**Table 2. Age-Adjusted Mean Values or Frequencies of Relevant Factors According to Brachial-Ankle Pulse Wave Velocity Quartiles in 1,577 Women**

| Variables | Brachial-ankle pulse wave velocity (cm/s) | | | | *p* for trend |
|---|---|---|---|---|---|
| | 900–1,269 (*n*=395) | 1,270–1,493 (*n*=392) | 1,494–1,821 (*n*=396) | 1,822–4,128 (*n*=394) | |
| Age (years) | 49.7±6.8 | 56.0±8.4 | 62.7±9.2 | 71.5±8.4 | <0.0001 |
| Heart rate (bpm) | 62.9±11.9 | 64.9±9.9 | 68.6±10.0 | 72.8±11.9 | <0.0001 |
| Systolic blood pressure (mmHg) | 107.5±17.9 | 121.7±15.8 | 135.2±15.9 | 150.5±19.9 | <0.0001 |
| Diastolic blood pressure (mmHg) | 63.9±11.9 | 73.5±9.9 | 80.2±10.0 | 87.4±11.9 | <0.0001 |
| Hypertension (%) | 3.2 | 16.9 | 50.2 | 85.5 | <0.0001 |
| Antihypertensive drugs (%) | 2.5 | 7.4 | 25.7 | 47.5 | <0.0001 |
| β-Blocker (%) | 0.2 | 1.4 | 2.6 | 6.0 | 0.0001 |
| Calcium channel blocker (%) | 1.9 | 6.1 | 20.5 | 38.1 | <0.0001 |
| ACE inhibitor (%) | 0.0 | 0.5 | 7.3 | 6.9 | <0.0001 |
| ARB (%) | 0.2 | 0.9 | 5.1 | 9.8 | <0.0001 |
| ECG abnormalities (%) | 3.0 | 8.4 | 10.3 | 30.9 | <0.0001 |
| Total cholesterol (mmol/L) | 5.2±1.0 | 5.5±0.9 | 5.6±0.9 | 5.4±1.0 | 0.01 |
| HDL cholesterol (mmol/L) | 1.8±0.5 | 1.8±0.4 | 1.7±0.4 | 1.6±0.5 | 0.0002 |
| LDL cholesterol (mmol/L) | 3.2±0.9 | 3.4±0.8 | 3.4±0.8 | 3.3±1.0 | 0.15 |
| Triglyceride (mmol/L) | 0.9±0.8 | 1.1±0.7 | 1.3±0.7 | 1.4±0.9 | <0.0001 |
| Dyslipidemia (%) | 49.2 | 53.5 | 58.8 | 68.4 | 0.0001 |
| Fasting plasma glucose (mmol/L) | 5.4±1.2 | 5.8±1.1 | 6.0±1.1 | 6.4±1.3 | <0.0001 |
| HbA1c (%) | 4.8±0.8 | 5.0±0.6 | 5.1±0.6 | 5.3±0.8 | <0.0001 |
| Diabetes (%) | 3.1 | 8.6 | 12.4 | 34.3 | <0.0001 |
| BMI | 21.7±4.0 | 22.8±4.0 | 23.6±4.0 | 24.0±4.0 | <0.0001 |
| Obesity (%) | 28.4 | 24.6 | 30.5 | 39.6 | 0.0004 |
| Serum calcium (mmol/L) | 2.3±0.1 | 2.3±0.1 | 2.3±0.1 | 2.3±0.1 | 0.01 |
| Serum potassium (mmol/L) | 4.3±0.4 | 4.3±0.4 | 4.3±0.4 | 4.2±0.4 | 0.003 |
| Alcohol intake (%) | 22.5 | 29.3 | 31.1 | 29.3 | 0.69 |
| Habitual smoking (%) | 21.0 | 6.3 | 9.4 | 4.7 | 0.49 |

Values are age-adjusted means±SD or frequencies. ACE, angiotensin-converting enzyme; ARB, angiotensin receptor blocker; HDL, high-density lipoprotein; LDL, low-density lipoprotein; BMI, body mass index.

## Measurements of QTc and baPWV

Standard, resting 12-lead ECG was performed using an ECG device (FCP-4266; Fukuda Denshi, Tokyo, Japan) in the supine position in the morning. Heart rate (bpm) and QT interval duration (ms) were determined automatically using the PI-10 ECG Analysis Program (Fukuda Denshi). The program calculated the QT interval duration from the beginning of QRS to the end of the T wave. The QT interval duration was corrected for heart rate by calculating QTc according to Bazett's equation (*18*).

$$QTc = QT \text{ interval duration [ms]}/(60/\text{heart rate})^{1/2}$$

The baPWV was measured in the supine position after at least 5 min of rest using a volume-plethysmographic apparatus (Form PWV/ABI; Colin, Komaki, Japan), as described previously (*19*). Briefly, cuffs to measure baPWV were wrapped on both brachia and ankles. PWV at the brachia and ankles were recorded using a semiconductor pressure sensor. Volume waveforms were stored with automatic gain analysis and quality adjustment. BaPWV was automatically calculated according to the following equation: $baPWV = (L_a - L_b)/T$, with $L_a$ being the distance from the heart to each ankle, $L_b$ the distance from the heart to the right upper arm, and $T$ the time delay from the right brachial waveform to each ankle waveform.

All clinical examinations including 12-lead ECG, measurement of baPWV and blood test were conducted on the same day.

## Relevant Factors

At baseline examination, a self-administrated questionnaire concerning current drug use including antihypertensive agents (*e.g.*, β-blocker, calcium channel blocker, angiotensin converting enzyme inhibitor or angiotensin receptor blocker), smoking, and alcohol intake was completed in advance by each participant and was checked by trained interviewers at the screening. These variables were classified as being either habitual or not. Blood pressure was measured three times

after the subject had rested for at least 5 min using a semiautomatic device (BP203RVIII; Colin) based on the cuff-oscillometric principle with the subject in the sitting position. The mean of the three measurements was used for the present analysis. Hypertension was defined as a systolic blood pressure ≥140 mmHg, a diastolic blood pressure ≥90 mmHg, or current use of antihypertensive agents. ECG abnormalities were defined as Q wave (Minnesota codes, 1–1, 2, 3), left ventricular hypertrophy (3–1) or ST depression (4–1, 2, 3). Body height and weight were measured in light clothing without shoes, and body mass index (BMI) was calculated as weight in kg divided by height in m squared. Blood samples were collected from an antecubital vein after an overnight fast for the determination of lipids, plasma glucose levels, serum calcium, and potassium. Serum total cholesterol, triglycerides, low-density lipoprotein (LDL)- and high-density lipoprotein (HDL)-cholesterol concentrations were determined enzymatically. Fasting blood glucose levels were measured by the glucose oxidase method. Hemoglobin A1c levels were measured by high-performance liquid chromatography. Dyslipidemia was defined as total cholesterol ≥5.68 mmol/L, LDL-cholesterol ≥4.13 mmol/L, HDL-cholesterol <1.03 mmol/L, triglycerides ≥1.69 mmol/L, or current use of lipid-lowering agents. Diabetes was defined according to the criteria recommended by the American Diabetes Association (*20*), in addition to a medical history of diabetes. Obesity was defined as BMI ≥25.0 kg/m$^2$.

## Statistical Analysis

The age-adjusted frequencies of relevant factors in quartile groups defined by baPWV were calculated by means of the direct method using the total study population as a standard and were compared using age-adjusted logistic regression models. The age-adjusted mean values of QTc and relevant factors in quartile groups defined by baPWV were calculated using covariance analysis and compared using multiple regression models. Multivariate-adjusted mean values of QTc in the four baPWV groups were estimated using multiple regression models including age, gender, hypertension, ECG abnormalities, dyslipidemia, diabetes, obesity, serum calcium and potassium levels, alcohol intake, and habitual smoking. Comparisons of the relationships of baPWV with QTc among subgroups were carried out by adding an interaction term to the statistical models. $p$ values less than 0.05 were considered statistically significant. Statistical analyses were performed using the SAS program package (SAS Institute, Cary, USA).

## Ethical Considerations

The ethics committee of Kyushu University approved this study, all participants provided written informed consent, and the procedures followed were in accordance with national guidelines.

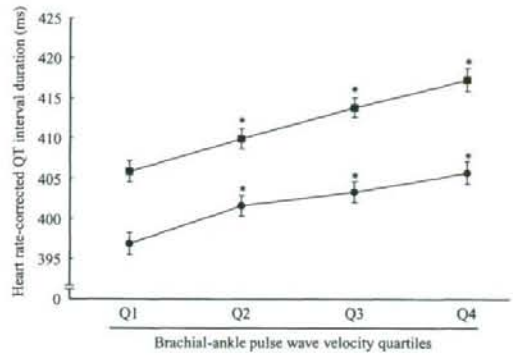


**Fig. 1.** *Age-adjusted mean values of heart rate–corrected QT interval duration according to quartiles of brachial-ankle pulse wave velocity levels for men (solid circles) and women (solid boxes). For men, the quartile groups were defined by baPWV values of less than 1,369, 1,370 to 1,560, 1,561 to 1,840, and 1,841 or greater; and for women, by baPWV of less than 1,269, 1,270 to 1,493, 1,494 to 1,821, and 1,822 or greater. The centers of the circles or boxes are placed at the estimates of mean values. Vertical lines represent SEM for mean values. *$p<0.01$ vs. the lowest quartile group. $p<0.0001$ for trend in both men and women.*

## Results

The mean value of QTc was 401.7 ms (SD, 21.5; range, 328.0–494.0) for men and 411.7 ms for women (SD, 23.3; range, 295.0–554.0). Baseline characteristics of male and female participants according to quartile groups defined by baPWV are shown in Tables 1 and 2, respectively. For men, the quartile groups were defined by baPWV values of less than 1,369, 1,370 to 1,560, 1,561 to 1,840, and 1,841 or greater cm/s; and for women, by baPWV of less than 1,269, 1,270 to 1,493, 1,494 to 1,821, and 1,822 or greater cm/s. The subjects with higher baPWV levels were significantly older. The frequencies of hypertension, dyslipidemia, diabetes, obesity, and alcohol intake increased with rising baPWV levels, while an inverse association was observed for the frequency of habitual smoking.

