosclerosis Society (Chapter 1) published in Japanese in June 2012.

Acknowledgements

We are grateful to the following societies for their

collaboration and valuable contributions: Dr. Hidenori Arai (The Japan Geriatrics Society), Dr. Kiminori Hosoda (Japan Society for the Study of Obesity), Dr. Hiroyasu Iso (Japan Epidemiological Association), Dr. Atsunori Kashiwagi (Japan Diabetes Society), Dr. Masayasu Matsumoto (The Japan Stroke Society), Dr. Hiromi Rakugi (The Japanese Society of Hypertension), Dr. Tetsuo Shoji (Japanese Society of Nephrology) and Dr. Hiroaki Tanaka (Japanese Society of Physical Fitness and Sports Medicine). We also thank Dr. Shinji Koba, Dr. Manabu Minami, Dr. Tetsuro Miyazaki, Dr. Hirotoshi Ohmura, Dr. Mariko Harada-Shiba, Dr. Hideaki Shima, Dr. Daisuke Sugiyama, Dr. Minoru Takemoto and Dr. Kazuhisa Tsukamoto for supporting this work.

Disclosures

T. Teramoto: Astellas Pharma Inc., MSD K.K., Kowa Pharmaceutical Co., Ltd., Kissei Pharmaceutical Co., Ltd., Daiichi-Sankyo Co., Ltd., Pfizer Co., Ltd., Bayer Yakuhin Ltd., Kobayashi Pharmaceutical Co., Ltd., GlaxoSmithKline K.K., Shionogi & Co., Ltd.; J. Sasaki: MSD K.K., Daiichi-Sankyo Co., Ltd., Bayer Yakuhin Ltd.; S. Ishibashi: Kowa Pharmaceutical Co., Ltd., Takeda Pharmaceutical Co., Ltd., MSD K.K.; S. Biro: none.; H. Daida: AstraZeneca K.K., MSD K.K, Mochida Pharmaceutical Co., Ltd., Kowa Pharmaceutical Co., Ltd., Astellas Pharma Inc., Daiichi-Sankyo Co., Ltd., Nippon Boehringer Ingelheim Co., Ltd., Pfizer Co., Ltd., Boston Scientific Japan K.K., Takeda Pharmaceutical Co., Ltd., Bayer Yakuhin Ltd., Mitsubishi Tanabe Pharma Corp., Dainippon Sumitomo Pharma Co., Ltd., Sanofi-Aventis K.K., Novartis Pharma K.K. Ltd., Shionogi & Co. Ltd.; S. Dohi: none.; G. Egusa: none.; T. Hiro: Kowa Pharmaceutical Co., Ltd.; K. Hirobe: none.; M. Iida: none.; S. Kihara: none.; M. Kinoshita: Daiichi-Sankyo Co., Ltd.; C. Maruyama: none.; T. Ohta: none.; T. Okamura: none.; S. Yamashita: MSD K.K., Bayer Yakuhin Ltd., Kowa Pharmaceutical Co., Ltd., Skylight Biotech Inc., Astellas Pharma Inc., Shionogi & Co., Ltd., Otsuka Pharmaceuticals Co., Ltd., Kissei Pharmaceuticals Co., Ltd.; M. Yokode: Otsuka Pharmaceuticals Co., Ltd., MSD K.K.; K. Yokote: MSD K.K., Ono Pharmaceutical Co., Ltd., Pfizer Co., Ltd., Astellas Pharma Inc., Takeda Pharmaceutical Co., Ltd., Kowa Pharmaceutical Co., Ltd., Novartis Pharma K.K., Daiichi-Sankyo Co., Ltd., Eli Lilly Japan K.K., Sanofi-Aventis K.K., Novo Nordisk Pharma Ltd., Dainippon Sumitomo Pharma Co., Ltd, Kyowa Hakko Kirin Co., Ltd., Shionogi & Co. Ltd.

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Supplementary Table. Relative Risk Charts for the Young, etc. with a Low Absolute Risk (based on the risk charts of the NIPPON DATA80)

			Nonsmokers			
Systolic blood pressure						
Second-degree or higher hypertension (≥160 mmHg)	2.2	2.8	3.6	4.6	5.8	7.4
First-degree hypertension (140-159 mmHg)	1.7	2.2	2.8	3.5	4.5	5.7
Normal (≤140)	1.0*	1.3	1.6	2.1	2.6	3.4
TC category (mg/dL)	160-179	180-199	200-219	220-239	240-259	260+
			Smokers			
Systolic blood pressure						
Second-degree or higher hypertension (≥160 mmHg)	3.2	4.1	5.2	6.6	8.4	10.7
First-degree hypertension (140-159 mmHg)	2.5	3.1	4.0	5.1	6.5	8.2
Normal (≤140 mmHg)	1.4	1.8	2.3	3.0	3.8	4.8
TC category (mg/dL)	160-179	180-199	200-219	220-239	240-259	260+

PLOS ONE

Association between Myocardial Triglyceride Content and Cardiac Function in Healthy Subjects and Endurance Athletes

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Abstract

Ectopic fat accumulation plays important roles in various metabolic disorders and cardiovascular diseases. Recent studies reported that myocardial triglyceride (TG) content measured by proton magnetic resonance spectroscopy (^{1}H-MRS) is associated with aging, diabetes mellitus, and cardiac dysfunction. However, myocardial TG content in athletes has not yet been investigated. We performed ^{1}H-MRS and cardiac magnetic resonance imaging in 10 male endurance athletes and 15 healthy male controls. Serum markers and other clinical parameters including arterial stiffness were measured. Cardiopulmonary exercise testing was also performed. There were no significant differences in clinical characteristics including age, anthropometric parameters, blood test results, or arterial stiffness between the two groups. Peak oxygen uptakes, end-diastolic volume (EDV), end-systolic volume (ESV), left ventricular (LV) mass, peak ejection rates and peak filling rates were significantly higher in the athlete group than in the control group (all $P<0.02$). Myocardial TG content was significantly lower in the athlete group than in the control group (0.60 ± 0.20 vs. $0.89\pm0.41\%$, $P<0.05$). Myocardial TG content was negatively correlated with EDV ($r=-0.47$), ESV ($r=-0.64$), LV mass ($r=-0.44$), and epicardial fat volume ($r=0.47$) (all $P<0.05$). In conclusion, lower levels of myocardial TG content were observed in endurance athletes and were associated with morphological changes related to physiological LV alteration in athletes, suggesting that metabolic imaging for measurement of myocardial TG content by ^{1}H-MRS may be a useful technique for noninvasively assessing the "athlete's heart".

