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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".

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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).

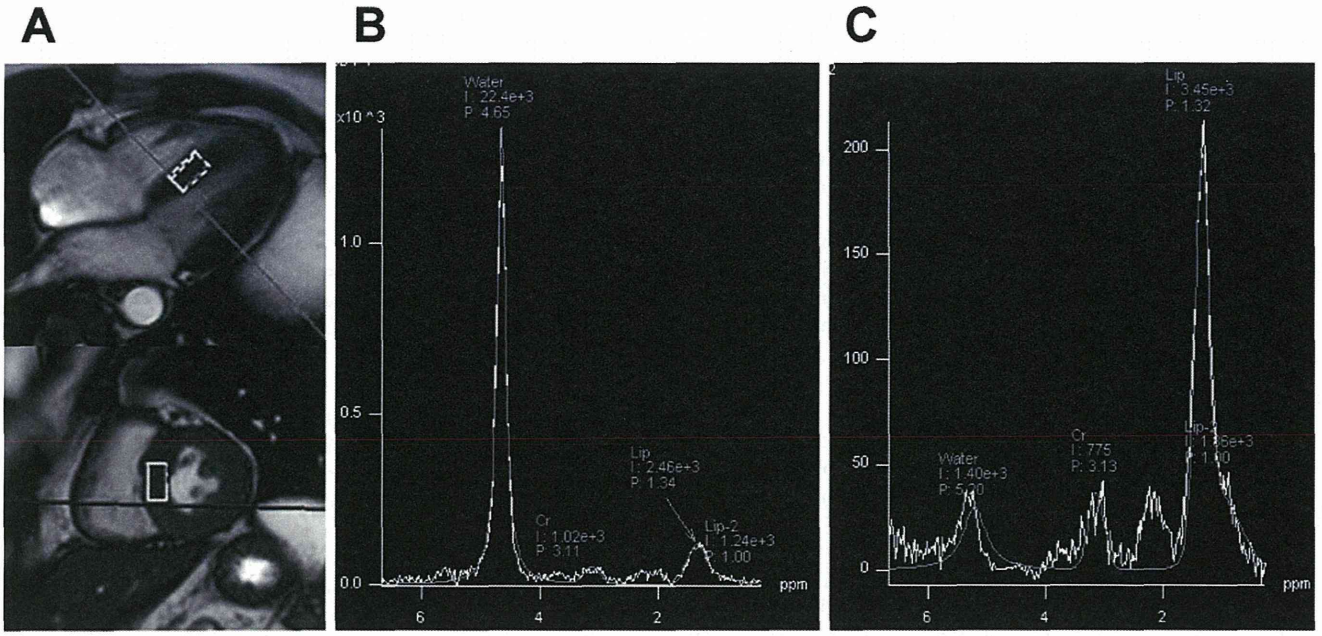

Figure 1. Representative results of H^1-MR spectra in a healthy subject. A: Myocardial voxel localization for H^1-MRS in 4-chamber and short axis views. B: H^1-MR spectra without water suppression. C: H^1-MR spectra without water suppression.
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Myocardial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

