most results are based on measurement of casual clinic blood pressure (CBP), which is less sensitive in detecting true changes in BP compared to home blood pressure (HBP) measurement [19,20]. HBP is measured by individuals themselves at home with a validated device over a long observation period, providing more reproducible and reliable values with less random error, without observer bias and without the white-coat effect [19,20]. Because of these advantages, HBP values have better predictive power for morbidity and mortality from cardiovascular diseases than CBP values [19–22], and HBP monitoring is now widely recommended in guidelines [19,21] and in a scientific statement [20].

To test the hypothesis that HBP measurements detect differences in BP between individuals exposed and those not exposed to ETS in the general population, the association between HBP values and ETS exposure was examined in a population-based, cross-sectional study.

Methods

Study population

The study was conducted as a part of the Ohasama study, a Japanese community-based, BP measurement project [23,24]. The total population of Ohasama was 7202 in 1998. Of this total population, 4964 were 35 years old or older. Of those, 1410 working outside of the town were considered ineligible and excluded from the study because they were not in the town during normal working hours. Of the remaining 3554 individuals, 213 were also excluded from the study because they were hospitalized, mentally ill, or bedridden. A total of 3341 participants were thus eligible for the study. A questionnaire was sent to each participant, and 1895 of the eligible participants gave their informed consent and responded to the questionnaire. Of those, 585 were excluded from the analysis because they were ex-smokers or current active smokers. Thus, the number of lifelong nonsmokers was 1310. Another 505 individuals with incomplete answers to the questions regarding demographic factors including ETS exposure were also excluded. Of the remaining 805 individuals, 754 who measured their HBP in the morning on at least three occasions (3 days) during the 4-week study period were included. This criterion was based on our previous observation that the average BP on the first three occasions was not significantly different from the mean for the entire study period [23]. Men ($n = 175$) were also excluded from the analysis because their number was small. Therefore, the study included 579 women [54.9% of the total number of lifelong nonsmoking women ($n = 1054$)].

Table 1 compares the characteristics of the included study participants with lifelong nonsmoking women who participated in the study but were ultimately excluded from the analysis due to incomplete data on ETS exposure (nonparticipants). The participants were characterized by a lower mean age, by lower percentages of participants taking antihypertensive medication and having a history of stroke and by higher percentages of participants being married and current drinkers.

Table 1 Characteristics of participants and nonparticipants in lifelong nonsmoking women ($n = 998$)

	Participants	Nonparticipants[a]	*P* value
N	579	419	
Mean age (years)	59.2 ± 13.1	64.1 ± 11.2	<0.0001
Marital status (married %)	71.0	61.3	0.0014
BMI (kg/m^2)	23.7 ± 3.3	23.7 ± 3.4	NS
Antihypertensive medication (%)	18.1	25.1	0.0081
History			
Diabetes mellitus (%)	8.6	9.3	NS
Stroke (%)	1.0	2.9	0.0323
Heart disease (%)	6.0	5.0	NS
Hyperlipidemia (%)	12.1	14.8	NS
Alcohol intake (current drinker %)	24.5	15.0	0.0003
Salt intake (≥12.28 g/day %)	50.1	43.9	NS
Time spent walking (≥1 h/day %)	79.8	81.1	NS

BMI, body mass index; ETS, environmental tobacco smoke. Student's *t*-test for continuous variables and χ^2-test for categorical variables. Continuous variables are expressed as mean ± SD. NS = $P > 0.05$. [a]Lifelong nonsmoking female participants who participated in the study but were ultimately excluded from the analysis due to incomplete data on ETS exposure.

Blood pressure and pulse rate measurement

The procedures used for HBP, pulse rate and CBP measurements, as well as the measuring devices, have been described elsewhere [23,25,26]. Briefly, physicians and public health nurses conducted health education classes to inform the participants about the HBP and pulse rate recording method, to teach them how to measure their own HBP and pulse rate, and to validate their ability to perform these tasks consistently. The women were then asked to measure their HBP and pulse rate every morning and evening and to record the results for 4 weeks. Measurements of morning HBP and pulse rate were made within 1 h of waking, before breakfast or taking any drugs, with the women seated and having rested for at least 2 min [27]. Measurements of evening HBP and pulse rate were obtained in a homologous way just before going to bed. The HBP and pulse rate of an individual were defined as the mean of all measurements obtained from that person. The mean ± SD numbers of morning HBP, morning pulse rate, evening HBP and evening pulse rate measurements were 22.6 ± 6.5 ($n = 579$), 22.4 ± 6.6 ($n = 567$), 22.8 ± 6.5 ($n = 577$) and 22.7 ± 6.6 ($n = 566$), respectively.

Two consecutive measurements of CBP were taken by a nurse or technician after the participant had been seated at rest for at least 2 min [23]. CBP was defined as the average of the two readings.