Figure 1 shows the age-adjusted mean values of QTc according to quartiles of the baPWV levels by sex. The age-adjusted mean values of QTc linearly increased with rising baPWV levels for men and women: for men, 396.7, 401.4, 403.2, and 405.6 ms for the 1st to 4th quartile groups, respectively ($p<0.0001$ for trend); for women, 405.7, 409.9, 413.8, and 417.4 ms for the 1st to 4th quartile groups, respectively ($p<0.0001$ for trend). When the Friedrich formula was used for estimation of QTc, similar associations were observed between baPWV and QTc in both men and women

**Table 3. Age- and Sex-Adjusted Mean Values of Heart Rate–Corrected QT Interval Duration According to Brachial-Ankle Pulse Wave Velocity Quartiles and Relevant Factors**

| | Quartiles of brachial-ankle pulse wave velocity | | | | *p* for trend | *p* for homogeneity |
|---|---|---|---|---|---|---|
| | Q1 | Q2 | Q3 | Q4 | | |
| Hypertension | | | | | | |
| No (*n*=1,618) | 402.4±0.9 | 405.8±0.9 | 408.8±1.3 | 410.8±1.9 | <0.0001 | 0.43 |
| Yes (*n*=1,048) | 402.8±3.9 | 408.8±2.0 | 409.7±1.3 | 412.8±1.2 | 0.01 | |
| Dyslipidemia | | | | | | |
| No (*n*=1,202) | 403.1±1.4 | 405.9±1.3 | 410.0±1.4 | 408.6±1.8 | 0.03 | 0.14 |
| Yes (*n*=1,464) | 401.3±1.4 | 407.0±1.2 | 408.8±1.1 | 414.5±1.2 | <0.0001 | |
| Diabetes | | | | | | |
| No (*n*=2,243) | 402.4±1.0 | 406.4±0.9 | 408.7±0.9 | 411.9±1.2 | <0.0001 | 0.39 |
| Yes (*n*=423) | 404.1±3.8 | 407.6±2.7 | 411.7±2.2 | 412.9±1.9 | 0.06 | |
| Obesity | | | | | | |
| No (*n*=1,938) | 402.7±1.1 | 406.4±1.0 | 408.7±1.0 | 411.2±1.2 | <0.0001 | 0.19 |
| Yes (*n*=728) | 401.7±2.0 | 406.9±1.7 | 410.5±1.6 | 414.8±1.8 | <0.0001 | |
| ECG abnormalities | | | | | | |
| No (*n*=2,196) | 402.2±1.0 | 405.8±0.9 | 409.1±0.9 | 411.8±1.2 | <0.0001 | 0.43 |
| Yes (*n*=470) | 402.3±3.7 | 411.1±2.9 | 410.6±2.3 | 413.7±2.1 | 0.04 | |
| Alcohol intake | | | | | | |
| No (*n*=1,504) | 404.3±1.4 | 408.1±1.2 | 410.6±1.2 | 413.6±1.3 | <0.0001 | 0.34 |
| Yes (*n*=1,162) | 399.8±1.4 | 404.4±1.3 | 407.6±1.3 | 410.9±1.6 | <0.0001 | |
| Habitual smoking | | | | | | |
| No (*n*=2,068) | 403.9±1.2 | 407.2±1.0 | 409.7±1.0 | 413.7±1.1 | <0.0001 | 0.21 |
| Yes (*n*=598) | 397.2±1.6 | 404.1±1.7 | 408.1±1.8 | 407.5±2.3 | 0.0003 | |

Values are age- and sex-adjusted means±SEM.

**Table 4. Age- and Sex-Adjusted Mean Values of Heart Rate–Corrected QT Interval Duration According to Brachial-Ankle Pulse Wave Velocity Quartiles and the Number of Relevant Factors**

| Number of relevant factors | Quartiles of brachial-ankle pulse wave velocity | | | | *p* for trend |
|---|---|---|---|---|---|
| | Q1 | Q2 | Q3 | Q4 | |
| 0–1 (*n*=903) | 406.0±1.6 | 404.0±1.4 | 408.1±1.4 | 411.6±1.7 | 0.001 |
| 2–3 (*n*=1,313) | 402.5±1.4 | 404.8±1.3 | 409.4±1.3 | 410.4±1.5 | 0.003 |
| 4–7 (*n*=450) | 407.4±2.3 | 407.7±2.2 | 413.9±2.2 | 413.5±2.4 | 0.02 |

Values are age- and sex-adjusted means±SEM. Relevant factors: hypertension, dyslipidemia, diabetes, obesity, ECG abnormalities, alcohol intake, and habitual smoking.

($p<0.0001$ for trend in both sexes). In the following analyses, male and female subjects were combined because the relationships of baPWV to QTc were comparable between men and women.

Table 3 shows the age- and sex-adjusted mean values of QTc according to quartiles of the baPWV levels for subgroups of participants defined on the basis of the presence or absence of hypertension, dyslipidemia, diabetes, obesity, ECG abnormalities, alcohol intake, or smoking habits. There were comparable relationships between baPWV and QTc for participants who were and were not hypertensive. Likewise, there were no interactions in the relationships of baPWV with QTc between subgroups defined by every other relevant factor (all $p$ values for interaction $>0.05$). There were also comparable relationships of baPWV with QTc between participants who were and were not taking antihypertensive agents or lipid-lowering agents ($p$ for interaction $>0.5$). We also estimated the age- and sex-adjusted mean values of QTc according to quartiles of the baPWV levels by the number of relevant factors (Table 4). There was a significantly positive relationship between baPWV and QTc in each of the groups defined by a number of cardiovascular risk factors of 0–1, 2–3, and 4–7.

Figure 2 shows the multivariate-adjusted mean values of QTc according to quartiles of the baPWV levels. The multivariate-adjusted mean values of QTc significantly increased with rising baPWV levels, even after controlling for age, sex, hypertension, ECG abnormalities, dyslipidemia, diabetes,

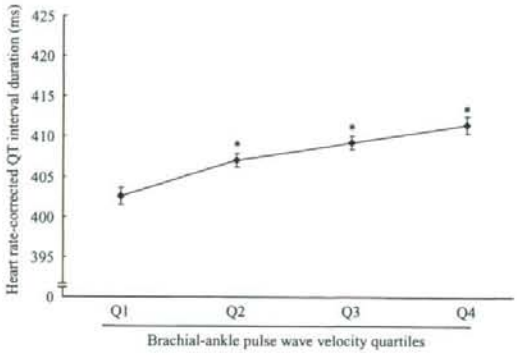


**Fig. 2.** *Multivariate-adjusted mean values of heart rate–corrected QT interval duration according to quartiles of brachial-ankle pulse wave velocity levels. The centers of the boxes are placed at the estimates of mean values. Other conventions are the same as in Fig. 1. Mean values and p values are adjusted for age, sex, hypertension, ECG abnormalities, dyslipidemia, diabetes mellitus, obesity, serum calcium and potassium, alcohol intake, and smoking habits. *$p<0.01$ vs. the lowest quartile group. $p<0.0001$ for trend.*

obesity, serum calcium, serum potassium, alcohol intake, and smoking habits ($p<0.0001$ for trend).

## Discussion

To our knowledge, this is the first study to address the associations between baPWV and QTc in a general population without preexisting cardiovascular disease. In the present analysis, the mean values of QTc increased with rising baPWV levels for both men and women. These associations remained strong and continuous, even after controlling for traditional cardiovascular risk factors, suggesting an independent relationship between subclinical arterial disease (atherosclerosis) and QT interval prolongation.

In the present study, there were strong and continuous relationships between QTc and baPWV, which has been shown to be a functional marker for subclinical atherosclerotic disease in central and peripheral arteries (*16*, *21*). Ours is the largest study to have investigated the association between subclinical arterial disease and QT interval prolongation, but there have been a few other cross-sectional studies addressing this question using other structural markers of subclinical arterial disease (*10–12*). The Insulin Resistance Atherosclerosis Study (IRAS) investigated the association between carotid intima media thickness and QTc in 912 nondiabetic subjects without coronary artery disease and found a close association between carotid atherosclerosis and QT interval prolongation (*10*). The Salzburg Atherosclerosis Prevention Program in Subjects at High Individual Risk also showed a positive correlation between carotid intima media thickness and QT interval duration in 1,199 clinically healthy subjects (*11*). These observational data support our hypothesis that subclinical arterial disease is associated with QT interval prolongation.

It is well known that the QT interval is affected by heart rate (*18*, *22*). In order to control for the confounding effects of heart rate, we used QTc, which was estimated by Bazett's formula, and found significant associations between baPWV and QTc. When the Friedrich formula was used for estimation of QTc instead of Bazett's formula, similar associations were observed. We also investigated the association between baPWV and crude QT interval duration and found significantly positive relationships even after adjustment for heart rate, ECG abnormalities, and other cardiovascular risk factors (data not shown). These results suggest that baPWV is significantly associated with QT interval duration and this association is independent of the effects of heart rate.

The mechanism underlying the association between subclinical arterial disease and the acquired form of QT interval prolongation has not been clearly defined. Subclinical arterial disease and subsequent arterial stiffness may increase ventricular load and, as a consequence, may promote myocardial and electrophysiological remodeling, resulting in QT interval prolongation (*23*, *24*). Another possible mechanism is that microvascular atherosclerosis in the coronary artery, which is strongly related to systemic arterial disease, may lead to subendocardial ischemia and thus extend QT interval duration (*25*).

One limitation of our study is that we have no information on subjects with congenital long QT syndrome. However, the prevalence of the congenital long QT syndrome has been reported to be less than 0.1% (*26*). Furthermore, in our subjects the relationship between baPWV and QTc was strong and continuous, even after excluding participants with QT intervals of 440 ms or more ($p<0.0001$ for trend). Thus, the influence of congenital long QT syndrome would seem to have been negligible. Another limitation is that information on repeated measurements of baPWV and QTc is limited. This fact made it difficult for us to conduct longitudinal analysis.

In conclusion, we found close associations between baPWV and QTc for men and women without histories of cardiovascular disease. These associations were independent of hypertension, ECG abnormalities, dyslipidemia, diabetes, obesity, alcohol intake, and smoking habits. Thus, subclinical arterial disease appears to contribute to the pathogenesis of QT interval prolongation. Future longitudinal studies are necessary to clarify the causal relationship between subclinical arterial disease and QT interval prolongation.

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# Fasting Plasma Glucose Cutoff for Diagnosis of Diabetes in a Japanese Population

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**Objective:** We examined the relationship between fasting plasma glucose (FPG) and 2-h post-load glucose (PG) levels, and the optimal FPG cutoff level to correspond to a 2-h PG of 11.1 mmol/liter, the gold standard diagnostic criterion, in a general Japanese population.

**Design:** Cross-sectional study populations of 2421 subjects in 1988 and 2698 subjects in 2002, aged 40–79 yr and without antidiabetic medication, were tested with an oral glucose tolerance test. The relationship between FPG and 2-h PG was investigated by various regression models and a receiver operating characteristic curve.

**Results:** The best-fit model for the relationship between FPG and 2-h PG was a quadratic regression model. The FPG cutoff levels corresponding to the 2-h PG of 11.1 mmol/liter by this model were 6.2 mmol/liter in 1988 and 6.3 mmol/liter in 2002. In the combined populations, the FPG cutoff point was 6.3 mmol/liter; the sensitivity and specificity of this cutoff point for detecting a 2-h PG of 11.1 mmol/liter were 75.2 and 88.6%, respectively. The receiver operating characteristic curve analysis confirmed that the corresponding FPG point was 6.2 mmol/liter in both the 1988 and 2002 populations. In a stratified analysis, the FPG cutoff level increased with increasing body mass index levels; however, even in subjects with body mass index more than or equal to 30 kg/m$^2$, the FPG cutoff level was lower than 7.0 mmol/liter.