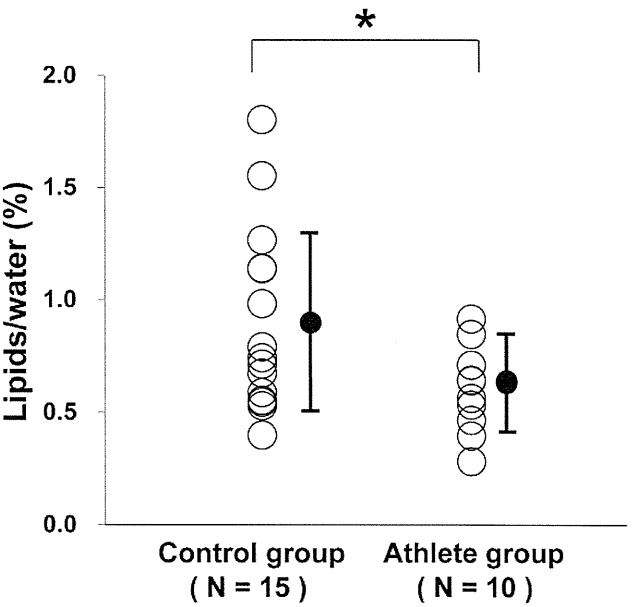

Figure 2. Comparison between myocardial TG content in the control group and the athlete group. * $P<0.05$ between the two groups.
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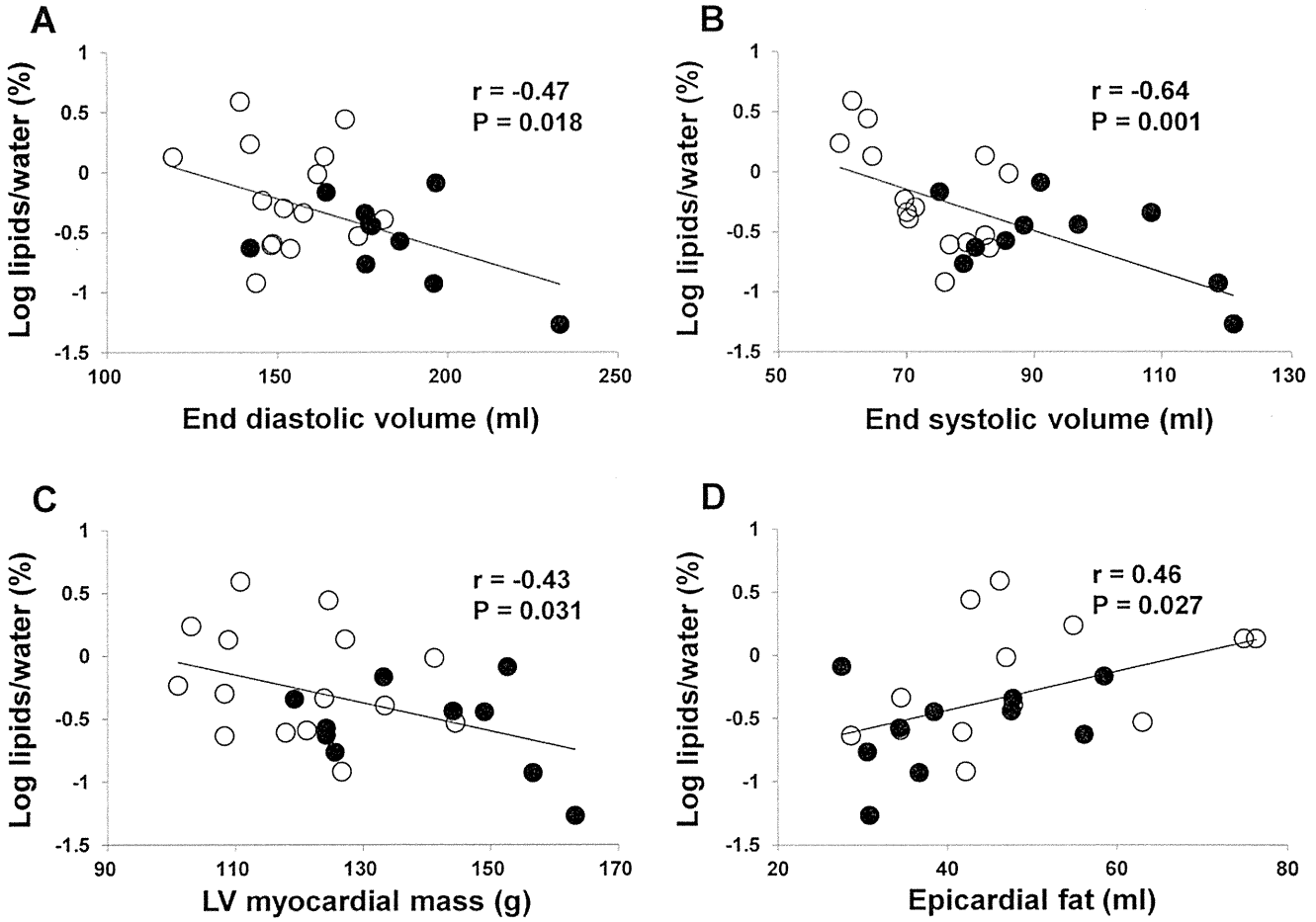