Blood pressure and pulse rate measuring device

HBP and pulse rate were measured with the HEM 701C (Omron Healthcare Co. Ltd, Kyoto, Japan), an automatic device based on the cuff-oscillometric method that

generates a digital display of systolic BP, diastolic BP and pulse rate. CBP was measured with a USM-700F (UEDA Electronic Works Co. Ltd, Tokyo, Japan), a fully automatic device based on the Korotkoff sound technique (a microphone method). The circumference of the arm was less than 34 cm in most cases, so a standard arm cuff was used for both BP measurements. All devices used in this study had been validated [25,26] and satisfied the criteria of the Association for the Advancement of Medical Instrumentation [28].

Definition of environmental tobacco smoke exposure

Environmental tobacco smoke exposure status was evaluated by the following two questions: 'How often are you exposed to smoke from cigarette smoking by other family members or guests at home?' and 'How often are you exposed to smoke from cigarette smoking by other persons at the workplace and/or other places?'. The women who responded 'hardly exposed' to both questions were categorized as those not exposed to ETS (non-ETS), whereas those who responded 'everyday', '3 or 4 days a week', '1 or 2 days a week' or 'occasionally' were categorized as those exposed to ETS. The exposed women were further classified into three categories according to their location of ETS exposure: those exposed to ETS at home [ETS(home)], those exposed to ETS at the workplace and/or other places [ETS(work/other)] and those exposed to ETS both at home and at the workplace and/or other places [ETS(both)]. For an additional analysis based on frequency of ETS exposure, the women who responded 'everyday' to either question were categorized as those exposed to ETS everyday [ETS(everyday)], whereas the remaining women who responded '3 or 4 days a week', '1 or 2 days a week' and 'occasionally' to either question were categorized as those exposed to ETS less frequently than everyday [ETS(occasionally)].

Data analysis

Information on smoking status, ETS exposure status, marital status, history of diabetes mellitus, history of stroke, history of heart disease, history of hyperlipidemia, alcohol intake, salt intake and activity levels (time spent walking per day) was obtained from the questionnaire. A standardized methodology was used to calculate dietary salt (NaCl) intake from a Japanese version of the food-frequency questionnaire. The reproducibility and validity of this version were previously reported in detail [29,30]. Information on age and use of antihypertensive medication was obtained from another questionnaire sent to each household at the time of the HBP measurements. Body mass index (BMI) information was obtained from medical records kept at Ohasama Hospital and from annual health check-up records.

The participants were stratified according to use of antihypertensive medication to avoid possible mitigation of pressor effect of ETS, because relatively small differences in BP between the participants exposed and those not exposed to ETS were expected to be detected from previous findings [17,18]. Variables were compared using the t-test, analysis of variance (ANOVA), χ^2-test, a logistic regression analysis adjusted for age (years) or analysis of covariance (ANCOVA) adjusted for age (years), marital status (married or single/divorced/widowed), BMI (kg/m^2), history of diabetes mellitus, history of stroke, history of heart disease, history of hyperlipidemia, alcohol intake (current drinker or not current drinker), salt intake (less than the median of 12.28 g/day or greater than or equal to the median) and time spent walking (less than 1 h/day or greater than or equal to 1 h/day), as appropriate. The level of statistical significance was set at $P < 0.05$. Data are presented as percentages or means ± SD (for the t-test and ANOVA) or means ± SE (for ANCOVA). All analyses were performed with SAS software version 9.1 (SAS Institute Inc., Cary, North Carolina, USA).

Results

Home blood pressure and pulse rate of the participants without antihypertensive medication

The characteristics of the study participants are presented in Table 2. Mean age, marital status and percentages of current drinkers were significantly different among the categories of ETS exposure status. This might have been due to the marked differences in age, because working women are usually younger than retirement age and their spouses may be comparatively younger and healthier. Younger women may also have more social opportunities to consume alcohol. A logistic regression analysis was performed to determine whether these factors are significantly different among the categories of ETS exposure status after adjusting for age. The results showed that marital status was not significantly different ($P = 0.40$), whereas percentages of current drinkers remained significantly different among the categories of ETS exposure status ($P = 0.01$).

Table 3 shows HBP and pulse rate levels by location of ETS exposure. The systolic morning HBP value in ETS(both) was approximately 4 mmHg higher than that in non-ETS ($P = 0.02$), and the systolic morning HBP value in ETS(home) and the systolic evening HBP value in ETS(both) were approximately 3 mmHg higher than those in non-ETS ($P = 0.04$ and $P = 0.03$, respectively). There was also a tendency for systolic morning HBP and systolic evening HBP values of all categories exposed to ETS to be higher than those in non-ETS. Systolic morning HBP and systolic evening HBP levels were not significantly different among the categories exposed to ETS, and diastolic HBP and pulse rate levels were not significantly associated with any ETS exposure status. There were no significant interactions between age and ETS exposure status on any HBP and pulse rate levels (all P for interaction >0.2).