**Conclusions:** Our findings suggest that the FPG cutoff level corresponding to the 2-h PG of 11.1 mmol/liter in the general Japanese population is lower than the current diagnostic criterion. ***(J Clin Endocrinol Metab* 93: 3425–3429, 2008)**

A 2-h post-load glucose (PG) cutoff level of 11.1 mmol/liter is considered to be the gold standard diagnostic criterion for diabetes mellitus. This cutoff point was originally adopted for several reasons (1). First, 11.1 mmol/liter has been found to approximate the cutoff point separating the two components of the bimodal distribution of 2-h PG levels. Second, according to several epidemiological studies, including our own, the prevalence of microvascular disease sharply increases in patients having a 2-h PG above 11.1 mmol/liter (1–4). Third, a great number of clinical and epidemiological studies have used this criterion. By contrast, fasting plasma glucose (FPG) has not been adequately justified as a diagnostic criterion. The FPG cutoff point for diagnosing diabetes was revised by the Expert Committee of the American Diabetes Association (ADA) (1) in 1997; namely, the cutoff point defining diabetes was reduced from more than or equal to 7.8 mmol/liter to more than or equal to 7.0 mmol/liter, though the ADA itself has recognized that this new cutoff point is not the best equivalent of the 2-h value of 11.1 mmol/liter (1, 5). The World Health Organization adopted an FPG of 7.0 mmol/liter as a diagnostic criterion of diabetes in 1998 (6). This lowering was based on the following findings from several studies, primarily with cohorts of high body mass index (BMI) subjects: 1) the prevalence and incidence of diabetic retinopathy increased at an FPG of approximately 7.0 mmol/liter (1, 3, 4); 2) the discrepancy in the detection rate of diabetes between FPG and 2-h PG values was reduced when an FPG of 7.0 mmol/liter was

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Abbreviations: ADA, American Diabetes Association; BMI, body mass index; FPG, fasting plasma glucose; OGTT, oral glucose tolerance test; PG, post-load glucose; ROC, receiver operating characteristic.

used; and 3) the prevalence of diabetes by a 2-h PG cutoff point of 11.1 mmol/liter was identical to that of an FPG of approximately 7.0 mmol/liter in several populations. However, the Diabetes Prevention Program Research Group has recently shown that the retinopathy characteristic of diabetes was present in persons whose FPG was below the diabetic range and who had no known history of diabetes (7). Furthermore, an integrated study of three general populations suggested that although the prevalence of retinopathy increased with FPG concentration, there was no clear diagnostic cutoff (8). These findings imply that data of diabetic retinopathy alone are not adequate to determine an FPG cutoff point. Thus, another approach, such as a regression analysis, is needed to validate the FPG cutoff point.

On the other hand, it remains controversial whether the FPG of 7.0 mmol/liter is adequately diagnostic for diabetes in Asian populations, which tend to be leaner than Western populations. For instance, FPG cutoff levels corresponding to a 2-h PG of 11.1 mmol/liter were also lower than 7.0 mmol/liter in other Asian populations (9–11). There have been very few reports on this issue in the Japanese population, in which the prevalence of diabetes has been increasing rapidly in recent years. The purposes of this study were to determine the FPG cutoff value corresponding to a 2-h PG of 11.1 mmol/liter, and to check whether this cutoff value varied according to changes in the society over time by examining the relationship between FPG and 2-h PG values in a general Japanese population at two different time points separated by an interval of 14 yr.

## Subjects and Methods

A population-based prospective study of cardiovascular disease has been underway since 1961 in the Town of Hisayama, a suburb in the Fukuoka metropolitan area on Kyushu Island, in Japan. Based on data from the national census, the age and occupational distributions for Hisayama have been almost identical to those of Japan as a whole from 1961 to the present. As a part of the study, two cross-sectional diabetes surveys of Hisayama residents were conducted in similar fashion in 1988 and 2002. A detailed description of the surveys has been published previously (12, 13); briefly, of the total of 3227 residents in 1988 aged 40–79 yr in the town registry, 2587 (participation rate, 80.2%) consented to take part in a comprehensive assessment, including a 75-g oral glucose tolerance test (OGTT) and an interview covering both medical histories (including items on diabetes, hypertension, and other chronic diseases) and current medical treatments with insulin and oral hypoglycemic agents. After excluding participants who had already had breakfast, those who were receiving insulin therapy for diabetes, and those who refused the OGTT due to complaints of nausea or general fatigue during the ingestion of glucose, we successfully completed the OGTT on 2480 subjects. An additional 59 subjects were excluded because they were taking oral hypoglycemic agents; thus, the final 1988 study group comprised 2421 subjects (1045 men and 1376 women) (Fig. 1). In 2002, we established another study population of 2698 (1162 men and 1536 women) using the same methods and criteria.

In both the 1988 and 2002 surveys, clinical evaluation and laboratory measurements were performed in a similar manner. The study subjects underwent the OGTT between 0800 and 1030 h after an overnight fast of at least 12 h. Blood for the glucose assay was obtained by venipuncture into tubes containing sodium fluoride at fasting and at 2-h post-load, and was separated into plasma and blood cells within 20 min. Plasma glucose levels were determined by the glucose-oxidase method. The between-assay and within-assay coefficients of variance of glucose measurement in our laboratory were 0.96 and 0.81% at 5.6 mmol/liter, and 0.81 and 0.56% at 16.7 mmol/liter, respectively. Total cholesterol and triglycerides were determined enzymatically. Blood pressure was obtained three times using a mercury sphygmomanometer with the subject in a sitting position; the average values were used in the analyses. Hypertension was defined as systolic blood pressure more than or equal to 140 mm Hg and/or diastolic blood pressure more than or equal to 90 mm Hg and/or current treatment with antihypertensive agents. The height and weight of each subject, wearing light clothes without shoes, were recorded, and the BMI (kg/m$^2$) was calculated. The interview investigated smoking habits and alcohol intake. Both were classified as either currently habitual or not. Subjects engaging in sports at least three times per week during their leisure time were classified into a regular exercise group.

SAS (SAS Institute Inc., Cary, NC) was used to perform all statistical analyses. Various regression models, including linear, quadratic, logarithmic, inverse, power, and exponential models, without covariates were examined to determine which best fit the relationship between FPG and 2-h PG levels. Furthermore, an FPG cutoff point corresponding to the 2-h PG of 11.1 mmol/liter was calculated from each regression equation. The sensitivity of the FPG cutoff point was defined as its ability to identify correctly individuals who had a 2-h PG of 11.1 mmol/liter or higher, and the specificity was its ability to identify correctly individuals who did not have a 2-h PG of 11.1 mmol/liter or higher. To compare the ability of FPG measurements to detect the presence or absence of a 2-h PG of 11.1 mmol/liter or higher across a range of values, we plotted receiver operating characteristic (ROC) curves. The diagnostic properties of specific cutoff levels of FPG were defined by maximizing the sensitivity and specificity to identify a 2-h PG of 11.1 mmol/liter or higher.

This study was conducted with the approval of the Ethics Committee of the Faculty of Medicine, Kyushu University, and written informed consent was obtained from the participants.

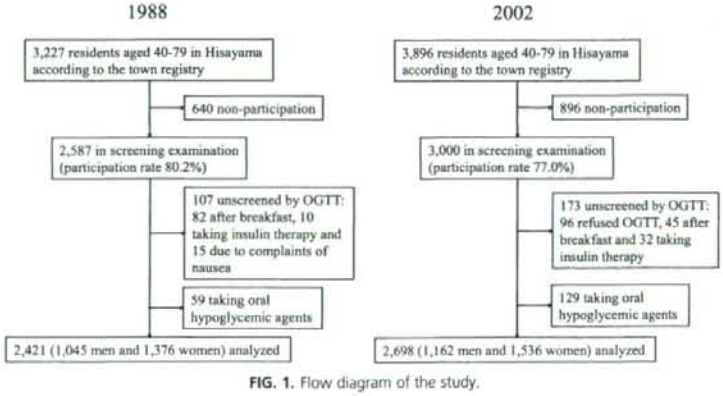


**FIG. 1.** Flow diagram of the study.

## Results

The clinical characteristics of the subjects in 1988 and 2002 are summarized in Table 1. Mean values of age, FPG, 2-h PG, and BMI were higher in 2002 than 1988, whereas the frequency of men was not different between the populations.

To elucidate the relationship between FPG and 2-h PG, we analyzed their interrelationships using the various regression models listed in Table 2. FPG values corresponding to a 2-h PG of 11.1 mmol/liter and $R^2$ values were calculated for the combined populations

**TABLE 1.** Clinical characteristics of subjects: the Hisayama study in 1988 and 2002

| | 1988 (n = 2421) | 2002 (n = 2698) | *P* value |
|---|---|---|---|
| Age (yr) | 57 (10) | 59 (11) | <0.001 |
| Men (%) | 43.2 | 43.1 | 0.94 |
| FPG (mmol/liter) | 5.7 (1.1) | 6.0 (1.0) | <0.001 |
| 2-h PG (mmol/liter) | 7.3 (3.2) | 7.7 (3.1) | <0.001 |
| BMI (kg/m$^2$) | 23.0 (3.1) | 23.3 (3.3) | <0.001 |

Values are means (SD).

of 1988 and 2002. The $R^2$ value was larger for the quadratic regression model, indicating that it is a better fit than the other models; the relevant FPG point in this model was 6.3 mmol/liter.

Figure 2 depicts the relationship between the FPG and 2-h PG in 1988 and 2002 considered separately. The quadratic model analyses were still the best fit among the various models for both the 1988 and 2002 populations (data not shown), with $R^2$ values of 64.0 in 1988 and 61.3 in 2002. The FPG point corresponding to a 2-h PG of 11.1 mmol/liter was 6.2 mmol/liter in 1988 and 6.3 mmol/liter in 2002.

To confirm the cutoff point of FPG corresponding to the 2-h PG of 11.1 mmol/liter, we plotted ROC curves and calculated the optimal cutoff points defined as the maximum combination of sensitivity and specificity, and their area under the ROC curves (Fig. 3). In the 1988 subjects, the corresponding FPG point was 6.2 mmol/liter. The sensitivity and specificity of this cutoff point were 81.2 and 88.7%, respectively; and the area under the curve was 91.0%. In the 2002 subjects, the cutoff point was 6.2 mmol/liter; the sensitivity, specificity, and area under the curve were 77.9, 81.3, and 86.7%, respectively.

Finally, we performed a stratified analysis by sex, age, and BMI levels in the combined population using both the quadratic regression model and ROC analysis (Table 3). The FPG level corresponding to the 2-h PG of 11.1 mmol/liter was slightly higher in men than women by both the quadratic regression model and ROC analysis. Higher FPG levels corresponding to a 2-h PG of 11.1 mmol/liter were observed in the younger age groups in the quadratic regression model analysis. However, in ROC analysis there was no association between age and FPG level. The FPG level corresponding to a 2-h PG of 11.1 mmol/liter increased with increasing BMI levels in both the quadratic regression model and ROC analysis. However, even in subjects with a BMI more than or equal to 30 kg/m$^2$, the FPG cutoff level was still lower than the diagnostic criterion of 7.0 mmol/liter.

## Discussion

We examined the association between FPG and 2-h PG levels in a general Japanese population at two time points separated by a 14-yr interval, and using the quadratic model, which proved to be the best fit for the data, demonstrated that the FPG level corresponding to a 2-h PG of 11.1 mmol/liter, the gold standard for diagnosis of diabetes, was 6.2 mmol/liter for the 1988 data and 6.3 mmol/liter for the 2002 data. The FPG points derived from the ROC analyses corroborated these findings. It has been reported that the corresponding FPG cutoff level by the quadratic model was 5.7 mmol/liter in Chinese (9) and 6.3 mmol/liter in Taiwanese (10). Together with the findings of these other studies, our results suggest that, in relatively lean Asian populations, including the Japanese, the FPG cutoff level is clearly lower than the FPG value of 7.0 mmol/liter, which is currently used in various diagnostic criteria for diabetes (1, 6), and that this situation did not change over the course of 14 yr in the Japanese population.

Although a method using FPG values corresponding to the gold standard of 2-h PG levels for diagnosis of diabetes has not yet been established, regression analysis appears to be a useful method for detecting the FPG cutoff value. Two previous epidemiological studies determined FPG cutoff points by analyzing the relationship between FPG and 2-h PG using linear or exponential models (14, 15). However, in our study the quadratic model showed the highest positive correlation between FPG and 2-h PG, and, thus, was the best-fitted model. This is consistent with the findings of studies in Taiwanese (9) and Chinese (10) populations.