Figure 3. Correlations between myocardial TG content and MRI parameters. A: A correlation between myocardial TG content and end-diastolic volume. B: A correlation between myocardial TG content and end-systolic volume. C: Correlation between myocardial TG content and left ventricular (LV) mass. D: Correlation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

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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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Regular Article

Patient Factors against Stable Control of Warfarin Therapy for Japanese Non-valvular Atrial Fibrillation Patients

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ABSTRACT

Introduction: Effectiveness and safety of warfarin therapy for non-valvular atrial fibrillation (NVAF) patients are strongly associated with its stability presented such as time in therapeutic range (TTR) of PT-INR. However, the factors that affect TTR have not been fully elucidated in Japan where majority of patients are controlled within the range of 1.6-2.6 of PT-INR irrespective of the age.
Methods: We retrospectively analyzed 163 NVAF patients taking warfarin to determine the factors that affect TTR including metabolic enzymes polymorphisms after TTR calculation with both the standard PT-INR range and the actual control range of 1.6-2.6.
Results: Overall TTR calculated using Japanese Guideline was 69.7 ± 25.1% (<70 and ≥70 years; 49.6 ± 24.8% and 77.8 ± 20.3%, respectively). After confirming that PT-INR values in patients <70 years distributed in the same range as in those ≥70 years, as in a Japanese large cohort, we recalculated TTR of those <70 years with 1.6-2.6 of PT-INR and found that it was 79.5 ± 20.1%. Poor control of this new TTR were significantly associated with the lower height, the higher serum creatinine, the lower creatinine clearance, female gender, and presence of congestive heart failure, ($p < 0.05$ respectively). Multivariate analysis revealed female gender and presence of congestive heart failure as independent predictor of the lower TTR ($p < 0.05$, $p < 0.01$, respectively). Polymorphism of CYP2C9 and VKORC1 were related to the dosage of warfarin but not determinant of TTR.
Conclusions: When evaluated using a range of PT-INR actually used in Japan, TTR is generally well controlled and female gender and presence of congestive heart failure significantly affected the poorer TTR control.

Introduction

Atrial fibrillation (AF) is a common and rapidly increasing arrhythmia in the current high-aged society. AF is highly associated with substantial morbidity and mortality particularly due to cerebral thromboembolism [1]. Though novel anti-coagulating agents have been recently developed especially for those with non-valvular AF (NVAF), warfarin still remains the most common and important anticoagulants used to reduce the risk of stroke in AF patients. The meta-analysis by Hart and co-workers reported that adjusted-dose warfarin reduced the risk of stroke by 64% (49%–74%) in NVAF patients [2]. Though the pharmacokinetics of warfarin is profoundly affected by many factors, it has not been fully understood what affect the actual control of international normalized ratio of prothrombin time (PT-INR) substantially. Recently, it has been known that the outcome of NVAF patients on warfarin depends on the length of the adequate PT-INR control presented as time in therapeutic range (TTR) [3,4]. It is reported that if the control of TTR value is <40%, the risk of stroke will increase even more than without using warfarin [4]. As many factors are suspected to affect TTR control but not determined yet, and to elucidate these factors should be of great importance, we decided to examine the relationship between the various factors and TTR among Japanese population. Particularly, as previous study by Okumura and co-workers reported that the dosage of warfarin significantly related to TTR value [5], we also investigated the relationship between warfarin dosage and polymorphism of

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metabolic enzymes (CYP2C9, CYP2C19, CYP4F2,and VKORC1), then we investigated the relationship between TTR and polymorphism of metabolic enzymes to evaluate final influence of these polymorphisms [6–8].