Citation: Sai E, Shimada K, Yokoyama T, Sato S, Miyazaki T, et al. (2013) Association between Myocardial Triglyceride Content and Cardiac Function in Healthy Subjects and Endurance Athletes. PLoS ONE 8(4): e61604. doi:10.1371/journal.pone.0061604

Editor: Anindita Das, Virginia Commonwealth University, United States of America

Received October 20, 2012; **Accepted** March 11, 2013; **Published** April 16, 2013

Funding: This work was partially supported by the Sportology Research Center grant from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and a grant from the Mizuno Sports Promotion Foundation. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

Ectopic fat accumulation is associated with various metabolic disorders and cardiovascular diseases [1–3]. Previous animal studies have shown that myocardial triglyceride (TG) accumulation triggers pathological changes, including myocardial apoptosis and ventricular systolic dysfunction [4,5]. However, the assessment of myocardial TG content is hampered by the difficulty of obtaining myocardial tissues in a clinical setting.

Recent studies have demonstrated that proton magnetic resonance spectroscopy (^{1}H-MRS) enables the noninvasive monitoring of TG accumulation in human myocardial tissue. Indeed, myocardial TG content, as measured by ^{1}H-MRS, has been associated with aging [6], diabetes mellitus [7], myocardial systolic dysfunction [4,8,9], and diastolic dysfunction [6,10]. In addition, caloric restriction induced a dose-dependent increase in myocardial TG content [11], whereas endurance training reduced myocardial TG content [12]. However, myocardial TG content in endurance athletes has not yet been investigated.

The purpose of this study is to evaluate the associations between myocardial TG content, cardiac morphology and left ventricular (LV) function assessed by ^{1}H-MRS and magnetic resonance imaging (MRI) in healthy subjects and endurance athletes.

Methods

Subjects

Fifteen healthy male subjects and 10 male endurance athletes were recruited by advertisements in a local area. All subjects were non-obese, aged 20–40 years, and without acute or chronic disease. Subjects receiving medical treatment, current smokers, and those with abnormal laboratory parameters were excluded. We defined an endurance athlete as a person who performed endurance training for more than 5 days a week, and was affiliated with a specific athletic association to participate in competitive sports such as cycling, track, or swimming. The international physical activity questionnaire (IPAQ) was used to assess each subject's activity level [13]. All protocols were approved by the ethical committee of the Juntendo University, and all participants

provided written informed consent before their participation in this study according to the guidelines established in the Declaration of Helsinki.

Measurements of Body Composition

Skeletal muscle mass and body fat weight were measured after overnight fasting by multi-frequency bioelectrical impedance analysis using eight tactile electrodes (MF-BIA8; In-Body 720, Biospace, Korea) [14] after overnight fasting. The subject stood on the footplate with barefoot and held the electrodes in both hands. This process takes 2 min, and measurement requires no specific skills. The apparatus then automatically displays measurements of fat-free mass, fat mass, and percentage body fat.

Blood Measurements

Standard laboratory tests including blood cell counts, fasting plasma glucose, lipids, creatinine, free fatty acid, and N-terminal pro-brain natriuretic peptide (NT-proBNP) were performed immediately before MRS after overnight fasting. Serum lipid profiles were measured using specific assays for total cholesterol (Symex Co, Kobe, Japan), triglyceride (Sekisui Medical, Tokyo, Japan), and high-density lipoprotein cholsterol (Sekisui Medical, Tokyo, Japan) by BioMajesty JCA-BM8060 analyzer (Japan Electron Optics Laboratory Ltd, Tokyo, Japan). Serum low-density lipoprotein cholesterol levels were calculated using the Friedewald's formula. Serum insulin was measured by chemiluminescent enzyme immunoassay using the Lumipulse presto II analyzer (Fujirebio Inc, Tokyo, Japan). A homeostasis model assessment index (HOMA-IR) was calculated to estimate insulin resistance from fasting insulin and glucose concentrations: insulin (μU/ml)×glucose (mmol/l)/22.5. Free fatty acid (FFA) was measured a standard enzymatic assay (Eiken chemical Co. Ltd, Tokyo, Japan) by BioMajesty JCA-BM2250 analyzer (Japan Electron Optics Laboratory Ltd, Tokyo, Japan). Serum NT-proBNP was determined using an electrostatic controlled linear inchworm actuator on Hitachi modular analytics (HITACHI Hi-Technologies Co. Ltd. Tokyo, Japan). HbA1c concentrations were measured in whole blood samples using latex-enhanced immunoassay (Fujirebio Co. Ltd. Tokyo, Japan).

MRI and MRS

All cardiac MRI and ^{1}H-MRS studies were performed using a MAGNETOM Avanto 1.5-Tesla MRI system (Siemens Medical Solution, Erlangen, Germany) with subjects resting in the supine position. To minimize the influence of breathing, a towel was strapped around the subject's upper abdomen. Dynamic cine images were used to determine LV mass, and LV functional parameters. Image analysis was performed using special evaluation software (Argus; Siemens Medical Systems, Erlangen, Germany) [15,16] on a separate work station. Endocardial and epicardial LV borders were traced manually at end-diastole and end-systole from short-axis cine images. End-diastolic volume (EDV), end-systolic volume (ESV), stroke volume, and ejection fraction were calculated by Simpson's method. In addition, the peak LV ejection and filling rates were automatically derived on the basis of LV volume-time curves. The area of epicardial fat was traced on consecutive end diastolic short axis images, beginning with the most basal slice at the level of the mitral valve, and moving apically through the stack until the most inferior margin of adipose tissue, as reported previously [17].

After the cine MRI imaging, myocardial TG content was determined by ^{1}H-MRS. A volume of interest (VOI = 2.0 cm^3 − 10×10×20 mm) was selected within the ventricular septum from cine dynamic cine-mode images of the heart (Figure 1). We adjusted the VOI size to the anatomy of the ventricular septum. The spectrum of water and lipid was acquired by point-resolved spectroscopy (PRESS) method using an echo time (TE) of 30 ms, and repetition time (TR) of at least 4,000 ms, myocardial TG signals were acquired at 1.4 ppm from spectra with water suppression, and water signals were acquired at 4.7 ppm from spectra without water suppression (Figure 1). Areas under the curves for water and lipid peaks were quantified using standard line-fitting procedures (Siemens Syngo Spectroscopy). Myocardial TG level was expressed as a ratio of lipid to water (%). Thus, ^{1}H-MRS evaluation of myocardial TG content was performed essentially as has been previously validated [18–21].

Measurement of Cardiopulmonary Fitness

All subjects underwent an incremental cycling test (Corival 400, Lobe B.V., Groningen, Netherlands) using an expiratory gas analyzer (Vmax-295, sensorMedics Co., Yorba Linda, CA, USA) to measure anabolic threshold (AT) and maximal oxygen consumption (VO_{2max}). After a 3-min rest period, a warm-up was performed for 3 minutes at 40 W, followed by ramp loading (15–30 W/min) until the subjective exhaustion, as described previously [22]. According to the ATS/ACCP guidelines, AT was determined by V-slope method. In cases when AT was not identified on the V-slope, we used the point at which V_E/VO_2 starts to increase while V_E/VCO_2 remains constant [23].