Table 2 Characteristics of the participants without antihypertensive medication by ETS location ($n=474$)

	non-ETS	ETS(work/other)	ETS(home)	ETS(both)	*P* value
N	143	47	129	155	
Mean age (years)	64.0 ± 10.7	47.7 ± 9.4	58.3 ± 12.8	52.3 ± 10.7	<0.0001
Marital status (married %)	64.3	85.1	72.9	83.2	0.0007
BMI (kg/m^2)	23.2 ± 3.2	23.4 ± 2.4	23.6 ± 3.5	23.5 ± 3.2	NS
History					
Diabetes mellitus (%)	6.3	8.5	8.5	7.1	NS
Stroke (%)	0.7	0.0	0.8	0.0	NS
Heart disease (%)	6.3	4.3	4.7	2.6	NS
Hyperlipidemia (%)	14.0	4.3	12.4	6.5	NS
Alcohol intake (current drinker %)	12.6	36.2	24.0	40.0	<0.0001
Salt intake (≥12.28 g/day%)	50.3	44.7	47.3	55.5	NS
Time spent walking (≥1 h/day %)	81.1	70.2	82.9	83.2	NS

BMI, body mass index; ETS, exposure to environmental tobacco smoke. Analysis of variance for continuous variables and χ^2-test for categorical variables. Continuous variables are expressed as mean ± SD. NS = $P > 0.05$.

Because percentages of current drinkers were significantly different among the categories of ETS exposure status after adjusting for age, subgroup analysis was performed in noncurrent drinkers. The results showed a similar tendency presented in Table 3 (data not presented).

Table 4 presents the results of the additional analysis based on frequency of ETS exposure. There was a similar tendency for systolic morning HBP and systolic evening HBP values of all categories exposed to ETS, including the values in ETS(occasionally), to be higher than those in non-ETS, as presented in Table 3. The results showed significant differences between the systolic morning HBP value in ETS(everyday) and that in non-ETS and between the systolic evening HBP value in ETS(everyday) and that in non-ETS ($P=0.02$ and $P=0.03$, respectively).

Home blood pressure and pulse rate of the participants with antihypertensive medication

Home blood pressure and pulse rate levels by location and frequency of ETS exposure ($n=105$) showed no significant differences in systolic HBP values between any ETS exposure group and the non-ETS group ($P>0.2$ and $P>0.5$, respectively). No other HBP and pulse rate levels were significantly associated with any ETS exposure status (data not presented).

Casual clinic blood pressure and pulse rate of the participants without antihypertensive medication

Table 5 shows mean CBP levels by location of ETS exposure. CBP values were available from 296 (62.4%) study participants without antihypertensive medication. The systolic and diastolic CBP values in ETS(home) were significantly higher than those in non-ETS ($P=0.02$ and $P=0.04$, respectively). No other significant differences in CBP values were seen between any ETS exposure group and the non-ETS group ($P>0.6$).

Discussion

The present results confirm that there is a relationship between increased HBP levels and ETS exposure in Japanese women without antihypertensive medication. HBP measurements detect approximately a 3–4 mmHg difference in BP between the ETS(home) and the ETS-(both) groups and the non-ETS group, whereas CBP measurements detect significant differences only between the ETS(home) group and the non-ETS group. Thus, HBP measurement is a more sensitive measurement for detecting small BP changes.

In the present study, systolic morning HBP values in ETS(home) and in ETS(both) and systolic evening HBP value in ETS(both) were significantly higher than those in non-ETS, whereas diastolic HBP and pulse rate levels were not significantly associated with any ETS exposure status. These findings are consistent with those of Heiss *et al.* [15] and Mahmud and Feely [16], who investigated the relationship between ETS exposure and BP levels in experimental studies. Makris *et al.* [17] investigated the association between ambulatory BP values and ETS exposure in 254 clinically normotensive nonsmokers who were self-referred to their outpatient

Table 3 HBP and PR of the participants without antihypertensive medication by ETS location

	non-ETS	ETS(work/other)	ETS(home)	ETS(both)
Systolic morning HBP (mmHg)	113.1 ± 1.08	114.7 ± 1.85	116.2 ± 1.07[a]	116.8 ± 1.01[a]
Diastolic morning HBP (mmHg)	71.0 ± 0.73	71.4 ± 1.24	71.6 ± 0.72	72.0 ± 0.68
Morning PR (beats/min)	66.2 ± 0.62	66.9 ± 1.06	66.9 ± 0.63	66.9 ± 0.59
Systolic evening HBP (mmHg)	111.9 ± 1.09	114.2 ± 1.86	114.3 ± 1.08	115.3 ± 1.02[a]
Diastolic evening HBP (mmHg)	69.0 ± 0.74	70.3 ± 1.26	69.4 ± 0.73	70.6 ± 0.69
Evening PR (beats/min)	68.7 ± 0.60	68.4 ± 1.02	68.7 ± 0.60	69.4 ± 0.57