The ADA recommends the use of the FPG instead of 2-h PG for diagnosing diabetes because it is difficult to perform an OGTT in routine clinical practice (1). Thus, it is very important to determine the appropriate FPG cutoff value for the diagnosis of diabetes in different populations. The FPG of 7.0 mmol/liter for diagnosing diabetes is based on several population studies examining the relationship between the glycemic threshold and diabetic retinopathy (1, 3, 4); however, optimal cutoff levels of plasma glucose for defining diabetes depend on ethnicity. In a Pima Indian study, the ROC curve analysis in a diabetic retinopathy study identified the optimal FPG cutoff level as 6.8 mmol/liter (3). The National Health and Nutrition Examination Survey III study of the U.S. population also reported that the prevalence of retinopathy increased dramatically at FPG levels of 6.7 mmol/liter (1). These findings were apparently confirmed by a similar study in Egypt (4), in which the optimal FPG cutoff level

**TABLE 2.** Relationship between FPG (Y) and 2-h PG (X) in various regression models for the combined population of 1988 and 2002

| Model | Equation | FPG corresponding to 2-h PG of 11.1 mmol/liter (mmol/liter) | $R^2$ (%) |
|---|---|---|---|
| Quadratic | $Y = 0.0149X^2 - 0.102X + 5.621$ | 6.3 | 62.3 |
| Linear | $Y = 0.243X + 4.024$ | 6.7 | 51.8 |
| Exponential | $Y = 2.718^{(0.0323X + 1.511)}$ | 6.5 | 48.6 |
| Power | $Y = 3.512X^{0.255}$ | 6.5 | 36.6 |
| Logarithmic | $Y = 1.831 \log X + 2.277$ | 6.7 | 35.6 |
| | $\log(Y) = 0.255 \log X + 1.256$ | 6.5 | 36.6 |
| | $\log(Y) = 0.243 (\log X)^2 - 0.748 \log X + 2.260$ | 6.5 | 50.2 |
| Inverse | $Y = 7.265 - 9.416/X$ | 6.4 | 20.1 |

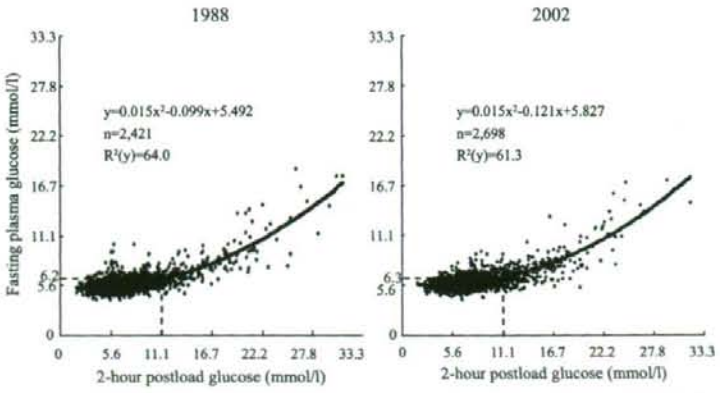


**FIG. 2.** The relationship between FPG and 2-h PG by a 75-g OGTT in Hisayama residents aged 40–79 yr in 1988 (*left panel*) and 2002 (*right panel*). *Solid line* represents the regression line by the quadratic regression model.

for detecting diabetic retinopathy was 6.9–7.2 mmol/liter. However, these three populations have higher BMI levels compared with Asian populations. We previously reported that although the glycemic threshold of 2-h PG for retinopathy in Japanese was 11.1 mmol/liter, that of FPG was only 6.4 mmol/liter (2). Other Asian population studies have reported optimal FPG cutoff levels for retinopathy ranging between 5.6 and 6.0 mmol/liter (16, 17). These findings suggest that FPG cutoff levels are lower in Asian populations than in other populations.

In our subjects the FPG cutoff levels corresponding to a 2-h PG of 11.1 mmol/liter increased with increasing BMI levels. However, even in subjects with a BMI more than or equal to 30 kg/m$^2$, the FPG cutoff level using the quadratic model was 6.4 mmol/liter, much lower than the diagnostic criterion of 7.0 mmol/liter. It is not clearly understood why FPG cutoff levels differ among ethnic groups. One possible explanation is that the capacity for acute insulin response to glucose load may influence the FPG cutoff level. The acute insulin response is known to be lower in Asian populations than other populations (18). In some clinical studies, the loss of acute insulin response by somatostatin was associated with a marked impairment in the initial suppression of hepatic glucose production, which led to higher 2-h PG concentrations (19, 20). Thus, impairment of acute insulin response may lead to a wide gap between FPG and 2-h PG; in other words, much lower FPG cutoff levels correspond to the 2-h PG diagnostic standard level. These findings might explain why the FPG cutoff level for the diagnosis of diabetes is lower in Asian populations, including ours, even in those with high BMI.

In the present study, the $R^2$ value in the quadratic model and the sensitivity, specificity, and area under the curve in the ROC analysis were all lower in 2002 than 1988. Although this phenomenon was not clearly understood, one possible reason may be that individuals in 2002 had more diverse lifestyles compared with those in 1988. Nevertheless, it is noteworthy that the FPG cutoff value corresponding to a 2-h PG of 11.1 mmol/liter was similar in the two populations.

Two limitations of our study should be discussed. First, in our study we determined the FPG cutoff level that corresponded to a 2-h PG of 11.1 mmol/liter, the gold standard for the diagnosis of diabetes, rather than that corresponding directly to diabetic complications. However, our previous study showed that the glycemic threshold of FPG for retinopathy is 6.4 mmol/liter (2), a result very similar to that of the present study. These findings suggest that the quadratic model precisely predicts the relationship between FPG and 2-h PG levels, making the FPG cutoff level nearly as accurate as the 2-h PG level, as well as more useful in clinical settings. Second, it is known that 2-h PG values in a 75-g OGTT have lower reproducibility than FPG (21, 22). It might be reasonable to propose FPG as the "gold standard." However, in the National Health and Nutrition Examination Survey III, 2-h PG was more specific for diabetic retinopathy than FPG (1). In several epidemiological studies, 2-h PG was also a stronger predictor of cardiovascular disease and total death compared with FPG (23–27). In addition, a 2-h PG of 11.1 mmol/liter was established in some revised processes for the diagnosis of diabetes. Based on these studies, then, a 2-h PG of 11.1 mmol/liter remains the "gold standard." Nevertheless, the present study found that two cross-sectional populations in 1988 and 2002 had nearly the same cutoff FPG values. This suggests that the high variability in 2-h PG values did not invalidate the present findings.

In conclusion, we have shown that the quadratic regression model is best fitted for the relationship between FPG and 2-h PG in a general Japanese population. The FPG cutoff level corresponding to a 2-h PG of 11.1 mmol/liter was 6.3 mmol/liter, and this result did not change over the course of 14 yr. Furthermore, the FPG cutoff levels were higher in subjects with higher BMI levels. The findings of the present study together with those of previous studies examining diabetic retinopathy sug-

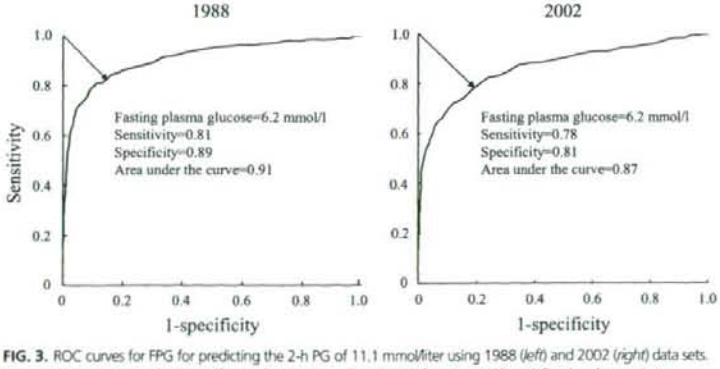


**FIG. 3.** ROC curves for FPG for predicting the 2-h PG of 11.1 mmol/liter using 1988 (*left*) and 2002 (*right*) data sets. The arrow shows the optimal cutoff point for detecting the 2-h PG of 11.1 mmol/liter defined as the maximum combination of sensitivity and specificity.

**TABLE 3.** FPG cutoff points corresponding to the 2-h PG of 11.1 mmol/liter by quadratic regression model and receiver operating curve analysis in the combined population of 1988 and 2002

| Factors | No. | Cutoff point defined by quadratic regression analysis (mmol/liter) | Cutoff point defined by ROC analysis (mmol/liter) |
|---|---|---|---|
| Sex | | | |
| Men | 2207 | 6.4 | 6.3 |
| Women | 2912 | 6.3 | 6.1 |
| Age (yr) | | | |
| 40–49 | 1341 | 6.4 | 6.0 |
| 50–59 | 1569 | 6.4 | 6.2 |
| 60–69 | 1363 | 6.3 | 6.2 |
| 70–79 | 846 | 6.2 | 6.1 |
| BMI (kg/m$^2$) | | | |
| <20 | 818 | 6.1 | 5.9 |
| 20–24.9 | 2978 | 6.3 | 6.1 |
| 25–29.9 | 1192 | 6.3 | 6.2 |
| ≥30 | 131 | 6.4 | 6.7 |

gest that in Asian populations, the FPG cutoff level corresponding to a 2-h PG of 11.1 mmol/liter is lower than 7.0 mmol/liter, the current diagnostic criterion for diabetes. Considering the growing importance of the FPG test in screening for diabetes, further investigations are required to clarify the optimal FPG cutoff level in Asian and other ethnic populations.

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Disclosure Statement: The authors have nothing to declare.

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# Secular Trends in the Incidence of and Risk Factors for Ischemic Stroke and Its Subtypes in Japanese Population

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***Background***—The study of long-term trends in the incidence of and risk factors for ischemic stroke subtypes could offer insights into primary and secondary prevention.

***Methods and Results***—We established 3 cohorts of residents ≥40 years of age in 1961, 1974, and 1988 in the Japanese community of Hisayama. Morphological examinations by autopsy or brain imaging were performed on most of the ischemic stroke cases developed in these cohorts. When 13-year follow-up data were compared, the age-adjusted incidence of ischemic stroke and lacunar infarction declined significantly from the first to the third cohort for both sexes, whereas the incidences of atherothrombotic and cardioembolic infarction did not change during this period. Hypertension was a powerful risk factor for the development of ischemic stroke, and improvement of hypertension control would have largely influenced this declining trend: The age- and sex-adjusted hazard ratio of hypertension decreased from 3.25 (95% CI 2.17 to 4.86) in the first cohort to 1.83 (1.29 to 2.58) in the third cohort. A rapid increase in the prevalence of metabolic disorders may have offset the impact of improvements in hypertension control and resulted in a slowdown of the decline in the incidence of ischemic stroke in the cohorts in the present study; however, hypertension still makes a large contribution to the development of ischemic stroke.