Evaluation of Atherosclerotic Parameters

The cardio ankle vascular index (CAVI) was measured as atherosclerotic parameters. CAVI was automatically calculated by VaSera VS-1500AN (Fukuda Denshi Co. Ltd., Tokyo, Japan) [24,25].

Statistical Analyses

Values are expressed as mean ± standard deviation (SD). For variables that did not show a normal distribution, the data were transformed into natural logarithmic values before statistical analyses. Correlations were calculated using Pearson's correlation co-efficient. Unpaired Student's *t*-test was used to compare groups. All statistical analyses were performed with SPSS version 20 (SPSS, Inc). A P value of less than 0.05 was considered significant.

Results

The clinical characteristics of study subjects are summarized in Table 1. There were no significant differences, in age, body composition, lipids, glucose, insulin levels, or NT-proBNP between the two groups. The levels of AT (29.2±6.6 ml/kg/min vs. 19.0±5.2 ml/kg/min, P = 0.0002), VO_{2max} (52.3±6.2 ml/kg/min vs. 43.2±8.0 ml/kg/min, P = 0.0057) and international physical activity questionnaire (IPAQ) score (2318±1605 vs. 5310±2869, P = 0.0048) were significantly higher in the athlete groups than in the control group.

MRI and MRS variables are shown in Table 2. The values of EDV (182±24 ml vs. 153±16 ml, P = 0.0011), ESV (96±16 ml vs. 73±8 ml, P = 0.0002), and LV mass (139±16 g vs. 120±13 g, P = 0.0034), were significantly higher in the athlete group than in the control group. Peak ejection rate (777±230 ml/sec vs. 551±206 ml/sec, P = 0.019) and peak filling rate (839±250 ml/sec vs. 619±177 ml/sec, P = 0.018) were significantly higher in the athlete group than in the control group. None of the subjects had an abnormal peak ejection or filling rate. Myocardial TG content was significantly lower in the athlete group than in the control group (0.60±0.20% vs. 0.89±0.41%, P = 0.045) (Figure 2).

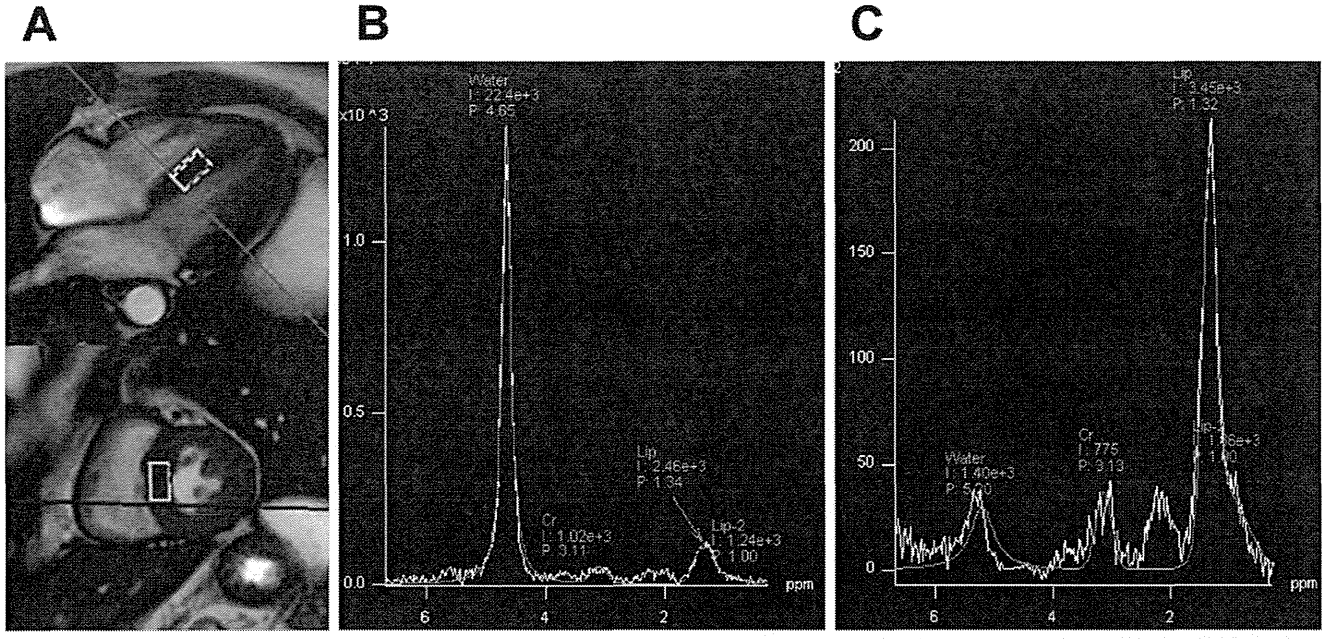

Figure 1. Representative results of H^1-MR spectra in a healthy subject. A: Myocardial voxel localization for H^1-MRS in 4-chamber and short axis views. B: H^1-MR spectra without water suppression. C: H^1-MR spectra without water suppression.
doi:10.1371/journal.pone.0061604.g001

Myocardial TG content was negatively correlated with EDV ($r=-0.47$, $P=0.018$), ESV ($r=-0.64$, $P=0.001$), LV mass volume ($r=-0.43$, $P=0.031$), and epicardial fat volume ($r=0.47$, $P=0.025$) (Figure 3). Although a significant correlation between myocardial TG content and VO_{2max} was not found ($r=-0.15$, $P=0.46$), epicardial fat volume was negatively correlated with EDV, a LV morphological parameter ($r=-0.44$, $P=0.022$).

Discussion

The present study demonstrated that myocardial TG content was significantly lower in the endurance athlete group than in the control group and that myocardial TG content was significantly correlated with EDV, ESV, LV mass, and epicardial fat volume. This study is, to the best our knowledge, the first report to demonstrate an association between TG content and physiological LV alteration in endurance athletes.

Much attention has been focused on the associations between ectopic fat accumulation, various metabolic disorders and cardiovascular diseases [1,2]. It has been reported that the myocardial TG content is associated with metabolic disorders [7,26]. The positive correlation between myocardial TG content and LV mass has also been reported among the diabetic patients as well as in obese individuals with insulin resistance [4,8]. Animal studies have demonstrated that myocardial TG content was associated with not only cardiovascular risk factors, but also with lipotoxicity-induced heart failure and premature death [20,27]. In addition, increased myocardial TG content induced pathological LV hypertrophy, cardiac dysfunction, and non-ischemic dilated cardiomyopathy [28]. However, the present study showed negative correlations between myocardial TG and LV mass as well as LV function. Several studies suggested that mitochondrial dysfunction in the myocardium exists in patients with diabetes and insulin resistance [29]. In contrast, the functional capacity of mitochondria in athlete's heart was reported to be increased by endurance training [30]. This difference in mitochondrial function may underlie the difference in myocardial TG content between the physiological modifications present in athlete's heart and the pathological changes that characterize the deteriorating heart in patients with diabetes and insulin resistance.