BMI, body mass index; ETS, exposure to environmental tobacco smoke; HBP, home blood pressure; PR, pulse rate. Analysis of covariance. Data were adjusted for age, marital status (married or single/divorced/widowed), BMI, history of diabetes mellitus, history of stroke, history of heart disease, history of hyperlipidemia, alcohol intake (current drinker or not current drinker), salt intake (≥12.28 g/day or <12.28 g/day) and time spent walking (≥1 h/day or <1 h/day). Data are expressed as mean ± SE.
[a] $P<0.05$ compared to non-ETS.

Table 4 HBP and PR of the participants without antihypertensive medication by ETS frequency

	Non-ETS	ETS(occasionally)	ETS(everyday)
N	143	155	176
Systolic morning HBP (mmHg)	113.0 ± 1.08	115.9 ± 0.98	116.7 ± 0.95[a]
Diastolic morning HBP (mmHg)	71.1 ± 0.72	72.0 ± 0.66	71.5 ± 0.64
Morning PR (beats/min)	66.2 ± 0.62	66.5 ± 0.57	67.2 ± 0.55
Systolic evening HBP (mmHg)	111.9 ± 1.08	114.2 ± 0.99	115.2 ± 0.96[a]
Diastolic evening HBP (mmHg)	69.1 ± 0.74	70.2 ± 0.67	69.9 ± 0.65
Evening PR (beats/min)	68.7 ± 0.60	68.6 ± 0.55	69.3 ± 0.53

BMI, body mass index; ETS, exposure to environmental tobacco smoke; HBP, home blood pressure; PR, pulse rate. Analysis of covariance. Data were adjusted for age, marital status (married or single/divorced/widowed), BMI, history of diabetes mellitus, history of stroke, history of heart disease, history of hyperlipidemia, alcohol intake (current drinker or not current drinker), salt intake (≥12.28 g/day or <12.28 g/day) and time spent walking (≥1 h/day or <1 h/day). Data are expressed as mean ± SE. [a] $P < 0.05$ compared to non-ETS.

hypertension clinic. Their results show that 24-h and daytime systolic BP, heart rate and daytime diastolic BP values are significantly higher in those with at least 1 h daily ETS exposure, compared with those with less exposure and those without ETS exposure. Although the study population and categories of ETS exposure status are different, the present results are consistent with their findings in that out-of-clinic BP measurements detect a difference in BP between individuals exposed and those not exposed to ETS.

Not only were the systolic HBP values of the ETS(home), the ETS(both) and the ETS(everyday) groups significantly higher than those in non-ETS, but systolic morning HBP and systolic evening HBP values of all categories exposed to ETS, including the ETS(work/other) and the ETS(occasionally) groups, tended to be higher than those in non-ETS in the present study. These findings indicate that ETS exposure may elevate systolic HBP regardless of location and frequency of exposure, which is consistent with the previous findings that even a small amount of ETS exposure causes detrimental effects at the clinical level [31,32]. Since systolic HBP is a strong predictive factor for morbidity and mortality from cardiovascular diseases [33,34], the present results may also reflect that a pressor effect, as well as other deleterious effects, of ETS exposure contribute to increased morbidity and mortality from cardiovascular diseases [1–9] in the general population.

Considering the fact that the pathophysiological and hemodynamic effects of ETS exposure last for 24 h after 30 min of ETS exposure at the experimental level [15], that the systolic HBP values of all categories exposed to ETS were consistently higher than those of the non-ETS group, and that the present results were obtained from multiple HBP measurements for a mean of 3 weeks, the present results may reflect a nonlinear persistent pressor effect caused by ETS exposure in the general population. Although there is a possibility that the present results may reflect a much shorter duration of pressor effects of ETS just after exposure, especially in the morning when many smokers tend to smoke just after waking, the present results are important from a prognostic hemodynamic standpoint. Since HBP measurement detects small BP changes, it may reflect persistent effects of ETS exposure and is more feasible to monitor a large population regularly, a further study using HBP measurement is necessary to clarify the chronic deleterious hemodynamic effects of ETS exposure at the population level, with more detailed data on ETS exposure status. HBP measurement may also be useful for future studies investigating the hemodynamic effects of other air pollutants, such as ambient particulate matter [35].

Differences in HBP between women exposed and those not exposed to ETS were not observed in women with antihypertensive medication. This might be because the relatively small pressor effect of ETS exposure was mitigated by the large BP-lowering effects of antihypertensive drugs. It is necessary to consider a pressor effect of ETS exposure at least when interpreting HBP data from normotensive or prehypertensive patients in clinical practice. The present results obviously raise concerns over public health. Achievement of smoke-free environments is thus also important from a hemodynamic standpoint.