***Conclusions***—These findings suggest that in the Japanese population, the incidence of ischemic stroke has declined significantly over the past 40 years, probably owing to better management of hypertension. There is a need for greater primary prevention efforts in the treatment of hypertension and metabolic disorders. **(*Circulation*. 2008;118:2672-2678.)**

**Key Words:** cerebral infarction ■ morbidity ■ risk factors ■ hypertension ■ trend

Stroke continues to be a major public health concern worldwide. In Japan, it is the third leading cause of death and a major neurological cause of long-term disability.[1] The increase in the elderly population that accompanies the improvement in life expectancy is expected to further increase stroke prevalence. On the other hand, there have been major advances in the identification and management of stroke risk factors and the treatment of acute stroke. The study of temporal trends in stroke incidence provides insights into the effect of these factors. Several epidemiological studies have reported that the declining or stable incidence of stroke is likely attributable to better treatment of risk factors over time.[2–8] On the basis of their 50 years of follow-up data, the authors of the Framingham Study recently showed that the age-adjusted incidence of stroke decreased significantly in men and women owing to the improved control of hypertension and smoking.[2] In Japan, the incidence of stroke declined by 60% from 1964 to 1983 in a rural population.[7] We also found in a Japanese urban area that the incidence of ischemic stroke declined markedly between the 1960s and 1970s as a result of hypertension control, but this declining trend was slowed in the late 1980s and 1990s, probably because of an increase in metabolic disorders.[8]

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Because the pathogenesis, prognosis, and treatment differ among ischemic stroke subtypes,[9,10] the evaluation of temporal trends in the incidence of and risk factors for ischemic stroke subtypes may contribute to more effective primary and secondary prevention of ischemic stroke. However, morphological features of the brain were not readily available before the widespread use of computed tomography and magnetic resonance imaging, and the definition of ischemic stroke subtypes was not determined until the early 1990s.[11–13] Therefore, there is little information on the effect of the changes in cardiovascular risk factors on secular trends in the incidence of ischemic stroke and its subtypes.

The Hisayama Study is a population-based study that has established several cohorts at times that correspond to periods of remarkable lifestyle changes in Japan.[8,14–16] One of the

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characteristics of this study is that most of the deceased study subjects underwent autopsy examination from the beginning of the study, and thus, the morphological features of the brains examined by autopsy or brain imaging are available for most of the stroke cases in each cohort.[8,14] Furthermore, study-team physicians performed physical and neurological examinations on the subjects who developed stroke and collected detailed clinical information throughout the study period. These characteristics of the study design enabled us to examine secular trends in the incidence of and risk factors for ischemic stroke subtypes. We previously reported the steadily declining incidence of lacunar infarction (LAI) using 12-year follow-up data of the first 3 cohorts.[17] In this article, we extend the follow-up period of these cohorts to 13 years and compare the impact of cardiovascular risk factors on the incidence of ischemic stroke subtypes.

## Methods

### Study Population

The Hisayama Study, an epidemiological study of cerebrovascular-cardiovascular diseases, was established in 1961 in Hisayama Town, a suburban community adjacent to Fukuoka City, a metropolitan area on Kyushu Island in southern Japan. The population of the town was ≈8000 in 2007, and full community surveys of the residents have been repeated since 1961. The study design and characteristics of the subject population have been described in detail elsewhere.[14–16] Briefly, we established 4 study cohorts from Hisayama residents ≥40 years of age in 1961, 1974, 1988, and 2002 after screening examinations. In 1961, a total of 1658 subjects in that age group consented to participate in the screening examination (participation rate 90.1%). After the exclusion of subjects with a history of stroke or myocardial infarction and subjects who died or moved out of town during the examination, 1618 subjects were enrolled as the first cohort. Similarly, after excluding subjects with a history of stroke or myocardial infarction, we established a second cohort consisting of 2038 subjects from 2135 participants (participation rate 81.2%) in 1974, a third cohort of 2637 subjects from 2742 participants (participation rate 80.9%) in 1988, and a fourth cohort of 3123 subjects from 3328 participants (participation rate 77.6%) in 2002. The health status of these cohort populations was followed up every year by repeated health examinations or by mail or telephone for any subjects who did not undergo a regular examination or who moved out of town. Only 2 subjects in the first cohort, 2 in the second cohort, and 1 in the third cohort were lost to follow-up. The development of cardiovascular diseases in the study populations was also checked by a daily monitoring system organized by the study team, local physicians, and members of the local health and welfare office. When the subjects died, autopsy examinations were performed at the Department of Pathology, Kyushu University.

### Measurement of Cardiovascular Risk Factors

Details of the measurement of cardiovascular risk factors in each cohort were published previously.[8,14–16] In brief, blood pressures were measured 3 times with subjects in a recumbent position in 1961 and in a sitting position in 1974, 1988, and 2002, and hypertension was defined as a mean systolic blood pressure ≥140 mm Hg, a mean diastolic blood pressure ≥90 mm Hg, or current use of antihypertensive agents. Glucose intolerance was defined by an oral glucose tolerance test in subjects with glycosuria in 1961, by fasting and postprandial glucose concentrations in 1974, and by a 75-g oral glucose tolerance test in 1988 and 2002, in addition to medical history of diabetes. Serum cholesterol levels were measured by the Zak-Henly method with the modification by Yoshikawa in 1961, by the Zurkowski method in 1974, and by the enzymatic method in 1988 and 2002. Hypercholesterolemia was defined as total cholesterol ≥5.7 mmol/L (220 mg/dL). Body height and weight were measured with subjects in light clothing without shoes, and obesity was defined as body mass index ≥25.0 kg/m$^2$. Information on antihypertensive treatment, alcohol intake, and smoking habits was obtained with the use of a standardized questionnaire and was categorized as current habitual use or not. Current drinking was also categorized as light (1 to 33 g/d) or heavy (≥34 g/d) drinking according to daily ethanol intake.

### Definition of Ischemic Stroke Subtypes

Stroke was defined as a sudden onset of nonconvulsive and focal neurological deficit that persisted for >24 hours and was classified as ischemic stroke, cerebral hemorrhage, subarachnoid hemorrhage, or undetermined type.[8] The diagnoses of ischemic stroke subtypes were made on the basis of the Classification of Cerebrovascular Disease III proposed by the National Institute of Neurological Disorders and Stroke,[11] as well as on the basis of the diagnostic criteria of the Trial of Org 10172 in Acute Stroke Treatment (TOAST) study[12] and Cerebral Embolism Task Force.[13] We classified ischemic stroke subtypes into 4 categories: LAI, atherothrombotic infarction (ATI), cardioembolic infarction (CEI), and undetermined subtype. Details of the diagnostic criteria of ischemic stroke subtypes have been published previously.[10] Briefly, LAI was diagnosed as the presence of a relevant brain stem or subcortical hemispheric lesion with a diameter of <1.5 cm demonstrated on brain imaging or autopsy and no evidence of cerebral cortical or cerebellar impairment. ATI was diagnosed when the subject had significant stenosis (>50%) or occlusion of a major cerebral artery with infarct size ≥1.5 cm on brain imaging or autopsy. The diagnosis of CEI was made on the basis of primary and secondary clinical features suggestive of CEI as reported by the Cerebral Embolism Task Force.[13] The category of undetermined stroke included all ischemic stroke cases for which the subtype could not be determined because of insufficient clinical or morphological information. We considered morphological findings significant and used clinical features as reference information.

During the 13-year follow-up period, first-ever ischemic stroke developed in 134 subjects (83 cases of LAI, 28 of ATI, 17 of CEI, and 6 of undetermined subtype) in the first cohort, in 142 subjects in the second cohort (76 cases of LAI, 29 of ATI, 34 of CEI, and 3 of undetermined subtype), and in 154 subjects in the third cohort (74 cases of LAI, 42 of ATI, 38 of CEI, and 0 of undetermined subtype). Among these, morphological examinations by autopsy or brain imaging were performed on 90.3% (autopsy rate 90.3%) in the first cohort, 97.2% (autopsy rate 87.5%) in the second cohort, and 100.0% (autopsy rate 72.4%) in the third cohort.

### Statistical Analysis

The prevalences of possible risk factors were adjusted for age by the direct method and were examined for trends across cohorts by the Cochran-Mantel-Haenszel $\chi^2$ test with 10-year age groupings. Age-adjusted mean values of risk factors were calculated by the covariance method, and their trends were tested by the linear regression model. The incidences of first-ever ischemic stroke and its subtypes were calculated by the person-year method with adjustment for age by the direct method. The world standard population was used as a standard population. The age-adjusted incidences among the first 3 cohorts were compared with the use of the Cox proportional hazards model. Age and sex-adjusted hazard ratios (HRs) and 95% CIs of cardiovascular risk factors for the development of ischemic stroke and its subtypes were estimated by the Cox proportional hazards model in each cohort, and the population attributable risk fraction of each risk factor was calculated.

### Ethical Considerations

The study protocol was approved by the Human Ethics Review Committee of the Graduate School of Medical Sciences, Kyushu University.

The authors had full access to the data and take full responsibility for its integrity. All authors have read and agree to the manuscript as written.

**Table 1. Trends in Age-Adjusted Prevalence of Cardiovascular Risk Factors Among 4 Examinations of the Hisayama Study by Sex**

| | Men | | | | | Women | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Variables | 1961 (n=705) | 1974 (n=855) | 1988 (n=1110) | 2002 (n=1315) | P for Trend | 1961 (n=913) | 1974 (n=1183) | 1988 (n=1527) | 2002 (n=1808) | P for Trend |
| Age, y | 55±11 | 56±11 | 57±12 | 60±12 | <0.001 | 57±12 | 58±12 | 59±12 | 62±13 | <0.001 |
| Hypertension, % | 38.4 | 43.1 | 44.1 | 42.0 | 0.25 | 35.9 | 40.1 | 35.1 | 31.3 | <0.001 |
| Antihypertensive agents, % | 2.0 | 8.4 | 13.2 | 18.2 | <0.001 | 2.1 | 7.4 | 13.4 | 16.6 | <0.001 |
| Systolic BP, mm Hg* | 162±18 | 157±18 | 151±18 | 148±18 | <0.001 | 163±19 | 161±19 | 154±19 | 149±19 | <0.001 |
| Diastolic BP, mm Hg* | 91±11 | 90±11 | 87±11 | 89±11 | 0.011 | 88±11 | 87±11 | 83±11 | 86±11 | <0.001 |
| Glucose intolerance, % | 11.6 | 14.1 | 39.3 | 54.5 | <0.001 | 4.8 | 7.9 | 30.0 | 35.5 | <0.001 |
| Obesity, % | 7.0 | 11.6 | 24.1 | 29.3 | <0.001 | 12.9 | 21.5 | 23.8 | 24.0 | <0.001 |
| Body mass index, kg/m$^2$ | 21.3±2.8 | 21.7±2.8 | 22.8±2.8 | 23.5±2.8 | <0.001 | 21.7±3.4 | 22.5±3.3 | 22.9±3.3 | 22.9±3.4 | <0.001 |
| Hypercholesterolemia, % | 2.8 | 12.2 | 26.9 | 25.8 | <0.001 | 6.6 | 19.9 | 41.6 | 41.6 | <0.001 |
| Total cholesterol, mmol/L | 3.9±0.9 | 4.7±0.9 | 5.1±0.9 | 5.1±0.9 | <0.001 | 4.2±1.0 | 5.0±1.0 | 5.5±1.0 | 5.4±1.0 | <0.001 |
| Atrial fibrillation, % | 0.7 | 1.6 | 1.6 | 1.1 | 0.84 | 0.5 | 0.4 | 0.9 | 0.6 | 0.55 |
| Current smoking, % | 75.0 | 73.3 | 50.4 | 46.9 | <0.001 | 16.6 | 10.2 | 6.9 | 8.5 | <0.001 |
| Current drinking, % | 69.6 | 63.8 | 61.5 | 71.7 | 0.043 | 8.3 | 5.7 | 9.5 | 29.1 | <0.001 |
| Light drinking, % | 43.4 | 31.9 | 29.5 | 37.7 | ... | 8.2 | 5.5 | 8.0 | 27.1 | ... |
| Heavy drinking, % | 26.3 | 31.9 | 32.0 | 34.0 | ... | 0.1 | 0.2 | 1.5 | 2.0 | ... |

BP indicates blood pressure. Hypertension was defined as systolic BP ≥140 mm Hg or diastolic BP ≥90 mm Hg or current use of antihypertensive agents. Hypercholesterolemia was defined as total cholesterol level ≥5.7 mmol/L (220 mg/dL). Obesity was defined as body mass index ≥25.0 kg/m$^2$. Current drinking was divided into light (1 to 33 g) and heavy (≥34 g) drinking according to daily ethanol intake.