After PT-INR range of 2.0-3.0 has been established as an appropriate therapeutic range according to the results of stroke prevention trials in AF [9] and cohort studies in Europe and North America [10], ACC/AHA/ESC guidelines recommend this PT-INR range for anticoagulant therapy with warfarin in NVAF patients. In Japan, as Yasaka et al found that the major ischemic or hemorrhagic events occurred in elderly Japanese NVAF patients when they were controlled out of 1.6-2.6 of PT-INR range, this PT-INR range has been generally considered as optimal for elderly patients ≥70 years to prevent such events [11] and accordingly it was documented in the Japanese Guidelines for Pharmacotherapy of Atrial Fibrillation [12]. Previous study from Japan reported that the control of TTR is good in the patients ≥70 years but poor in those <70 years [5,13]. This fact seems to have derived from the fact that most Japanese cardiologists tend to keep PT-INR within 1.6-2.6 even in those <70 years. The result a recent large Japanese cohort study backed up this notion [13]. Therefore, it seems particularly important to know the actual range of PT-INR of the controlling doctors to elucidate the real influence of each potential factor on TTR in NVAF patients.

Methods

From July 2011 to March 2012, we collected data of 163 NVAF outpatients ≥40 years undergoing warfarin therapy for ≥6 months, who had been followed up at four institutes and retrospectively analyzed the relationship between TTR and the candidate factors including age, height, body weight, gender, serum creatinine, creatinine clearance (CCr; Cockcroft-Gault method), serum albumin, components of CHADS2 score (congestive heart failure, hypertension, age >75 years, diabetes mellitus, and prior stroke/transient ischemic attack), dosage of warfarin at enrollment, co-administration of antiplatelet drug. Patient was eligible only after confirming that at least 2 points were kept within appropriate PT-INR range in order to exclude those within unstable initiation period of warfarin therapy.

All the attending physician were familiar to the Japanese Guidelines for Pharmacotherapy of Atrial Fibrillation (JCS2008); that is, between 2.0 and 3.0 for patients <70 years and between 1.6 and 2.6 for those ≥70 [12]. However, the actual dosage of warfarin was determined by the discretion of each physician. Administration of warfarin in patients with low CHADS2 score [14] of 0 or 1was left to the decision of the attending physician.

TTR was determined using the method of linear interpolation between consecutive PT-INR values in each patient reported by Rosendaal method [3,4,15] We also investigated the relationship between warfarin dosage and polymorphism of metabolic enzymes (CYP2C9, CYP2C19, CYP4F2,and VKORC1), then we investigated the relationship between TTR and polymorphism of metabolic enzymes to evaluate the final influence of these polymorphisms [6–8]. Genetic variation analysis was carried out according to the previous report [8]. In brief, genomic DNA was extracted from whole blood. The VKORC1-1639 G > A, CYP2C9*2, CYP2C9*3, CYP2C19*2, CYP2C19*3 and CYP4F2-1347 C > T polymorphisms were determined using the PCR–restriction fragment length polymorphism method. Nine patients were dropped out because of informed consent or technical error, and then we examined 154 patients in genetic variability analysis.

All data are expressed as mean ± standard deviation. For comparison of TTR between 2 groups Mann Whitney test was used. For comparison of TTR among 3 or more groups, 1-way analysis of variance (Kruskal-Wallis test) was used followed by post-hoc Dunns test. For correlation of TTR with the candidate factors, we performed univariate-analyses using Spearman rank correlation test, then multiple stepwise regression analysis using JMP Version 9 was performed to detect the independent predictors of TTR. In order to avoid inclusion of factors that are strongly mutually correlated, we excluded the factors such as creatinine clearance, age >75 years which are components of CHADS2 score, from the multivariate analysis. Thus, height, body weight, gender, creatinine, congestive heart failure, and hypertension, which p values were within 0.1 by univariate-analyses, were used in multiple stepwise regression analysis (backward elimination method). $P < 0.05$ was considered significant.

The study protocol was approved by the ethics committee of each institution and written informed consent was given by each patient.

Results

Patients' Clinical Characteristics

There were 101 male and 62 female patients, and their mean age was 74.4 ± 8.8 years, ranging from 49 to 93. Mean warfarin dose administered at the enrollment was 2.9 ± 1.2 mg/day, ranging from 1.0 to 10 mg/day. Antiplatelet drug were co-administrated in 25 patients (15.3%) (Table 1).