Several limitations of the present study need to be discussed. First, as more detailed data on time, duration

Table 5 CBP of the participants without antihypertensive medication by ETS location ($n = 296$)

	non-ETS	ETS(work/other)	ETS(home)	ETS(both)
N	102	21	96	77
Systolic CBP (mmHg)	126.6 ± 1.46	125.4 ± 3.24	131.5 ± 1.46[a]	126.5 ± 1.68
Diastolic CBP (mmHg)	71.6 ± 0.89	70.8 ± 1.98	74.2 ± 0.89[a]	72.4 ± 1.03

BMI, body mass index; CBP, casual clinic blood pressure; ETS, exposure to environmental tobacco smoke. Analysis of covariance. Data were adjusted for age, marital status (married or single/divorced/widowed), BMI, history of diabetes mellitus, history of stroke, history of heart disease, history of hyperlipidemia, alcohol intake (current drinker or not current drinker), salt intake (≥12.28 g/day or <12.28 g/day) and time spent walking (≥1 h/day or <1 h/day). Data are expressed as mean ± SE. [a] $P < 0.05$ compared to non-ETS.

and quantity of ETS exposure were unavailable in our study population, the dose–response relationship between HBP levels and ETS exposure is unknown. A further study using HBP measurement is necessary with more detailed data on ETS exposure status. Second, although age distribution of the categories of ETS exposure status was uneven, age did not significantly interact with ETS exposure status on the present results. Third, as the study was cross-sectional, the results do not show a causal relationship between ETS exposure and BP elevation or development of hypertension. A longitudinal study is necessary to investigate this causal relationship in the Ohasama study, as well as in other populations. Fourth, the study excluded men due to the small number of lifelong nonsmoking men. It remains to be investigated whether a positive association between ETS exposure and BP is present in men. Lastly, since a biological marker of ETS exposure, such as cotinine concentration, was not measured, there may be misclassification of ETS exposure status. However, ETS exposure status in a self-administered questionnaire is shown to be generally accurate in a large-scale cohort study in a Japanese population, with a slightly higher rate of passive smokers falsely reporting themselves to be nonpassive smokers compared to Western studies [36]. Therefore, we believe that the present results are acceptable, but they may underestimate the true magnitude of the hemodynamic effects of ETS exposure due to these misclassifications.

In conclusion, this is the first population-based study demonstrating a significant association between increased HBP and ETS exposure. HBP measurement is recommended to investigate the effects of ETS exposure in the general population. ETS exposure may increase BP levels, which may synergistically contribute to unfavorable cardiovascular outcomes, along with the other deleterious effects of ETS.

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References

1 US Department of Health and Human Services. *The health consequences of involuntary exposure to tobacco smoke: a report of the Surgeon General.* Rockville, MD: US Department of Health and Human Services; 2006.
2 Sandler DP, Comstock GW, Helsing KJ, Shore DL. Deaths from all causes in nonsmokers who lived with smokers. *Am J Public Health* 1989; **79**:163–167.
3 You RX, Thrift AG, McNeil JJ, Davis SM, Donnan GA. Ischemic stroke risk and passive exposure to spouses' cigarette smoking. *Am J Public Health* 1999; **89**:572–575.
4 Bonita R, Duncan J, Truelsen T, Jackson RT, Beaglehole R. Passive smoking as well as active smoking increases the risk of acute stroke. *Tobacco Control* 1999; **8**:156–160.
5 Iribarren C, Darbinian J, Klatsky AL, Friedman GD. Cohort study of exposure to environmental tobacco smoke and risk of first ischemic stroke and transient ischemic attack. *Neuroepidemiology* 2004; **23**:38–44.
6 Wen W, Shu XO, Gao YT, Yang G, Li Q, Li H, Zheng W. Environmental tobacco smoke and mortality in Chinese women who have never smoked: prospective cohort study. *BMJ* 2006; **333**:376.
7 He Y, Lam TH, Jiang B, Wang J, Sai X, Fan L, *et al.* Passive smoking and risk of peripheral arterial disease and ischemic stroke in Chinese women who never smoked. *Circulation* 2008; **118**:1535–1540.
8 Glymour MM, DeFries TB, Kawachi I, Avendano M. Spousal smoking and incidence of first stroke: the Health and Retirement Study. *Am J Prev Med* 2008; **35**:245–248.
9 McGhee SM, Ho SY, Schooling M, Ho LM, Thomas GN, Hedley AJ, *et al.* Mortality associated with passive smoking in Hong Kong. *BMJ* 2005; **330**:287–288.
10 Celermajer DS, Adams MR, Clarkson P, Robinson J, McCredie R, Donald A, Deanfield JE. Passive smoking and impaired endothelium-dependent arterial dilatation in healthy young adults. *N Engl J Med* 1996; **334**:150–154.
11 Sumida H, Watanabe H, Kugiyama K, Ohgushi M, Matsumura T, Yasue H. Does passive smoking impair endothelium-dependent coronary artery dilation in women? *J Am Coll Cardiol* 1998; **31**:811–815.
12 Raitakari OT, Adams MR, McCredie RJ, Griffiths KA, Celermajer DS. Arterial endothelial dysfunction related to passive smoking is potentially reversible in healthy young adults. *Ann Intern Med* 1999; **130**:578–581.
13 Woo KS, Chook P, Leong HC, Huang XS, Celermajer DS. The impact of heavy passive smoking on arterial endothelial function in modernized Chinese. *J Am Coll Cardiol* 2000; **36**:1228–1232.
14 Otsuka R, Watanabe H, Hirata K, Tokai K, Muro T, Yoshiyama M, *et al.* Acute effects of passive smoking on the coronary circulation in healthy young adults. *JAMA* 2001; **286**:436–441.
15 Heiss C, Amabile N, Lee AC, Real WM, Schick SF, Lao D, *et al.* Brief secondhand smoke exposure depresses endothelial progenitor cells activity and endothelial function: sustained vascular injury and blunted nitric oxide production. *J Am Coll Cardiol* 2008; **51**:1760–1771.
16 Mahmud A, Feely J. Effects of passive smoking on blood pressure and aortic pressure waveform in healthy young adults: influence of gender. *Br J Clin Pharmacol* 2004; **57**:37–43.
17 Makris TK, Thomopoulos C, Papadopoulos DP, Bratsas A, Papazachou O, Massias S, *et al.* Association of passive smoking with masked hypertension in clinically normotensive nonsmokers. *Am J Hypertens* 2009; **22**:853–859.
18 Whincup PH, Gilg JA, Emberson JR, Jarvis MJ, Feyerabend C, Bryant A, *et al.* Passive smoking and risk of coronary heart disease and stroke: prospective study with cotinine measurement. *BMJ* 2004; **329**:200–205.