*Mean systolic and diastolic BPs among hypertensive subjects in each examination.

## Results

### Trends in Cardiovascular Risk Factors

We compared the age-adjusted prevalence of cardiovascular risk factors at baseline examination among the 4 cohorts by sex (Table 1). During the 40-year period from 1961 to 2002, the populations grew 5 years older in both sexes. The age-adjusted prevalence of hypertension was stable at ≈40% in men ($P$ for trend=0.25) and decreased significantly in women ($P$ for trend <0.001), whereas the proportion of individuals using antihypertensive agents increased consistently with time in both men and women. As a result, age-adjusted mean blood pressures among hypertensive men and women decreased significantly throughout the study period. In contrast, the age-adjusted prevalence of glucose intolerance and obesity increased greatly over the study period for both sexes. More than half of men and one third of women had glucose intolerance in 2002. The age-adjusted prevalence of hypercholesterolemia increased 10-fold in men and 6-fold in women from 1961 to 1988 but was unchanged in 2002. The age-adjusted prevalence of current smoking for men was 4-fold higher than that for women in 1961, and it decreased significantly with time for both sexes. The prevalence of current drinking increased significantly for both sexes in 2002.

### Trends in Incidence of Ischemic Stroke Subtypes

We then compared the age-adjusted incidence of ischemic stroke using the results of a 13-year follow-up in the first 3 cohorts (1st, 2nd, and 3rd cohort). The age-adjusted incidence of ischemic stroke declined significantly for both sexes throughout the cohorts: It significantly declined by 56% for men and by 40% for women from the first to the third cohort ($P$ for trend <0.001 for either sex; Table 2). In regard to ischemic stroke subtypes, the age-adjusted incidence of LAI for men declined significantly by 54% from the first to the second cohort, and it continued to decline by 39% from the second to the third cohort ($P$ for trend <0.001). The age-adjusted incidence of LAI for women also declined by 25% from the first to the second cohort, and it continued to decline by 17% from the second to the third cohort ($P$ for trend=0.003). The age-adjusted incidence of ATI and CEI did not change significantly among the cohorts for either sex.

### Trends in Proportion of Ischemic Stroke Subtype

The proportions of ischemic stroke subtypes among the cohorts are shown by sex in the Figure. For men, the proportion of subjects with LAI decreased steadily from the first to the third cohort, whereas the proportions with ATI and CEI increased. For women, the proportion of the subjects with CEI increased slightly from the first to the third cohort, but the proportions of those with the other subtypes were constant among the cohorts.

### Trends in the Effect of Cardiovascular Risk Factors on Ischemic Stroke

Because both cardiovascular risk factors and the incidence of ischemic stroke changed dramatically, we compared the impact of cardiovascular risk factors on the development of ischemic stroke among the first 3 cohorts (Table 3). In the first cohort, hypertension was a powerful risk factor for ischemic stroke (age- and sex-adjusted HR 3.25, 95% CI 2.17 to 4.86) and largely contributed to its occurrence (population attributable risk fraction 51%). The impact of hypertension gradually declined during the study period; however, hyper-

**Table 2. Age-Adjusted Incidence Rate (per 1000 Person-Years) of Ischemic Stroke and Its Subtypes Among 3 Cohorts of the Hisayama Study by Sex, With a 13-Year Follow-Up in Each Cohort**

| | Men | | | | Women | | | |
|---|---|---|---|---|---|---|---|---|
| | 1st Cohort (7456 PY) | 2nd Cohort (9655 PY) | 3rd Cohort (12 333 PY) | *P* for Trend | 1st Cohort (10 294 PY) | 2nd Cohort (13 762 PY) | 3rd Cohort (17 953 PY) | *P* for Trend |
| Ischemic stroke | | | | | | | | |
| No. of events | 72 | 70 | 70 | | 62 | 72 | 84 | |
| Incidence rate | 8.73 | 5.44 | 3.85 | <0.001 | 4.28 | 3.06 | 2.57 | <0.001 |
| LAI | | | | | | | | |
| No. of events | 48 | 34 | 30 | | 35 | 42 | 44 | |
| Incidence rate | 5.68 | 2.59 | 1.59 | <0.001 | 2.41 | 1.81 | 1.50 | 0.003 |
| ATI | | | | | | | | |
| No. of events | 14 | 14 | 22 | | 14 | 15 | 20 | |
| Incidence rate | 1.88 | 1.03 | 1.23 | 0.27 | 0.96 | 0.61 | 0.54 | 0.084 |
| CEI | | | | | | | | |
| No. of events | 9 | 21 | 18 | | 8 | 13 | 20 | |
| Incidence rate | 1.08 | 1.74 | 1.03 | 0.43 | 0.58 | 0.56 | 0.53 | 0.86 |
| Undetermined subtype | | | | | | | | |
| No. of events | 1 | 1 | 0 | | 5 | 2 | 0 | |
| Incidence rate | 0.09 | 0.09 | 0.00 | 0.20 | 0.33 | 0.08 | 0.00 | 0.004 |

PY indicates person-years.

tension was still a significant risk factor and made the largest contribution to the development of ischemic stroke even in the third cohort (HR 1.83, 95% CI 1.29 to 2.58, population attributable risk fraction 30%). Glucose intolerance was also a significant risk factor for ischemic stroke in the first cohort. The effect of glucose intolerance on the occurrence of ischemic stroke was reduced and was not significant in the second cohort, but it appeared to be a significant risk factor in the third cohort. The population attributable risk fraction for glucose intolerance decreased from 13% in the first cohort to 4% in the second cohort and then increased to 13% in the third cohort. Obesity appeared to be a significant risk factor for ischemic stroke in every cohort, and its population attributable risk fraction was increased gradually from 6% in the first cohort to 9% in the third cohort. Hypercholesterolemia, smoking habits, and alcohol intake were not significant risk factors for ischemic stroke in any of the cohorts. In the multivariate analysis that included all risk factors, hypertension was a significant risk factor for ischemic stroke, and its HR decreased from 2.92 (95% CI 1.93 to 4.41) in the first cohort to 1.71 (95% CI 1.20 to 2.45) in the third cohort. Glucose intolerance was an independent risk factor for ischemic stroke in the first cohort (HR 1.91, 95% CI 1.23 to 2.95) but was not significant in the third cohort (HR 1.28, 95% CI 0.93 to 1.78). Obesity was not a significant risk factor in any of the cohorts after adjustment for other risk factors. We tried to investigate the effect of cardiovascular risk factors on ischemic stroke subtypes, but we could not find reliable evidence of an effect of these risk factors on the development of each subtype, probably because of the small number of events.

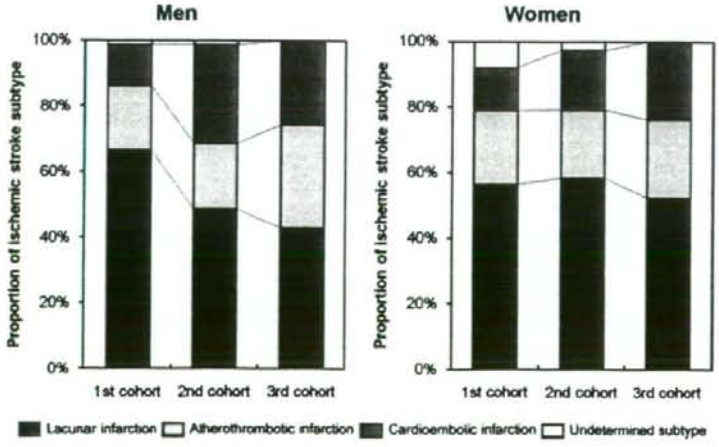


**Figure.** Proportion of ischemic stroke subtypes among the 3 cohorts of the Hisayama Study by sex.

**Table 3. Age- and Sex-Adjusted HRs of Cardiovascular Risk Factors for Ischemic Stroke Among 3 Cohorts of the Hisayama Study**

| | 1st Cohort | | | 2nd Cohort | | | 3rd Cohort | | |
|---|---|---|---|---|---|---|---|---|---|
| | HR (95% CI) | *P* | PAF | HR (95% CI) | *P* | PAF | HR (95% CI) | *P* | PAF |
| Hypertension | 3.25 (2.17–4.86) | <0.001 | 0.51 | 3.09 (2.05–4.65) | <0.001 | 0.53 | 1.83 (1.29–2.58) | <0.001 | 0.30 |
| Glucose intolerance | 2.45 (1.60–3.74) | <0.001 | 0.13 | 1.38 (0.87–2.17) | 0.17 | 0.04 | 1.41 (1.02–1.94) | 0.036 | 0.13 |
| Obesity | 1.83 (1.12–3.00) | 0.017 | 0.06 | 1.63 (1.04–2.57) | 0.034 | 0.07 | 1.54 (1.07–2.21) | 0.021 | 0.09 |
| Hypercholesterolemia | 1.07 (0.50–2.29) | 0.87 | 0.00 | 1.42 (0.95–2.12) | 0.085 | 0.07 | 0.96 (0.68–1.35) | 0.80 | −0.02 |
| Current smoker | 1.27 (0.85–1.90) | 0.24 | 0.10 | 0.83 (0.55–1.24) | 0.36 | −0.08 | 1.33 (0.89–1.98) | 0.16 | 0.07 |
| Current drinker | 0.99 (0.65–1.51) | 0.94 | −0.01 | 1.45 (0.96–2.19) | 0.081 | 0.12 | 1.09 (0.72–1.64) | 0.70 | 0.02 |

PAF indicates the population attributable risk fraction.

## Discussion

By comparing the incidence of ischemic stroke subtypes among 3 cohorts established at different times in a Japanese community, we demonstrated that the incidence of LAI declined significantly from the first to the third cohort for both sexes, whereas the incidence of ATI and CEI remained stable. During the study period, blood pressure levels among hypertensive subjects decreased significantly with time as a result of the popularization of antihypertensive medication. The prevalence of smoking habits declined steadily for both sexes. Contrary to these declining trends, the prevalence of metabolic disorders, namely, obesity, glucose intolerance, and hypercholesterolemia, increased steeply with time. These changes in cardiovascular risk factors might affect the incidence of ischemic stroke and its subtypes.