Previous studies reported the relationship between exercise and lipid content in skeletal muscle. High levels of intra-myocellular lipid (IMCL) were reported in the skeletal muscles of patients with diabetes mellitus [31] and elderly subjects [32]. On the other hand, it has also been reported that similar high levels of IMCL occur in skeletal muscles of athletes, despite the marked insulin sensitivity and the high oxidative capacity of these muscles, this is the so-called "athlete's paradox" [33]. Increases in IMCL content provide a substrate for energy metabolism during exercise [34]. A high availability of fatty acids is needed to augment TG resynthesis in skeletal muscle during and after exercise [34]. Diacylglycerol and/or ceramide, but not TG, may be directly associated with the development of insulin resistance [35,36]. In the present study, no "athlete's paradox" was observed in the subjects' cardiac muscles. Several potential reasons have been raised. One possibility is the difference in mitochondrial function with regard to fatty acid metabolism between skeletal muscle and cardiac muscle. Fatty acid metabolism may be more efficient in cardiac muscles, which has more abundant mitochondria than in skeletal muscles [37]. Another reason relates to the differences in regulation of fatty acid β-oxidation between the two types of muscle. To sustain contractile function in the heart requires a greater energy supply [38]. Therefore, the fatty acid β-oxidation system in cardiac muscle is very dynamic and sufficient to meet the energy demands of the heart. Alterations in lipoprotein lipase (LPL) synthesis as well as the activation, secretion, transportation, capillary luminal binding, and the degradation of fats in cardiac myocytes, contribute to myocardial fatty acid supply, uptake and fatty acid β-oxidation [38]. In addition, the heart muscle is reported to be less susceptible to developing insulin resistance than skeletal

Table 1. Clinical Characteristics.

	Control group (n = 15)	Athlete group (n = 10)	P value
Age, years	28.8±4.5	26.4±4.4	0.20
Body height, m	1.735±0.051	1.732±0.047	0.88
Body weight, kg	67.9±7.4	67.8±4.2	0.94
Body mass index, kg/m^2	22.5±1.9	22.6±1.9	0.90
Skeletal muscle mass, kg	30.7±2.6	32.5±2.0	0.083
Body fat weight, kg	13.6±3.8	10.6±3.6	0.066
Percent of body fat, %	18.6±5.0	15.4±4.8	0.14
Neck circumference, cm	36.9±2.4	36.8±1.8	0.92
Waist circumference, cm	80.5±6.8	78.1±4.0	0.36
Total cholesterol, mg/dl	174.6±26.3	182.5±24.5	0.45
Triglyceride, mg/dl	74.6±27.0	61.1±15.8	0.16
LDL-cholesterol, mg/dl	104.2±26.4	111.1±29.0	0.53
HDL-cholesterol, mg/dl	55.7±11.3	59.2±12.7	0.47
Fasting free fatty acid, μEq/L	299.1±132.3	364.7±211.5	0.32
Fasting blood glucose, mg/dl	90.7±8.6	90.9±5.0	0.93
Insulin, μU/ml	5.6±3.0	4.4±1.4	0.22
HOMA-IR	1.3±0.6	1.0±0.3	0.22
HbA1c, %	4.7±0.3	4.7±0.2	0.51
Creatinine, mg/dl	0.84±0.10	0.84±0.05	0.85
eGFR, ml/min/m^2	91.6±12.2	92.1±6.7	0.91
NT-proBNP, ng/l	18.6±18.0	10.1±3.9	0.15
Urinary acid, mg/l	6.0±0.9	5.4±1.3	0.15
Anaerobic threshold, ml/kg/min	19.0±5.2	29.2±6.6	0.0002
VO_2max, ml/kg/min	43.2±8.0	52.3±6.2	0.0057
CAVI	6.5±0.7	6.2±0.6	0.53
IPAQ score	2318±1605	5310±2869	0.0048

Values are mean ± SD. bpm = beats per minutes, LDL = low-density lipoprotein; HDL = high-density lipoprotein; eGFR = estimated glomerular filtration rate; HOMA-IR = homeostasis model assessment of insulin resistance, NT-proBNP = N-terminal pro brain natriuretic peptides, VO_2max = maximal oxygen intake, CAVI = cardio ankle vascular index, IPAQ = international physical activity questionnaire.
P value denotes significance of unpaired *t* test between athlete group and healthy control.
doi:10.1371/journal.pone.0061604.t001

muscle [39]. Therefore, insulin responsiveness and its consequences in the heart may be relatively high in endurance athletes.

A recent study has shown that acute endurance exercise leads to increased myocardial TG content depending on elevated plasma free fatty acid concentrations and the uptake of free acids in the heart. The mechanism is considered to be related to the increased availability of fatty acid during exercise in fasting healthy males [40]. The level of circulating free fatty acids concentration was low in the present study. Thus, fatty acid availability must be relatively low in these individuals. Indeed, myocardial TG content was not reported to change even after exercise in subjects with a suppressed state of free fatty acid synthesis [40]. In addition, endurance training regulates the activity of LPL [41], which provides the major source of free fatty acids derived from TG content lipoproteins. Endurance athletes manifesting physiological LV adaptations may be augmented to drive alterations in fatty acid metabolism on fasting state.

Table 2. MRI variables.

	Control group (n = 15)	Athlete group (n = 10)	P value
LV ejection fraction, %	50.6±5.5	48.1±6.3	0.32
LV end diastolic volume, ml	153±16	182±24	0.0011
LV end systolic volume, ml	73±8	95±16	0.0002
Stroke volume, ml	80±14	88±17	0.22
Cardiac output	4.8±0.8	5.2±1.2	0.29
LV myocardial mass, g	120±13	139±16	0.0034
Peak ejection rate, ml/sec	551±206	777±230	0.019
Peak filling rate, ml/sec	619±177	839±250	0.018
Epicardial fat volume, ml	48.8±14.8	38.3±8.2	0.057

Values are mean ± SD. LV = left ventricular.
P value denotes significance of unpaired *t* test between athlete group and healthy control.
doi:10.1371/journal.pone.0061604.t002

We measured several TG-associated enzymes and proteins, including adiponectin, pre-heparin LPL, apolipoprotein (apo) CII, and apo CIII. No significant difference was observed between the two groups for each parameter (data not shown). One of the major reasons, why these enzyme and proteins were not significantly different, is supposed to the study subjects consisting with healthy lean young men without any metabolic disorder. Myocardial lipid metabolism is regulated by a complex balance between fatty acid supply to the heart, competing energy substrates, energy demand and oxygen supply to the heart, uptake and esterification of fatty acid, and control of mitochondrial functions such as fatty acid oxidation and electron transport chain activity [38]. In addition, epicardial fat, which stores free fatty acid during excessive circulating free fatty acid accumulation and releases fatty acid when energy is needed, is directly connected to the myocardium.