19 Parati G, Stergiou GS, Asmar R, Bilo G, de Leeuw P, Imai Y, *et al.* European Society of Hypertension guidelines for blood pressure monitoring at home: a summary report of the Second International Consensus Conference on Home Blood Pressure Monitoring. *J Hypertens* 2008; **26**:1505–1530.

20 Pickering TG, Miller NH, Ogedegbe G, Krakoff LR, Artinian NT, Goff D, American Heart Association; American Society of Hypertension; Preventive Cardiovascular Nurses Association. Call to action on use and reimbursement for home blood pressure monitoring: a joint scientific statement from the American Heart Association, American Society of Hypertension, and Preventive Cardiovascular Nurses Association. *Hypertension* 2008; **52**:10–29.

21 Imai Y, Otsuka K, Kawano Y, Shimada K, Hayashi H, Tochikubo O, *et al.* Japanese society of hypertension (JSH) guidelines for self-monitoring of blood pressure at home. *Hypertens Res* 2003; **26**:771–782.

22 Ohkubo T. Prognostic significance of variability in ambulatory and home blood pressure from the Ohasama study. *J Epidemiol* 2007; **17**:109–113.

23 Imai Y, Satoh H, Nagai K, Sakuma M, Sakuma H, Minami N, *et al.* Characteristics of a community-based distribution of home blood pressure in Ohasama in northern Japan. *J Hypertens* 1993; **11**:1441–1449.

24 Tsuji I, Imai Y, Nagai K, Ohkubo T, Watanabe N, Minami N, *et al.* Proposal of reference values for home blood pressure measurement: prognostic criteria based on a prospective observation of the general population in Ohasama, Japan. *Am J Hypertens* 1997; **10**:409–418.

25 Imai Y, Abe K, Sasaki S, Minami N, Munakata M, Sakuma H, *et al.* Clinical evaluation of semiautomatic and automatic devices for home blood pressure measurement: comparison between cuff-oscillometric and microphone methods. *J Hypertens* 1989; **7**:983–990.

26 Imai Y, Nishiyama A, Sekino M, Aihara A, Kikuya M, Ohkubo T, *et al.* Characteristics of blood pressure measured at home in the morning and in the evening: the Ohasama study. *J Hypertens* 1999; **17**:889–898.

27 Ogihara T, Kikuchi K, Matsuoka H, Fujita T, Higaki J, Horiuchi M, *et al.* The Japanese Society of Hypertension Guidelines for the Management of Hypertension (JSH 2009). *Hypertens Res* 2009; **32**:3–107.

28 Association for the Advancement of Medical Instrumentation. *American National Standards for Electronic or Automated Sphygmomanometers.* Washington, DC: Association for the Advancement of Medical Instrumentation; 1987.