Status of PT-INR Control and Influential Factors

Averaged TTR value under current Japanese Guideline was 69.7 ± 25.1%, (49.6 ± 24.8% and 77.8 ± 20.3%, <70 and ≥70 years, respectively, $p < 0.0001$) (Fig. 1A,B). We analyzed the factor that influenced TTR value and found that age was only and strongly associated with TTR value ($p < 0.0001$). However, we noticed that PT-INR in patients <70 years distributed within the same range and with a same shape as those in patient ≥70 years (Fig. 2) as was suggested by a large Japanese cohort study [13]. The average of PT-INR values in patients <70 years and ≥70 years were 2.1 ± 0.6, and 2.0 ± 0.6, respectively. As shown in Fig. 1C, TTR distributed in the very high range even in patients <70 years when TTR was calculated using the "actual" PT-INR

Table 1
Characteristics of the patients.

Total n (%)	163
Age	74.4 ± 8.8
<70 years	47(28.8%)
≥70 years	116(71.2%)
Gender Male	101(62%)
Female	62(38%)
Body weight (kg)	58.8 ± 11.2
Creatinine (umol/L)	88.4 ± 35.4
Serum albumin (mg/L)	42 ± 3
Creatinine clearance (ml/min)	58. ± 24.6
CHADS2 score	
0	26(15.9%)
1	61(37.4%)
2	41(25.2%)
3	24(14.7%)
4	8(4.9%)
5	3(1.8%)
6	0(0%)
Congestive heart failure	13(8.0%)
Hypertension	78(47.9%)
Age (≥75 years old)	89(54.6%)
Diabetes mellitus	44(27.0%)
Stroke	22(13.5%)
TTR[§](guideline)	69.7 ± 25.1%
<70 years old	49.6 ± 24.8%
≥70 years old	77.8 ± 20.3%
TTR (1.6-2.6)	79.5 ± 20.1%
Warfarin dosage (mg)	2.9 ± 1.2
Co-administration of antiplatelet agent	25(15.3%)

Data are expressed as means ± SD.
§ TTR: time in therapeutic range.

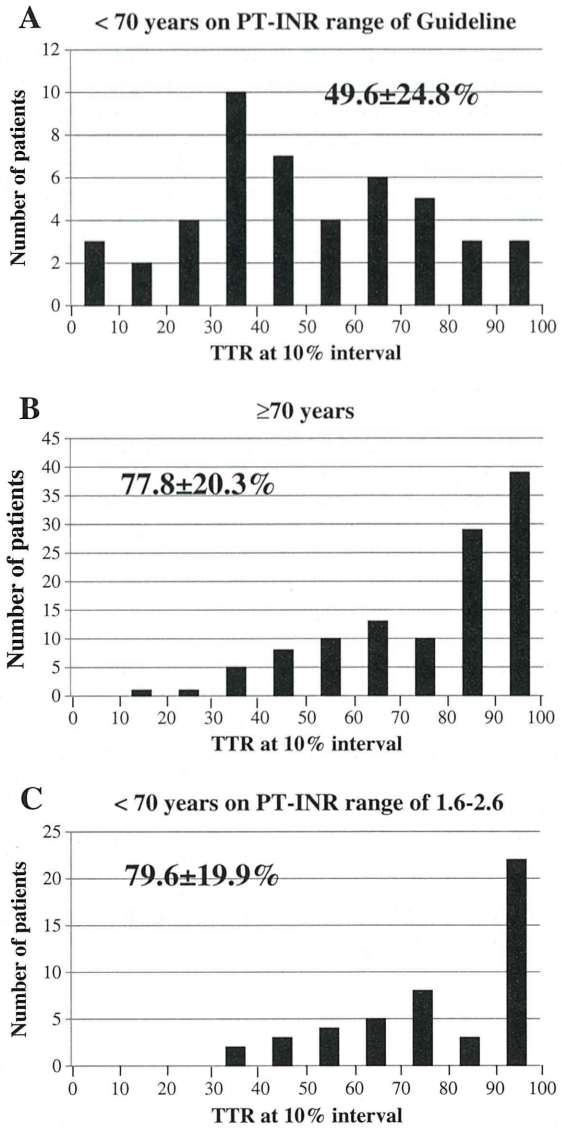

Fig. 1. TTR Distribution. Distribution of number of patients with time in the therapeutic range (TTR) from 0 to 100% at every 10% for patients aged <70 years on Japanese guideline (A), for patients aged ≥70 years (B), and for patients aged <70 years on INR range 1.6-2.6 (C). Data are expressed as means ± SD.