Hypertension is the most powerful risk factor for ischemic stroke.[9] In the first cohort, hypertension contributed to approximately half of the occurrence of ischemic stroke. During the study period, the age-adjusted prevalence of hypertension declined in women, and the proportion of all participants receiving hypertensive treatment increased steeply in both sexes. This improvement of hypertension control resulted in a decrease in age-adjusted mean systolic blood pressure level of 14 mm Hg among hypertensive subjects in both sexes. Because of this improved control of hypertension, the impact of the disease on the development of ischemic stroke was seen to weaken in the third cohort. The Framingham Study also showed a decline in the annual incidence of nonembolic stroke during a follow-up period of 50 years or more.[2] During this period, the mean systolic blood pressure level, prevalence of hypertension, and proportion of all participants receiving treatment for hypertension improved significantly. These reductions in the incidence of ischemic stroke and improvements in treatment for hypertension were similar to the findings of the present study. Our previous study showed that the impact of hypertension was similar for all ischemic stroke subtypes.[10] These results suggest that better management of hypertension might have made the biggest contribution to the declining trend in the incidence of ischemic stroke, especially of LAI; however, hypertension was still a significant risk factor in the third cohort and had a large attributable risk fraction for ischemic stroke. Because half of the hypertensive subjects did not undergo treatment for hypertension in the third cohort, there is a need for greater primary prevention efforts to improve the treatment of hypertension.

In subjects in the present study, the age-adjusted prevalence of metabolic disorders, such as obesity, hypercholesterolemia, and glucose intolerance, increased greatly during the past 40 years, probably owing to the westernization of the Japanese lifestyle. When we examined the impact of these metabolic disorders on the development of ischemic stroke, glucose intolerance was a significant risk factor in the first and the third cohort, and the impact of obesity was constant throughout the study period. Both glucose intolerance and body mass index have been shown to be significant risk factors for ischemic stroke and LAI.[10,18] Moreover, obesity is closely related to other cardiovascular risk factors and jointly increases the risk of ischemic stroke.[19] Our previous study also showed that the accumulation of metabolic disorders (that is, metabolic syndrome) was a significant risk factor for the development of ischemic stroke in our third cohort.[20] We speculate that the improved management of hypertension and the worsening of metabolic disorders cancelled each other out and resulted in the slowdown of the declining trend of the incidence of LAI and the sustained incidence of ATI.

Smoking is a widely accepted risk factor for ischemic stroke in Western populations, but this relationship is controversial for Japanese.[10,21,22] In the present study cohorts, the declining prevalence of smoking habits closely mirrored the declining trend in the incidence of ischemic stroke; however, smoking habits had little impact on the incidence of ischemic stroke in the present study cohorts. One possible explanation is that the association between smoking and the risk of ischemic stroke is only evident in populations with moderate to high levels of serum cholesterol.[23] A recent review of cardiovascular mortality trends in Japan[23] showed that the increase in serum cholesterol appeared mainly in young to middle-aged people. In contrast, elderly people, a high-risk group for ischemic stroke, continued to maintain a lower cholesterol level. However, the prevalence of smoking habits is still high in Japanese men, and therefore, the adverse influence of smoking might appear in the current generation of younger men, with a higher cholesterol level to be seen in the future.

LAI is the most common subtype of ischemic stroke in the Japanese population, unlike in Western populations.[1] Among subjects in the present study, because of the decreased incidence of LAI and the sustained incidences of ATI and CEI, the proportion of ischemic stroke subtypes has become closer to that of Western populations in men (Figure).

However, the pattern of ischemic stroke subtypes differed from that of Western populations, with subjects in the present study showing a high proportion of LAI even in recent years (43% for men and 52% for women in the third cohort). A recent hospital-based registration study in an urban area[24] and a study of 16 992 patients with acute ischemic stroke from rural areas in Japan[25] also showed a higher prevalence of LAI than of other subtypes. One possible explanation for this is the racial difference in the genetic susceptibility of LAI. We recently found 2 susceptibility genes for ischemic stroke, *PRKCH* and *AGTRL1*, in a genome-wide association study.[26,27] A single-nucleotide polymorphism in the *PRKCH* gene increased the risk of LAI, but this single-nucleotide polymorphism is specific to Asian populations.[27]

The present study has several limitations. First, the number of events of subtypes other than LAI was relatively small, and therefore, the power to assess trends in the incidence of and risk factors for ischemic stroke subtypes was weak. Second, there were a large number of subjects overlapping among the cohorts. Indeed, 916 of the subjects in the first cohort also accounted for 45% of the population of the second cohort. In addition, a total of 1229 subjects in the second cohort also participated in the third cohort (47% of the third cohort). However, we treated the overlapping subjects as in any life table analysis, establishing every cohort after excluding subjects with prior stroke or myocardial infarction at baseline. Therefore, these overlapping populations were not considered to distort the incidence trends in the present study. Third, the measurement of blood glucose and the criteria for glucose intolerance were different among the cohorts, which suggests an underestimation of the prevalence of glucose intolerance in the former cohorts. Nevertheless, the rapid changes in other risk factors in the present study are in accordance with the results of the National Nutritional Survey and other surveys of Japan.[23] Finally, the methods of case ascertainment and the diagnostic sensitivity of imaging techniques changed dramatically during the study period. The proportion of case subjects with of incident ischemic stroke who received diagnostic imaging tests increased over time. Echocardiography and carotid scanning were rarely performed in the former cohorts (3.0% and 0% in the first cohort, 29.6% and 4.2% in the second cohort, and 61.7% and 27.3% in the third cohort, respectively). Therefore, it is possible that the trends in the incidence of ATI and CEI were less accurate than the trends for LAI. Nonetheless, we believe that the findings of the present study reflect the actual secular trends in the incidence of ischemic stroke subtypes and their risk factors in the Japanese population, because we performed comprehensive surveillance, including autopsy examinations, in most of the cases.

## Conclusions

By comparing the incidence of and risk factors for ischemic stroke subtypes among 3 cohorts established at different times in a Japanese community, we demonstrated that the incidence of LAI declined significantly from the 1960s to the late 1990s, but LAI remained the most frequent subtype of ischemic stroke in the Japanese. The improvement in hypertension control might have had a major influence on this declining trend. However, hypertension still has a large impact on ischemic stroke, and the increasing prevalence of metabolic disorders might emerge as an additional risk in future cohorts. The present study indicates the need for continued primary prevention efforts, particularly with respect to hypertension and metabolic disorders.

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## Disclosure

None.

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## CLINICAL PERSPECTIVE

Stroke continues to be a major public health concern worldwide. Several epidemiological studies have reported that the declining or stable incidence of stroke is most often attributed to better treatment of risk factors over time. Here, by comparing the incidence of and risk factors for ischemic stroke subtypes among 3 cohorts established at different times in a Japanese community, we demonstrate that the age-adjusted incidence of ischemic stroke and of lacunar infarction declined significantly from the 1960s to the late 1990s, but lacunar infarction remains the most frequent subtype of ischemic stroke in the Japanese. Hypertension was a powerful risk factor for the development of ischemic stroke, and improvement of hypertension control would have largely influenced this declining trend: The age- and sex-adjusted hazard ratio of hypertension decreased from 3.25 (95% CI 2.17 to 4.86) in the first cohort to 1.83 (1.29 to 2.58) in the third cohort. However, hypertension still has a large impact on ischemic stroke, and the increase in metabolic disorders might emerge as an additional risk in the third cohort. The present study indicates the need for continued primary prevention efforts, particularly with respect to hypertension and metabolic disorders.

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# Inhibitory effects of aripiprazole on interferon-γ-induced microglial activation via intracellular $Ca^{2+}$ regulation *in vitro*

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**Abstract**

The activation of the inflammatory/immunological response system is suggested to be related to the pathophysiology of schizophrenia. Aripiprazole is a novel atypical antipsychotic, which is a high-affinity dopamine $D_2$ receptor partial agonist. Atypical antipsychotics, all of which have dopamine $D_2$ receptor antagonism, have recently reported to have significantly inhibitory effects on interferon (IFN)-γ-induced microglial activation *in vitro*. In the present study, we investigated whether or not aripiprazole also has anti-inflammatory effect on IFN-γ-induced microglial activation. Not quinpirole, dopamine $D_2$ full agonist, but aripiprazole significantly inhibited the generation of nitric oxide (NO) and tumor necrosis factor (TNF)-α from IFN-γ-activated microglia and suppressed the IFN-γ-induced elevation of intracellular $Ca^{2+}$ concentrations ($[Ca^{2+}]_i$) in murine microglial cells. Increased $[Ca^{2+}]_i$ has been reported to be required, but by itself not sufficient, for the release of NO and certain cytokines. As a result, we can speculate that aripiprazole may inhibit IFN-γ-induced microglial activation through the suppression of IFN-γ-induced elevation of $[Ca^{2+}]_i$ in microglia. Our results demonstrated that not only antipsychotics which have dopamine $D_2$ receptor antagonism but also aripiprazole have anti-inflammatory effects via the inhibition of microglial activation. Antipsychotics may therefore have a potentially useful therapeutic effect on patients with schizophrenia by reducing the microglial inflammatory reactions.

**Keywords:** atypical antipsychotic drug, inflammatory cytokine, intracellular $Ca^{2+}$ regulation, microglia, nitric oxide, schizophrenia.

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Microglial activation plays an important role in the pathophysiology of neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease via the release of proinflammatory cytokines, nitric oxide (NO) or reactive oxygen species (McGeer *et al.* 1993; Stoll and Jander 1999). Recent neuroimaging studies suggest the possibility that even schizophrenia may be a kind of neurodegenerative diseases like Alzheimer's disease and Parkinson's disease (Lieberman 1999; Lieberman *et al.* 2005; Salisbury *et al.* 2007). Postmortem brain studies using class II human leucocyte antigen (HLA-DR) have revealed microglial activation in the brains of patients with schizophrenia (Bayer *et al.* 1999; Radewicz *et al.* 2000; Steiner *et al.* 2006, 2008). The activation of the inflammatory/immunological response has been increasingly reported in patients with schizophrenia (Lin *et al.* 1998; Zhang *et al.* 2004; Bernstein *et al.* 2005; Drzyzga *et al.* 2006). Immunomodulatory drugs such as cyclooxygenase-2 inhibitors and minocycline, a potent inhibitor of microglial activation, have shown beneficial effects on symptoms of schizophrenia (Akhondzadeh *et al.* 2007; Miyaoka *et al.* 2007). Moreover, atypical antipsychotics, such as clozapine and risperidone, have reported to decrease serum levels of IL-2, IL-6 and tumor necrosis factor (TNF) −α in patients with schizophrenia (Lu *et al.* 2004).

For long time, dopamine $D_2$ receptor antagonism has been believed to be the primary therapeutic target for schizophre-

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*Abbreviations used*: $[Ca^{2+}]_i$, intracellular $Ca^{2+}$ concentration; BAPTA, 1,2-Bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid; BIM I, bisindolylmaleimide I; DMSO, dimethyl sulfoxide; ERK, extracellular signal regulated kinase; IFN-γ, interferon-γ; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetra-zolium bromide; NO, nitric oxide; PKC, protein kinase C; TNF-α, tumor necrosis factor-α.

 

nia because almost all antipsychotics have dopamine $D_2$ receptor antagonism (Kapur and Mamo 2003; Miyamoto *et al.* 2005). Aripiprazole is a novel atypical antipsychotic, which is a high-affinity dopamine $D_2$ receptor partial agonist (Burris *et al.* 2002; Shapiro *et al.* 2003). In spite of a different pharmacological profile, aripiprazole is effective against the positive and negative symptoms of patients with schizophrenia like other antipsychotics with lower side effects (Potkin *et al.* 2003; Tandon *et al.* 2006; Kane *et al.* 2007; Taylor *et al.* 2007).