29 Ogawa K, Tsubono Y, Nishino Y, Watanabe Y, Ohkubo T, Watanabe T, *et al.* Validation of a food-frequency questionnaire for cohort studies in rural Japan. *Public Health Nutr* 2003; **6**:147–157.

30 Tsubono Y, Ogawa K, Watanabe Y, Nishino Y, Tsuji I, Watanabe T, *et al.* Food frequency questionnaire and a screening test. *Nutr Cancer* 2001; **39**:78–84.

31 Glantz SA, Parmley WW. Even a little secondhand smoke is dangerous. *JAMA* 2001; **286**:462–463.

32 Pechacek TF, Babb S. How acute and reversible are the cardiovascular risks of secondhand smoke? *BMJ* 2004; **328**:980–983.

33 Hozawa A, Ohkubo T, Nagai K, Kikuya M, Matsubara M, Tsuji I, *et al.* Prognosis of isolated systolic and isolated diastolic hypertension as assessed by self-measurement of blood pressure at home: the Ohasama study. *Arch Intern Med* 2000; **160**:3301–3306.

34 Inoue R, Ohkubo T, Kikuya M, Metoki H, Asayama K, Kanno A, *et al.* Stroke risk of blood pressure indices determined by home blood pressure measurement. The Ohasama Study. *Stroke* 2009; **40**:2859–2861.

35 Brook RD. Why physicians who treat hypertension should know more about air pollution. *J Clin Hypertens* 2007; **9**:629–635.

36 Ozasa K, Higashi A, Yamasaki M, Hayashi K, Watanabe Y. Validity of self-reported passive smoking evaluated by comparison with smokers in the same household. *J Epidemiol* 1997; **7**:205–209.

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Original Article

New reference growth charts for Japanese girls with Turner syndrome

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Abstract ***Background***: Currently used growth charts for Japanese girls with Turner syndrome (TS) were constructed with auxological data obtained before the secular trend in growth reached a plateau. These charts were published in 1992 and may no longer be valid for the evaluation of stature and growth in girls with TS in clinical settings. Thus, we need to establish new clinical growth charts.

Methods: The samples for analysis were obtained by a retrospective cohort study. A total of 1867 Japanese girls with TS were registered between 1991 and 2004 for growth hormone (GH) treatment and their pretreatment anthropometric measurements were obtained. Reference growth charts were newly constructed using the LMS method from 1447 girls' cross-sectional data after exclusion of measurements derived from those with the presence of puberty, with previous growth-promoting treatment, or without cytogenetic evidence of TS.

Results: The new clinical reference growth charts differ from the old charts. Secular trends can be detected in both height and weight. Mean adult height on the new chart is 141.2 cm, 3.0 cm taller than the old data. This result seems attributable to the secular trend observed during the same period in Japanese women.

Conclusions: The newly constructed clinical reference growth charts for Japanese girls with TS seem to be better for the evaluation of growth in girls with TS born after approximately 1970, although selection bias and some other limitations in the present study should be kept in mind.

Key words growth chart, LMS method, secular trend, Turner syndrome.

Background

Turner syndrome (TS) is the most common chromosomal disorder in girls and affects about one in 1500 to 2500 live-born female infants.[1] One of the most significant features of the syndrome is short stature. Untreated girls are reported to be approximately 20 cm shorter than normal girls within their respective populations.[2] Growth hormone (GH) has been used to accelerate growth, and it is known to increase adult height.[3]

Growth patterns of girls with TS are different from those in normal populations mainly because of the short stature homeobox-containing gene on the X chromosome (SHOX) haploinsufficiency and their ovarian insufficiency. TS-specific growth curves have been published in various countries[4–11] including Japan,[12] and they have been clinically used for the evaluation of stature and growth. Those of the Japanese were constructed with data from subjects whose body measurements were obtained by sending questionnaires to their follow-up hospitals. The data consisted of 6255 measurements from 705 girls born between 1955 and 1989.

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Japan has experienced extremely rapid changes in eating habits together with vast socioeconomic changes since the end of the Second World War, and these changes have affected Japanese children's growth. The physical size of Japanese children has increased along with these environmental changes, and nutrition is thought to be the most important contributing factor. In Japan the food supply has been sufficient or even excessive since 1970. The acceleration of growth is reported to have been most prominent between 1955 and 1970,[13] but it has reached a plateau since around 1990, as discussed later. Thus the subjects analyzed in the currently used charts were born before the secular trend approached the recent plateau. Therefore, use of the presently available growth charts may be inadequate for the evaluation of recent cases of TS. In this context, construction of new reference charts and their validation have become necessary.