range of 1.6-2.6 even for these patients <70 years. Therefore, we judged that most of the participating cardiologists unconsciously tried to keep PT-INR values within 1.6-2.6 irrespective of the age of their patients. Hence, we reevaluated the association of TTR under this new range of PT-INR irrespective of age of the patients to more properly elucidate the patient's factor(s), which actually regulates the variation of TTR. TTR under 1.6-2.6 of PT-INR range in the patients <70 years was 79.5 ± 20.1% with this method. Univariate analysis using this new calculation revealed that lower height($R^2 = 0.026$, $p = 0.039$), female gender (male 82.1 ± 19.0 vs. female 75.2 ± 21.3, $p = 0.0275$), the higher serum creatinine ($R^2 = 0.03$, $p = 0.026$), the lower CCr($R^2 = 0.027$, $p = 0.036$), and presence of congestive heart failure ($p = 0.0018$), were significantly associated with the lower TTR (Table 2). However, factors such as age, body weight, serum albumin, dosage of warfarin, were not associated with TTR (Table 2). Further multivariate analysis revealed that female gender and presence of congestive heart failure were the independent predictors of poor control of TTR($p = 0.045$, $p = 0.0003$, respectively) (Table 3).

The Genotype Analysis of Metabolizing Enzymes

The genotype frequency of CYP2C9 was 147 of 1*/1* (95.5%), 7 (4.5%) of 1*/3* and none of 3*/3*. The genotype frequency of VKORC1 was 130 (84.4%) of AA, 23 (14.9%) of GA, and 1 (0.6%) of GG. The genotype frequency of CYP4F2 was 85 95 (55.0%) of CC, 52 (33.8%) of CT, and 7 (11.0%) of TT. The genotype frequencies of CYP2C19 2* was 76 (49.4%) of CC, 50 (32.5%) of CT, and 28 (18.2%) of TT. The genotype frequencies of CYP2C19 3* was 117 (76.0%) of CC, 35 (22.7%) of CT, and 2 (1.3%) of TT (Fig. 3). Because the genetic variability in the patients under warfarin therapy plays an important role in determining the dosage of warfarin, we also evaluated the association between the genetic variability and warfarin dosage at the enrollment. About CYP2C9, compared with patients with 1*/1*, dosage of warfarin of the patients with 1*/3* were significantly lower (2.9 ± 1.2 vs. 2.1 ± 0.43 mg; $p < 0.05$). As for VKORC1, compared with patients with AA, dosage of warfarin of the patients with GA were significantly higher (4.0 ± 1.6 vs. 2.9 ± 1.0 mg; $p < 0.01$) (Fig. 3). The mean dosage of warfarin in CYP4F2 CC, CT, and TT are (2.7 ± 1.3, 2.9 ± 1.0, 3.4 ± 1.2, respectively, P = ns). The mean dosage of warfarin in CYP2C19*2 CC, CT, and TT are (2.9 ± 1.3, 2.8 ± 1.0, 2.9 ± 1.1, respectively, P = ns) and in CYP2C19*3 CC, CT, and TT are (2.8 ± 1.2, 2.9 ± 1.0, 3.0, respectively, P = ns).

We investigated the relationship between TTR and polymorphism of metabolic enzymes, CYP2C9 and VKORC1 because those might have influenced dosage of warfarin. However there is no significant association between TTR and those polymorphism of metabolic enzymes, CYP2C9 and VKORC1 (Table 2).