We recently demonstrated that atypical antipsychotics such as risperidone, perospirone, quetiapine and ziprasidone, all of which have $D_2$ antagonism, have a significantly inhibitory effect on interferon (IFN) -γ-induced microglial activation *in vitro* (Kato *et al.* 2007; Bian *et al.* 2008). In the present study, we therefore investigated whether or not aripiprazole also has an inhibitory effect on IFN-γ-induced microglial activation. Intracellular $Ca^{2+}$ regulation plays an important role in microglial activation (Hoffmann *et al.* 2003). We therefore also investigated the effect of aripiprazole on the intracellular $Ca^{2+}$ regulation in IFN-γ-activated microglia.

## Materials and methods

### Materials

Aripiprazole was kindly donated by Otsuka Pharmaceutical Co., Ltd. (Tokushima, Japan). Recombinant IFN-γ and mouse granulocyte macrophage colony stimulating factor were purchased from R&D systems (Minneapolis, MN, USA). Bisindolylmaleimide I (BIM I) was purchased from BIOMOL (Plymouth Meeting, PA, USA). Quinpirole, SB203580, PD98059, 1,2-Bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid (BAPTA) AM and all other main chemicals were purchased from Sigma (St. Louis, MO, USA).

Aripiprazole was initially dissolved into 20 mM with dimethyl sulfoxide (DMSO) and then diluted to 1 mM with phosphate-buffered saline (PBS) for experiments. The final concentrations of aripiprazole were 5–20 μM. DMSO at the highest concentration (0.1%) under the experimental conditions was not toxic to cells.

### Cell cultures

The murine microglial cell line, 6-3, was kindly gifted from Prof. M. Sawada of Nagoya University. The 6-3 cells were established from neonatal C57BL/6J (H-2b) mice using a non-enzymatic and non-virus-transformed procedure (Kanzawa *et al.* 2000). The 6-3 cells closely resemble primary cultured microglia (Sawada *et al.* 1998; Kanzawa *et al.* 2000). The 6-3 cells were cultured in Eagle's minimal essential medium, 0.3% $NaHCO_3$, 2 mM glutamine, 0.2% glucose, 10 g/mL insulin and 10% fetal calf serum, and then were maintained at 37°C in a 10% $CO_2$ and 90% air atmosphere. One ng/mL mouse recombinant granulocyte macrophage colony stimulating factor was supplemented in the culture medium to maintain the 6-3 cells because these cells stopped proliferating in its absence (Kanzawa *et al.* 2000). The culture media were renewed twice per week.

Primary mixed cells were prepared from the whole brain of the 3-day-postnatal Sprague-Dawley rats, using Cell Strainer (BD Falcon, Franklin Lakes, NJ). Primary rat microglial cells were selected after attachment to Aclar film (Nisshin EM, Tokyo, Japan) for 2 h in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (10% FBS/Dulbecco's modified Eagle's medium). Aclar films were slightly washed by phosphate-buffered saline and then transferred to fresh 10% FBS/Dulbecco's modified Eagle's medium, and the fresh microglia was expanded for 1–2 days. The purity of the isolated microglia was assessed by immunocytochemical staining for microglial marker, Iba-1, and > 99% of cells were stained positively.

Rat pheocromocytoma PC12 cells were cultured in Eagle's minimal essential medium, 0.3% $NaHCO_3$, 2 mM glutamine, 0.2% glucose, 10 g/mL insulin and 10% fetal calf serum, and then were maintained at 37°C in a 10% $CO_2$ and 90% air atmosphere.

### Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from the 6-3 murine microglial cells using the RNeasy Mini Kit (Quiagen). RT-PCR was performed with RNA and gene-specific primer for dopamine $D_2$ receptors (forward: 5′-GCAGCCGAGCTTTCAGGGCC-3′ and reverse: 5′-GGGATGTT-GCAGTCACAGTG-3′) as previously described by Lemmer *et al.* (Lemmer *et al.* 2002) and reagents included in the SuperScript III RT-PCR System (invitrogen). Reverse transcription and Amplification was carried out in a gradient cycler (Biometra). The reaction mixture was incubated at 94°C for 2 min to fully activate the Taq DNA polymerase, then followed by a touchdown protocol of denaturing at 94°C for 15 s, annealing from 94°C down to 66°C for 30 s, and extension at 68°C for 1 min in 30 cycles. Finally, a 5-min extension at 68°C was conducted. PCR products were resolved by electrophoresis in 2% agarose gels, stained with ethidium bromide, and photographed. The predicted size was checked by a 100 bp DNA ladder (Biobeer).

### NO and TNF-α release assessment

The 6-3 cells were plated on 96-well tissue culture plates at $1 \times 10^5$ cells per 200 μL per well and then were pre-incubated in the presence or absence of aripiprazole or $D_2$ receptor full agonist, quinpirole for 12 h and then incubated in the presence or absence of 50 U/mL IFN-γ or 1 μg/mL LPS at 37°C. After 48 h, the collected media were assayed for NO or TNF-α accumulation. NO or TNF-α release into the culture medium was measured using a Griess reaction assay kit (Dojindo, Kumamoto, Japan) or a mouse TNF-α enzyme-linked immunosorbent assay (ELISA) kit (Biosource International, Camarillo, CA, USA), respectively. The absorbance of Griess reaction or ELISA was read at 540 nm or 450 nm, respectively, using a plate reader (Labsystems Multiscan MS, Frankfurt, Germany). Rat primary microglial cells were plated on 96-well tissue culture plates at $1 \times 10^4$ cells per 100 μL per well and then were pre-incubated in the presence or absence of aripiprazole or quinpirole for 12 h and then were incubated in the presence or absence of 50 U/mL IFN-γ or 1 μg/mL LPS at 37°C. After 48 h, the collected media were assayed for NO accumulation as described above.

### Intracellular $Ca^{2+}$ imaging

The experiments were performed in HEPES buffer (150 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose and 10 mM HEPES, pH 7.4 with Tris-OH) at room temperature (25°C).

Intracellular $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) was monitored using fura-2 (AM) (Grynkiewicz *et al.* 1985; Mizoguchi *et al.* 2002). The 6-3 cells plated on glass-base dish were loaded with 5 μM fura-2 AM (Dojindo, Wako, Japan) for 20 min and washed three times with HEPES buffer before the measurement. During the measurement using an inverted microscope (20×; Olympus IX70-22FL, Olympus Co. Tokyo, Japan), external HEPES buffer was constantly perfused (10 mL/min). For fura-2 excitation, the cells were illuminated with two alternating wavelengths, 340 and 380 nm using a computerized system. The emitted light was collected at 510 nm using a cooled CCD camera (C4742-95ER, Hamamatsu Photonics, Hamamatsu, Japan) and images were stored every 5 s. These series of sequential data were analyzed using the AquaCosmos software package (Hamamatsu photonics, Hamamatsu, Japan). The $[Ca^{2+}]_i$ was calculated from the ratio (R) of fluorescence recorded at 340 and 380 nm excitation wavelengths for each pixel within a cell boundary (AquaCosmos software). Calibrations (conversion of R340/380 values into calcium concentrations) were performed as described previously (Grynkiewicz *et al.* 1985). Basal $[Ca^{2+}]_i$ was determined from the initial 10 images of each cell recording. A $[Ca^{2+}]_i$ signal was defined as an increase in R 340/380 with clear time correlation to the application of IFN-γ.

### Cell viability

Cell viability was determined by colorimetric measurements of the reduction product of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT). After treatment with or without aripiprazole, the original medium was removed from the 96-well plates, and the cells were incubated for 2 h at 37°C in the presence of phenol red free minimum essential medium (Invitrogen Corporation, NY.) containing 0.5 mg/mL MTT. A 100 μL MTT lysis buffer (5% sodium dodecylsulfate and 5 mM HCl) was then added to each well, and the plates were incubated at 37°C overnight to dissolve the formazan that had formed in the wells. MTT is reduced to formazan in the mitochondria of living cells. Reduced MTT was measured by means of a plate reader (Labsystems Multiscan MS, Frankfurt, Germany) at a wavelength of 570 nm.

### Statistics

All data are represented as the means ± SEM and they were analyzed by a one-way analysis of variance (ANOVA) followed by Fisher's PLSD *post hoc* test for specific comparisons. Significance was established at a level of $p < 0.05$.

## Results

### The effect of aripiprazole on NO and TNF-α release by IFN-γ-activated microglia

We found the PCR products of the dopamine $D_{2L}$ receptors mRNA, thus indicating that the 6-3 murine microglial cells express dopamine $D_2$ receptors (Fig. 1).

Interferon-γ significantly induced NO and TNF-α release from the 6-3 murine microglial cells as which is consistent with previous reports (Kato *et al.* 2007). The 6-3 cells were pre-treated with DMSO (0.1%), aripiprazole (5, 10 and 20 μM) or $D_2$ receptor full agonist, quinpirole (5, 10 and 20 μM) for 12 h, then the cells were treated with each drug and IFN-γ (50 U/mL) for 48 h. Aripiprazole significantly inhibited the NO release dose-dependently in comparison with the positive control (DMSO + IFN-γ group), while quinpirole did not inhibit the NO release (Fig. 2a). In order to confirm whether these effects are specific to IFN-γ–induced microglial activation or not, we measured the effect of aripiprazole on LPS-induced microglial activation. Aripiprazole significantly inhibited NO release, while, quinpirole did not inhibit such NO release by LPS-activated 6-3 microglia (Fig. 2b). The degree of inhibition of NO release was less in LPS- than in IFN-γ– activated 6-3 microglia. These results suggest that the inhibitory effects of aripiprazole were not specific to IFN-γ receptor-mediated signaling. In addition, we also prepared rat primary microglial cells in order to confirm the relevance of our results in these cells. Similar to what was observed in the 6-3 murine microglial cells, aripiprazole significantly inhibited NO release from LPS-activated rat primary microglia, while, quinpirole did not inhibit NO release by LPS-activated rat primary microglia (Fig. 2c). The treatments of aripiprazole or quinpirole with or without LPS did not have any effect on the cell viability in these experiments (data not shown). These results suggest that the inhibitory effects of aripiprazole on microglial activation were not specific to the 6-3 microglia.

The 6-3 cells were pre-treated with DMSO (0.1%), aripiprazole (5, 10 and 20 μM) or quinpirole (5, 10 and 20 μM) for 12 h, then the cells were treated with each drug and IFN-γ (50 U/mL) for 48 h. Aripiprazole strongly inhibited the release of TNF-α dose-dependently. On the other hand, quinpirole had no inhibitory effect on the release

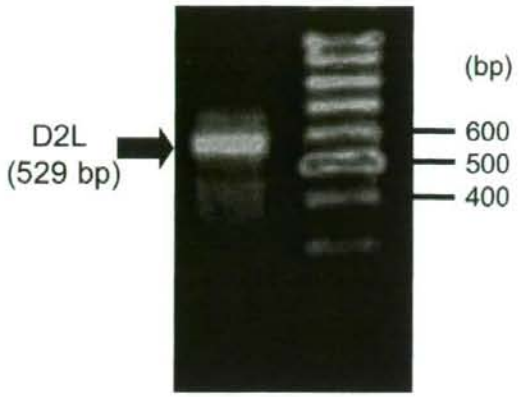


**Fig. 1** The expression of dopamine $D_2$ receptor mRNA in murine microglial cells. PCR using cDNA from the murine 6-3 microglial cells was performed. Agarose gel electrophoresis of PCR amplified products of dopamine $D_2$ receptors. The band corresponds to mRNA of the dopamine $D_{2L}$ receptors (529 bp).