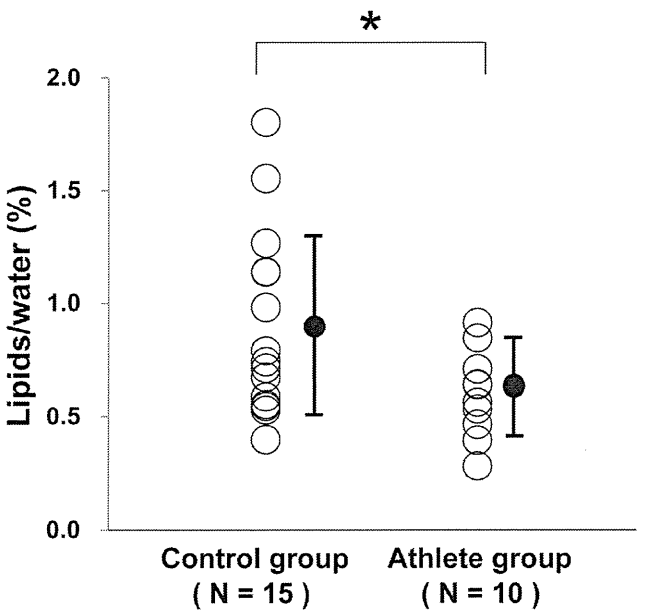

Figure 2. Comparison between myocardial TG content in the control group and the athlete group. * $P<0.05$ between the two groups.
doi:10.1371/journal.pone.0061604.g002

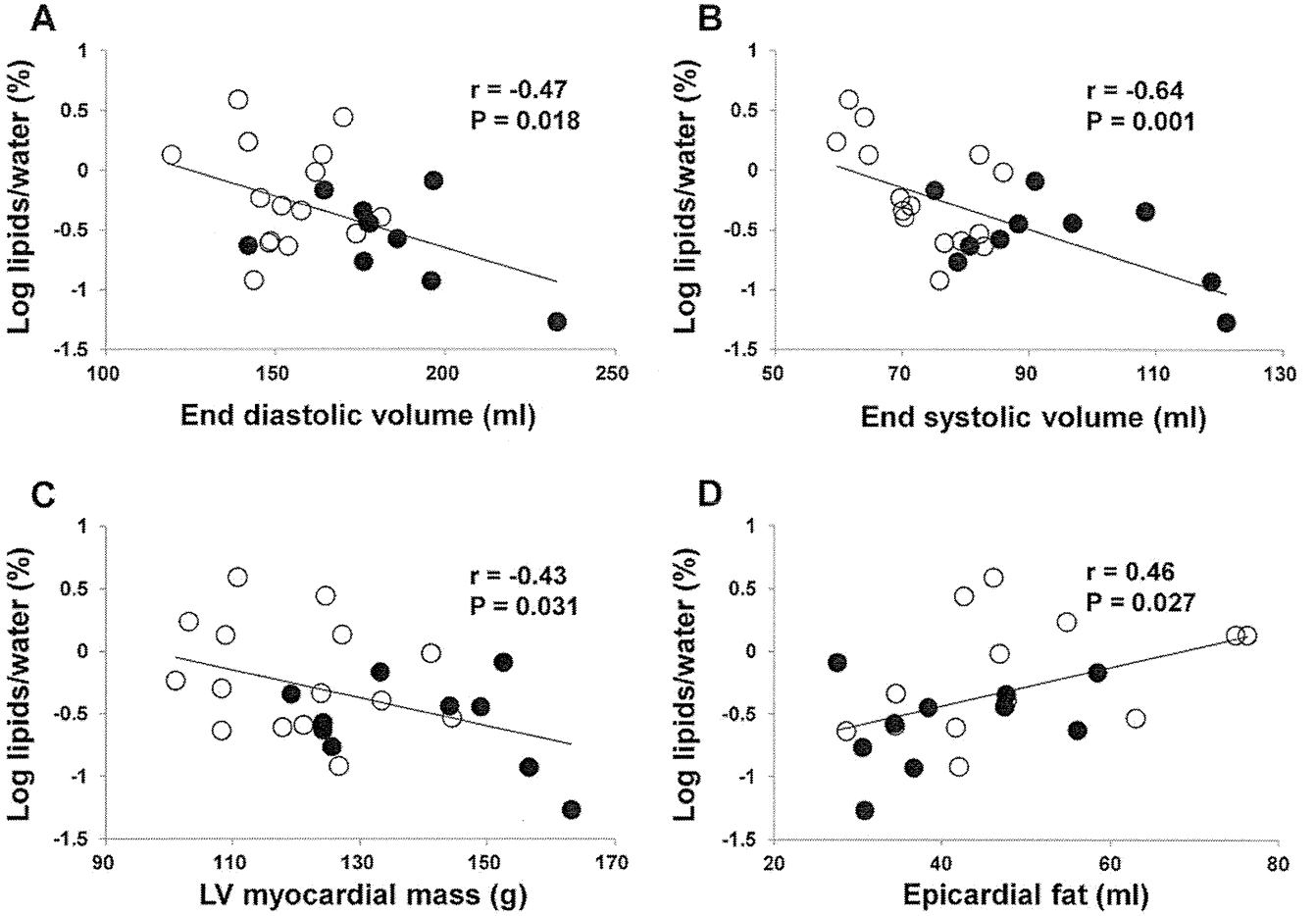

Figure 3. Correlations between myocardial TG content and MRI parameters. A: A correlation between myocardial TG content and end-diastolic volume. B: A correlation between myocardial TG content and end-systolic volume. C: Correlation between myocardial TG content and left ventricular (LV) mass. D: Correlation between myocardial TG content and epicardial fat volume. Open circle; control group. Closed circle; athlete group.
doi:10.1371/journal.pone.0061604.g003

Accordingly, we report a significant positive correlation between epicardial fat volume and myocardial TG content. It has reported that the metabolic rates of lipolysis and lipogenesis are 2-fold higher in epicardial fat than in other fat deposits. Indeed, we detected a negative correlation between epicardial fat volume and EDV, a LV morphology parameter. The precise mechanism underlying the low myocardial TG content in endurance athletes remains elusive. However, the significant positive correlation between epicardial fat volume and myocardial TG content may be related to the increase of utilizing fatty acid in endurance athletes. In our next step, we plan to clarify the impact of exercise on myocardial TG content and LV alterations in endurance athletes.

Limitations

The present study has several limitations. First, this was a single center study with a small sample size, studies of larger sample size are required to confirm these findings. Second, this study included only male subjects. Third, a previous study has demonstrated that a negative relationship between myocardial TG content and cardiopulmonary fitness in obese women [26]. This correlation between myocardial TG content and VO_{2max} was not found in our study. This discrepancy may have resulted from the difference between the subjects in these studies, as in the present study, all subjects of the present study were healthy males without metabolic disorders. Finally, athlete's heart is considered to be reversible [42], therefore, we will next evaluate the effect of detraining on myocardial TG content.