Discussion

Major Findings

The major findings in this study are 1) female gender and presence of congestive heart failure were the independent predictor of lower TTR when their relationship was analyzed under the assumption that participating cardiologists tried to keep PT-INR of 1.6-2.6 even if the patient was younger than 70 years. 2) Genetic variability of metabolizing enzymes plays an important role in determining the dosage of warfarin but variability of PT-INR (TTR) was not affected by the variability. Recently two articles reported the results of analysis about the factors that influenced TTR variation [5,17]. Okumura and co-workers reported that age and warfarin dosage were two independent predictors of TTR in Japanese NVAF patients [5]. In their study, TTR in patients <70 and ≥70 years was 46 ± 23% and 77 ± 17% [5], which are almost identical to our results. They concluded that TTR is strongly influenced by age presumably because Japanese physicians administered warfarin so as to attain the lower PT-INR value than what is recommended in the guideline due to an anxiety against the hemorrhage accident even knowing the recommendation in Japanese guideline: PT-INR control of 2.0-3.0 in patients <70 years and 1.6-2.6 ≥ 70 years. Because TTR value mainly depends on which PT-INR range was used for the calculation by definition, TTR in those <70 years becomes inevitably lower when actual PT-INR range is dissociated from the recommended PT-INR range. We found that PT-INR values in patients <70 years distributed in the same range in patient age ≥70 years in this study, which is the same result as in a recent large Japanese cohort, J-RHYTM study, about warfarin control for NVAF [13]. Furthermore, recently a report from Kotani et al. suggested that PT-INR level between 1.6 and 2.6 was permitted as a proper range irrespective of the patients' ages in Japanese population [16]. After confirming these points, we reevaluated the association of TTR using this actual range of PT-INR of 1.6-2.6 irrespective of age of the patients. Interestingly enough, this new calculation could elucidate that height, gender, serum creatinine, CCr, and presence of congestive heart failure were significantly associated with TTR, but age, body weight, hypertension, diabetes mellitus, prior stroke/transient ischemic attack, serum albumin, dosage

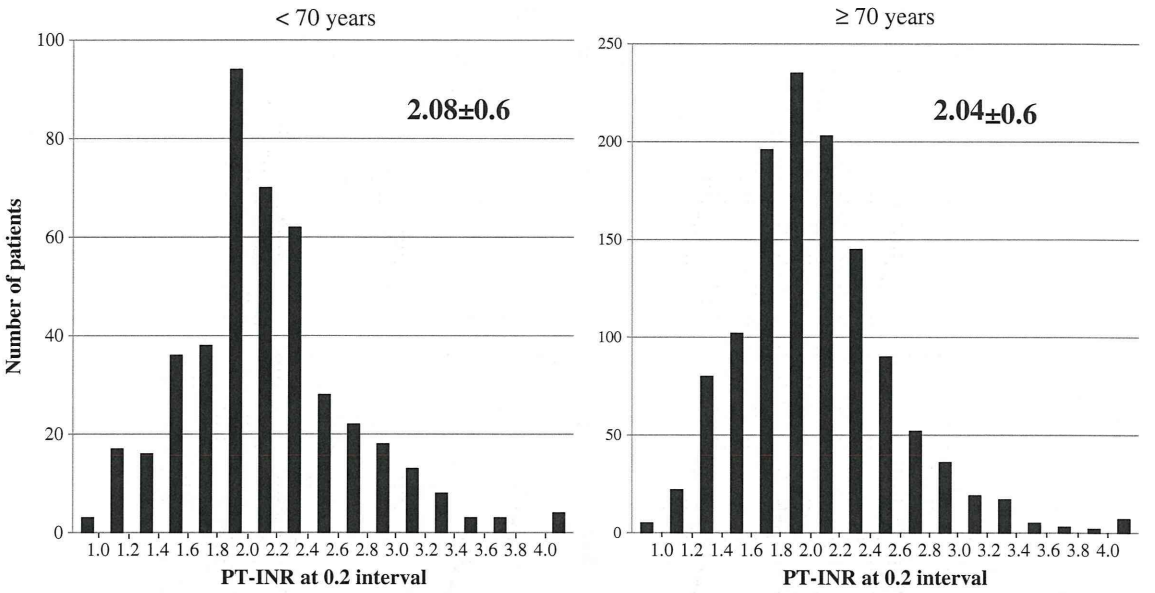

Fig. 2. PT-INR distribution in patients with < 70 and ≥ 70 years. Distribution of number of patients with PT-INR from < 1.0 to ≥ 4.0 at every 0.2 for patients aged < 70 and ≥ 70 years. Data are expressed as means ± SD.

of warfarin, genetic factors, and antiplatelet drug administration were not. Further multivariate analysis confirmed that gender and presence of congestive heart failure were the independent predictor of TTR. We consider that it was only possible to uncover the hidden relationships between the factors and TTR control by changing range of PT-INR control from that in guideline to actual one.