Conclusions

Low levels of myocardial TG content were observed in endurance athletes and were associated with the morphology of physiological LV alteration. These data suggest that metabolic imaging for measurement of myocardial TG content by ^{1}H-MRS may be a useful technique for noninvasively assessing the "athlete's heart".

Acknowledgments

The authors thank Tomo Onishi (Teikyo University Faculty of Medical Technology, Tokyo, Japan), Nozomi Hamasaki (Department of Radiology, Juntendo University Hospital, Tokyo, Japan), Dr. Kosuke Fukao, Dr. Kuniaki Hirose, Dr. Ryoko Ichikawa, Dr. Masaki Maruyama (Juntendo University Department of Cardiovascular Medicine Juntendo University Hospital, Tokyo, Japan), and Meng-kyu Kim (Kyungpook National University, Daegu, South Korea) for their great support in clinical measurements.

Author Contributions

Interpreted results of experiments: ES KS YT SA HW RK HD. Prepared figures: ES KS TM. Approved final version of manuscript: ES KS TY SS TM MH YT SA HW RK HD. Conceived and designed the experiments: ES KS TY. Performed the experiments: ES TY SS TM MH. Analyzed the data: ES KS. Contributed reagents/materials/analysis tools: ES KS TY. Wrote the paper: ES KS TY.

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Diabetes Research and Clinical Practice

journal homepage: www.elsevier.com/locate/diabres

International Diabetes Federation

High levels of very long-chain saturated fatty acid in erythrocytes correlates with atherogenic lipoprotein profiles in subjects with metabolic syndrome

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ARTICLE INFO

Article history:
Received 12 June 2012
Received in revised form
29 September 2012
Accepted 22 October 2012
Published on line 10 November 2012

Keywords:
Atherogenic lipoproteins
Inflammation
Metabolic syndrome
Peroxisomal dysfunction
Saturated very long-chain fatty acid

ABSTRACT

Aim: Very long chain saturated fatty acid (VLCFA) levels in erythrocytes are associated with metabolic syndrome (MS). However, the relationship between levels of the VLCFA ligonoceric acid (C24:0) in erythrocytes and the atherogenic lipoprotein profiles and inflammatory state in MS remain unclear.

Methods: Based on the International Diabetes Federation (IDF) definition of MS, 195 apparently healthy males were assigned to either an MS group ($n = 38$) or a non-MS group ($n = 157$). Fatty acid composition of erythrocytes was determined by gas liquid chromatography.

Results: Erythrocytes from the MS group had a significantly higher level of C24:0 than cells from the non-MS group ($4.06 \pm 0.48\%$ versus $3.88 \pm 0.34\%$; $p = 0.03$). C24:0 levels were significantly correlated with several components of MS. The C24:0 levels showed a significant negative correlation with LDL and HDL particle size. Multivariate linear regression analysis showed that C24:0 levels were independently correlated with LDL particle size after adjusting for age and each MS criterion. C24:0 levels were also positively correlated with log-transformed high-sensitivity CRP levels ($p = 0.04$).

Conclusion: C24:0 levels in erythrocytes are associated with specific atherogenic lipoprotein profiles and inflammation status in subjects with MS.

1. Introduction

Metabolic syndrome (MS) is a constellation of metabolic risk factors that includes increased waist circumference, atherogenic dyslipidemia, elevated blood pressure, and elevated blood glucose associated with insulin resistance [1,2]. Several meta-analyses have shown that MS is associated with an approximately 2-fold increased risk of cardiovascular disease [3–5]. One of the characteristic phenotypes of MS is the accumulation of fat in adipose tissue and release of free fatty acids (FFAs) into the circulation. An excessive influx of FFAs

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0168-8227/$ – see front matter © 2012 Elsevier Ireland Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.diabres.2012.10.025

into muscles and the liver leads to insulin resistance. Several studies have suggested a relationship between plasma fatty acid composition and each of the MS components, including insulin resistance, glucose intolerance, hypertension, and serum lipoprotein disorders [6,7].

Although saturated very long chain fatty acids (VLCFAs) are minor fatty acid components in human tissues and the bloodstream, associations between C26:0 levels, a VLCFA, in erythrocytes and risks of cardiovascular diseases have been observed [8]. We have also reported that absolute C26:0 levels in whole blood were significantly associated with several features of MS [9]. However, levels of C26:0 in the circulation are so low that it is relatively complicated to measure C26:0 levels in clinical settings. Therefore, we confirmed the association between MS and the levels of another saturated VLCFA, lignoceric acid (C24:0), which were measured by a simple established method [10].

Few studies have investigated possible correlations between saturated VLCFA levels and precise atherogenic lipoprotein profiles and systemic inflammatory states, both of which are important MS atherogenic features. Here, we found that a high level of C24:0, but not other fatty acids, in erythrocytes was significantly correlated with small LDL and HDL particles, which are specific components of atherogenic lipoprotein profiles, and high levels of high-sensitivity C-reactive protein (hs-CRP), which indicates systemic inflammation. These results suggest that measuring the level of C24:0 VLCFA in erythrocytes may be a useful marker to evaluate MS atherogenicity.

2. Materials and methods

2.1. Study subjects

We studied 195 consecutive and apparently healthy male subjects who underwent a medical check-up at the Nagasu-Kashiwado Clinic from December 2004 to January 2005. All subjects gave informed consent and the study was approved by the local ethical committee. We excluded patients who were receiving any medicines for diabetes mellitus or dyslipidemias and subjects with high levels of hs-CRP (more than 1 mg/l). Blood pressure (BP) was measured with a standard mercury sphygmomanometer after the subjects had rested for more than 5 min. The mean of two measurements of systolic and diastolic BP while sitting was used. Height and weight were measured using an automated scale, and body mass index was calculated as the weight in kilograms divided by the square of height in meters. Waist circumference was determined by measurements around the umbilical area while standing straight and after expiration.

Study subjects were divided into an MS group and a non-MS group according to the International Diabetes Federation (IDF) definition of MS [2]. Briefly, subjects with MS were defined as having a waist circumference of ≥85 cm plus two or more of the following factors: (1) elevated concentration of triglycerides (TG > 150 mg/dl) or specific treatment for this lipid abnormality; (2) reduced concentration of high density lipoprotein cholesterol (HDL-C < 40 mg/dl) or specific treatment for this lipid abnormality; (3) elevated BP: systolic BP > 130 mmHg or diastolic BP > 85 mmHg or treatment for previously diagnosed hypertension; and (4) elevated fasting plasma glucose (FPG concentration > 100 mg/dl) or previously diagnosed type 2 diabetes.

2.2. Blood sampling

Whole blood samples were drawn after overnight fasting. Serum levels of total cholesterol (TC), TG, and HDL-C were measured by standard enzymatic methods (Kainos, Tokyo, Japan) and low-density lipoprotein cholesterol (LDL-C) values were calculated using the Friedewald formula [11]. Plasma glucose concentrations were determined by the glucose oxidase method (Kainos, Tokyo, Japan) and serum insulin levels were measured according to a double antibody technique (Dainabot, Tokyo, Japan). HbA1c (%) was measured with previously standardized Japanese HbA1c and measurement methods (NGSP). The homeostasis model assessment for insulin resistance (HOMA-IR) was calculated using the following formula: fasting glucose (mmol/l) × fasting insulin (mU/l)/22.5, which was rearranged from the formula originally proposed by Matthews et al. [12].

2.3. Measurement of fatty acid composition in erythrocytes

Total lipids were extracted from erythrocytes by the method of Folch, and fatty acids were directly transmethylated with 14% boron trifluoride methanol solution (Sigma–Aldrich Japan, Tokyo, Japan) at 90 °C for 90 min. Fatty acids were measured using a GC-FID system (6890 N; Agilent Technologies, Tokyo, Japan) equipped with a fused silica capillary column (Omegamax 250; 30 m × 0.25 mm i.d.; 0.25 μm film thickness; Supelco, USA) using tricosanoic acid (C23:0) methyl ester as an internal standard. The injector and detector temperatures were both set at 270 °C and the column temperature was held at 205 °C. Helium was used as the carrier gas at a flow rate of 2.0 ml/min with a split ratio of 50:1 [10].

2.4. Measurement of lipoprotein profiles

Average LDL and HDL particle diameters (nm) were obtained from LDL and HDL peak times with a dual detector, high performance liquid chromatography (HPLC) system with two tandem-connected TSKgel LipopropakXL columns (300 mm × 7.8 mm; Tosoh, Japan) from Skylight Biotech, Inc. (Akita, Japan), as previously described [13,14].

2.5. Statistical analysis

Continuous variables were expressed as mean ± SD and categorical variables were reported as percentages. Statistical differences between the groups were analyzed by Welch's test and chi-square tests. Levene's test was used to assess the equality of variances in different samples. Correlations between two variables were determined by simple linear regression analysis. Multiple linear regression analysis was used to determine the associations between erythrocyte C24:0

levels and LDL particle sizes, HDL particle size or hs-CRP levels independently related to the MS components. Statistical analysis used StatView software (Version 5.0 for Windows, SAS Institute, Cary, NC). p-Values < 0.05 were considered statistically significant.

3. Results

3.1. Characteristics of the study subjects

The characteristics of the subjects in the present study are shown in Table 1. The two groups were not significantly different in terms of age. Compared with the non-MS group, the MS group had significantly higher body mass index (BMI) ($p < 0.001$), waist circumference ($p < 0.001$), systolic BP ($p < 0.001$), diastolic BP ($p < 0.001$), and mean BP ($p < 0.001$). In the MS group, plasma TG levels were significantly increased ($p = 0.005$) and HDL-C levels were significantly decreased ($p < 0.001$) compared with the non-MS group. Plasma TC and LDL-C levels were not significantly different between the two groups. In the MS group, FPG ($p < 0.001$), insulin ($p < 0.001$), HbA1c ($p = 0.01$), HOMA-IR ($p < 0.001$), and hs-CRP ($p = 0.03$) were significantly increased compared with the non-MS group.

3.2. Comparison of erythrocyte fatty acid composition in the MS and non-MS groups

Table 2 shows the fatty acid composition of erythrocytes. In the MS group, erythrocyte levels of C18:0 (stearic acid), and C24:0 (lignoceric acid) were significantly higher than in the non-MS group ($17.6 \pm 1.4\%$ versus $17.2 \pm 1.0\%$, $p = 0.04$; and $4.06 \pm 0.48\%$ versus $3.88 \pm 0.34\%$, $p = 0.03$, respectively). Conversely, MS group erythrocytes had significantly lower levels of C18:1n-7 (vaccenic acid) than erythrocytes from the non-MS group ($1.30 \pm 0.16\%$ versus $1.38 \pm 0.15\%$, $p = 0.005$).

3.3. Correlations between C24:0 and MS risk factors

The correlations between erythrocyte C24:0 levels and the components of MS are shown in Table 3. C24:0 levels were positively correlated with BMI ($r = 0.227$, $p < 0.001$) and systolic BP ($r = 0.158$, $p = 0.03$), plasma LDL-C ($r = 0.167$, $p = 0.02$), and TG ($r = 0.176$, $p = 0.01$). C24:0 levels were negatively correlated with, HDL-C ($r = -0.186$, $p = 0.009$). There was no significant correlation between C24:0 levels and age, plasma TC, fasting plasma glucose, fasting insulin levels, or HOMA-IR.

3.4. Correlation of C24:0 with LDL and HDL particle size

We explored the correlation between erythrocyte C24:0 levels and LDL and HDL particle size (Fig. 1). C24:0 levels were inversely correlated with both LDL and HDL particle diameter. The levels of other fatty acids however, were not significantly correlated with LDL and HDL particle size. After adjusting for each MS criterion (waist circumference, systolic BP, FPG, TG, and HDL-C) and age, C24:0 levels were still independent variables associated with LDL particle size ($p = 0.04$), but not HDL particle size ($p = 0.12$).

3.5. Correlation between C24:0 and hs-CRP

Fig. 2 shows that the level of C24:0 in erythrocytes was significantly correlated with log-transformed hs-CRP levels ($r = 0.15$, $p = 0.04$). After adjusting for each MS criterion (waist circumference, systolic BP, FPG, TG, and HDL-C) and age, there was no significant association between log-transformed C24:0 levels and hs-CRP levels ($p = 0.36$).

4. Discussion

The level of C24:0 was significantly higher in erythrocytes from the MS group than the non-MS group, and C24:0 levels

Table 1 – Characteristics of study subjects.

	Non-MS	MS	F statics	p value
	n = 157	n = 38		
Age (years)	50.2 ± 9.9	50.1 ± 8.6	0.11	NS
Body mass index (kg/m^2)	23.2 ± 2.5	27.2 ± 3.3	0.29	<0.001
Waist circumference (cm)	83.4 ± 6.5	95.0 ± 6.3	0.32	<0.001
Systolic blood pressure (mmHg)	127 ± 15	139 ± 15	0.58	<0.001
Diastolic blood pressure (mmHg)	80 ± 10	87 ± 11	0.53	<0.001
Current smoker (%)	69 (44)	16 (43)		NS
Total cholesterol (mg/dl)	194 ± 35	190 ± 39	0.05	NS
Triglycerides (mg/dl)	111 ± 44	130 ± 32	0.33	0.005
HDL-cholesterol (mg/dl)	58 ± 12	47 ± 8	0.009	<0.001
LDL-cholesterol (mg/dl)	77 ± 18	82 ± 15	0.15	NS
Blood glucose (mg/dl)	99 ± 13	108 ± 14	0.46	<0.001
Insulin (mU/l)	4.6 ± 2.7	9.4 ± 6.7	<0.001	<0.001
HOMA-IR	1.2 ± 0.9	2.6 ± 2.1	<0.001	<0.001
HbA1c (NGSP)(%)	5.7 ± 0.4	6.0 ± 0.8	0.004	0.01
Hs-CRP (mg/l)	0.86 ± 1.41	1.66 ± 2.09	0.002	0.03

Values are mean ± SD. MS, metabolic syndrome; HDL, high density lipoprotein; LDL, low density lipoprotein; HOMA-IR, homeostasis model assessment for insulin resistance; Hs-CRP, high sensitive C reactive protein.