Sub-analysis of RE-LY trial recently reported that the degree of adherence, regardless whether it was intentional or not, to a simple warfarin dosing algorithm predicted improved TTR and accounted for considerable part of TTR variation between centers and countries rather than patient's factors [17]. This result might come from the nature of RE-LY trial that it is a large scale multi-country and multicenter trial and the enrolled patients might have been controlled more strictly than usual from the initiation period of anti-coagulation because it was well supervised study. In the article, the patient's factors such as higher age, female gender, smoking, congestive heart failure, diabetes mellitus, co-administration of amiodarone, and usage of insulin were associated with the lower TTR. These results are partially consistent with our results where multivariate analysis revealed female gender and presence of congestive heart failure, as independent predictors of TTR.

Although clinically available warfarin is a racemic mixture, (S)-warfarin is five times more potent as an anticoagulant than (R)-warfarin. (S)-warfarin is primarily metabolized into 7-hydroxylated form in humans, principally by cytochrome P450, subfamily IIC, polypeptide 9 (CYP2C9). So far, it is reported that CYP2C9 has two types of variants (CYP2C9*2 (R144C) and CYP2C9*3 (I359L)), though there is no report of CYP2C9*2 allele in the Asian people. Previous studies have demonstrated that the warfarin 7-hydroxylase activity of the CYP2C9*3 variant allele is approximately 1/10 of wild type of CYP2C9 (CPY2C9*1) and such carriers of the variant allele required a lower maintenance dose of warfarin due to the decreased metabolism [6]. In the present study, we also showed that the dosage of warfarin in the patients with CYP2C9 1*/3* were significantly lower compared with patients with 1*/1*, which coincided with previous reports [6–8]. However, we did not find any significant association between TTR and CYP2C9 genotypes. Warfarin exerts its anticoagulant effects by interfering with regeneration of vitamin K by reduction of its 2, 3-epoxide in the vitamin K cycle, leading to inhibition of gamma-carboxylation of vitamin K-dependent clotting factor II (prothrombin), VII, IX and X. This vitamin K epoxide reductase is encoded by vitamin K epoxide reductase complex subunit 1 (VKORC1). In this study we showed that patients with VKORC1 GA were administrated with significantly higher dosage of warfarin compared with patients with VKORC1 AA. These results also coincided with previous reports [6–8]. However, again, there is no significant association between TTR and VKORC1 genotypes. We concluded that genetic variability, such as CYP2C9 and VKORC1, among patients plays an important role in determining the dosage of warfarin but not variation of TTR after once it comes to a maintenance phase.

Table 2
Candidate factors related to the better TTR value (as of PT-INR range 1.6-2.6 irrespective of age).

	R square	P-value	number of patients
Height	0.026	0.039*	163
Body Weight		0.09	163
Age		0.39	163
Gender		0.0275*	163
Creatinine	0.03	0.026*	163
Creatinine clearance	0.027	0.0355*	163
Serum albumin		1.07	163
Component of CHADS2 score			
Congestive heart failure		0.0018*	163
Hypertension		0.12	163
Age		0.41	163
Diabetes Mellitus		0.41	163
Stroke		0.22	163
Warfarin dosage		0.51	163
CYP2C9 genotype variants		0.12	154
VKORC 1 genotype variants		0.12	154
Antiplatelet co-administration		0.67	163

Significant p value expressed * symbol.

Table 3
Multiple stepwise regression analysis of the variables that were related to TTR (as of PT-INR range 1.6-2.6 irrespective of age).

	β	SEM	t	P value
Congestive heart failure	10.17	2.77	3.66	0.0003*
Gender (female)	3.13	1.54	2.02	0.045*

Significant p values are expressed with asterisk.