Table 2 – Proportion of fatty acid (%) in erythrocytes from MS and non-MS subjects.

	Non-MS $n = 157$	MS $n = 38$	F statics	p value
C14:0 (myristic acid)	0.21 ± 0.05	0.22 ± 0.06	0.04	NS
C16:0 (palmitic acid)	20.4 ± 1.3	20.9 ± 1.6	0.12	0.08
C16:1*n*-7 (palmitoleic acid)	0.44 ± 0.07	0.46 ± 0.08	0.70	NS
C18:0 (stearic acid)	17.2 ± 1.0	17.6 ± 1.4	0.004	0.04
C18:1*n*-9 (oleic acid)	12.4 ± 0.82	12.3 ± 0.79	0.80	NS
C18:1*n*-7 (vaccenic acid)	1.38 ± 0.15	1.30 ± 0.16	0.87	0.005
C18:2*n*-6 (linoleic acid)	8.30 ± 0.97	8.05 ± 1.06	0.57	NS
C18:3*n*-3 (α-linolenic acid)	0.13 ± 0.03	0.12 ± 0.03	0.86	NS
C20:0 (arachic acid)	0.35 ± 0.04	0.34 ± 0.04	0.78	NS
C20:1	0.42 ± 0.09	0.43 ± 0.10	0.28	NS
C20:3*n*-6 (dihomo-γlinolenic acid)	1.16 ± 0.18	1.22 ± 0.17	0.80	NS
C20:4*n*-6 (arachidonic acid)	10.9 ± 1.6	10.5 ± 1.8	0.44	NS
C20:5*n*-3 (eicosapentaenotic acid)	1.66 ± 0.77	1.57 ± 0.74	0.78	NS
C22:0 (behenic acid)	1.22 ± 0.15	1.25 ± 0.16	0.58	NS
C22:1	0.13 ± 0.15	0.11 ± 0.12	0.63	NS
C22:4*n*-6	1.56 ± 0.41	1.53 ± 0.51	0.03	NS
C22:5*n*-6 (*n*-6 docosapentaenoic acid)	0.28 ± 0.11	0.26 ± 0.07	0.71	NS
C22:5*n*-3 (*n*-3 docosapentaenoic acid)	2.27 ± 0.38	2.16 ± 0.42	0.69	NS
C22:6*n*-3 (docosahexaenoic acid)	7.01 ± 1.43	6.84 ± 1.78	0.23	NS
C24:0 (lignoceric acid)	3.88 ± 0.34	4.06 ± 0.48	0.02	0.03
C24:1*n*-9 (nervonic acid)	4.17 ± 0.39	4.19 ± 0.44	0.37	NS

Values are mean ± SD.

were significantly associated with several components of MS. Additionally, we found that the increased level of C24:0, but not other fatty acids, in erythrocytes was significantly correlated with small LDL and HDL particle size, which are specific components of atherogenic lipoprotein profiles. Increased C24:0 levels were also positively correlated with systemic inflammation as indicated by hs-CRP levels.

Fatty acid beta-oxidation occurs in both mitochondria and peroxisomes. Long chain fatty acids (C16–C20) are primarily oxidized in mitochondria, whereas peroxisomes are involved in the beta-oxidation of VLCFAs (>C20) [15]. Peroxisomal dysfunction gives rise to an over-accumulation of VLCFAs in the body as a whole [16]. VLCFAs accumulate in the plasma, membranes of erythrocytes, and/or tissues of patients with inherited peroxisomal diseases, which are characterized by progressive demyelination and adrenal insufficiency [17–19]. X-adrenoleukodystrophy (X-ALD), the most common peroxisomal disorder, is associated with increased levels of saturated VLCFAs (>C22:0) [16]. Treatment with the potent and selective histone deacetylase inhibitor normalized the levels of VLCFAs in skin fibroblasts from X-ALD patients by increasing the peroxisomal C24:0 beta-oxidation activity [20]. Peroxisomal dysfunction plays an important role in aging-related diseases [21], and, furthermore, a recent report suggested that peroxisome-related alterations and increased VLCFAs may contribute to the progression of Alzheimer's disease [22].

Table 3 – Correlations of the proportion of C24:0 with risk factors of metabolic syndrome.

	r	t-Score	p-Value
Age	0.084	1.17	NS
Body mass index	0.227	3.24	0.001
Waist circumference	0.258	3.71	<0.001
Systolic blood pressure	0.158	2.23	0.03
Total cholesterol	0.139	1.95	NS
HDL-cholesterol	−0.186	−2.62	0.009
LDL-cholesterol	0.167	2.31	0.02
Triglycerides	0.176	2.49	0.01
Fasting plasma glucose	0.066	0.92	NS
Fasting serum insulin	0.125	1.75	NS
HOMA-IR	0.099	1.38	NS

HDL, high density lipoprotein, LDL, low density lipoprotein, HOMA-IR, homeostasis model assessment for insulin resistance.

The expression of enzymes involved in fatty acid synthesis and elongation may contribute to the accumulation of VLCFAs. In particular, ELOVL1 and ELOVL3 have chain length specificity toward VLCFA [23,24], and silencing of ELOVL1 reduces elongation of C22:0–C26:0 and lowers C26:0 levels in X-ALD fibroblasts [23]. ELOVL3 mediates the elongation of C22:0–C24:0 and C24:0–C26:0 in vivo [24]. It has been reported that ELOVL3 expression regulates diet-induced obesity, hepatic lipogenic gene expression, and hepatic TG content [25]. Several studies, including our own, have demonstrated the association of saturated VLCFA levels with the risks of cardiometabolic syndrome [8,9]. In this study, the accumulation of C24:0 was also associated with MS. Taken together, MS may be associated with an imbalance between the synthesis and metabolism of saturated VLCFAs.

LDL and HDL particles associated with MS tend to be small and dense [26]. Smaller LDL particles are more atherogenic than larger LDL as they may filter more readily into the arterial wall and are more prone to atherogenic modifications [27]. Small, dense HDL sub-fractions are increased in MS and are associated with elevated oxidative stress and insulin resistance [28]. However, the association between various fatty acid