Methods

Population

The samples were obtained from a database compiled by the Foundation for Growth Science, Japan. The Foundation has been controlling the use of GH through its registration system in Japan, which judges candidates' eligibility for supplemental GH treatment according to the diagnostic criteria for GH deficiency established by the Ministry of Health, Labor and Welfare's Study

Table 1 Age distribution

Age (years)	Number
0	1
1	9
2	14
3	41
4	74
5	104
6	105
7	104
8	113
9	152
10	160
11	168
12	131
13	75
14	68
15	52
16	38
17	22
18	11
19	2
20	3
Total	1447

Group for Hypothalamo-Pituitary Disorders.[14] Medical doctors are encouraged to have each candidate registered for GH treatment at the Foundation using an application form that includes his/her pre-treatment anthropometric measurements, karyotype (in the case of TS), presence or absence of puberty, and evidence of informed consent from each subject regarding the use of the data for scientific purposes.

Between 1991 and 2004, 1867 girls were registered as TS subjects in this cohort. The diagnosis of TS was confirmed by reviewing all the reported karyotypes of cultured peripheral blood lymphocytes. In this study TS was defined as a karyotype that contains a cell line of monosomy lacking at least a distal major part in the short arm of the X chromosome. Subjects having no evidence of such karyotypic features, missing a description regarding puberty status, with secondary pubertal signs, with a history of previous growth-promoting therapy, or whose age was over 20 were excluded.

Statistical analysis

Data were cleaned in several stages. Bivariate plots of height and weight were used to identify gross disproportions. Data points were scrutinized, going back to the source data if necessary, and transcription errors were corrected. If a value was deemed highly unlikely (more than 5 standard deviation scores [SDS] from the mean), such a point was deleted, even in the absence of any evidence of a transcription error.

Reference growth charts were obtained using the LMS method,[15] which assumes that the data can be transformed to normality by a suitable power transformation (L); the distribution is then summarized by the median (M) and the coefficient of variation (S). The values of L, M, and S are constrained to change smoothly with age, and fitted values can be used to construct any required centile curves. The karyotypes of 45,X and non-45,X were compared for body height using analysis of covariance (ANCOVA) with covariates of age and age-karyotype interaction. This analysis was performed using JMP 6.0.3 (SAS Institute Inc., Cary, NC, USA.) and *P*-values less than 0.05 were considered statistically significant.

Results

In total, 420 subjects were excluded because of insufficient or inadequate cytogenetic evidence for the diagnosis (31 subjects), secondary pubertal signs (107 subjects), lack of records about puberty (14 subjects), previous growth-promoting treatment (264 subjects), age over 20 (one subject) and highly unlikely measurements (three subjects). The remaining 1447 subjects were analyzed. Table 1 lists the number of subjects according to age. Their birth years range from 1970 to 2002 (median: 1985). Perinatal information and their parents' anthropometric measurements were collected whenever possible. Gestational age is 39.6+/–1.6 weeks (n = 1268), birth length 46.8+/–2.7 cm (n = 633), birthweight 2.68+/–0.44 kg (n = 1322), and target height 157.6+/–7.2 cm (n = 1289). Target height was calculated by the formula adjusted for the Japanese before the secular trend reached a

Table 2 Karyotypes of 1447 subjects

	Non-Mosaic	Number of subjects	Mosaic	Number of subjects
Aneuploidy	45,X	432	45,X/46,XX	87
			45,X/47,XXX	91
			45,X/46,XY	16
			45,X/46,XX/47,XXX	6
		432		200
Structural abnormality	46,X,i(Xq)	128	45,X/46,X,i(Xq)	309
	46,X,del(Xp)	55	45,X/46,X,del(Xp)	22
	46,X,r(X)	3	45,X/46,X,r(X)	106
	others	10	45,X/46,X,+mar	109
			others	73
		196		619
Total		628		819

Table 3 LMS values of height and weight for the Japanese girls with Turner syndrome

Height				Weight			
Age (years)	L	M	S	Age (years)	L	M	S
1	1	66.75	0.024	1	1.63	6.92	0.094
1.5	1	71.25	0.025	1.5	1.37	8.02	0.094
2	1	75.44	0.026	2	1.11	9.10	0.094
2.5	1	79.1	0.026	2.5	0.86	10.06	0.095
3	1	82.39	0.027	3	0.64	10.90	0.096
3.5	1	85.46	0.028	3.5	0.44	11.65	0.097
4	1	88.38	0.028	4	0.24	12.37	0.099
4.5	1	91.11	0.029	4.5	0.06	13.04	0.102
5	1	93.68	0.029	5	−0.12	13.71	0.106
5.5	1	96.23	0.030	5.5	−0.32	14.44	0.111
6	1	98.75	0.030	6	−0.51	15.24	0.117
6.5	1	101.24	0.031	6.5	−0.69	16.10	0.124
7	1	103.81	0.031	7	−0.87	17.12	0.131
7.5	1	106.39	0.032	7.5	−1.03	18.32	0.139
8	1	108.79	0.032	8	−1.14	19.58	0.147
8.5	1	111.02	0.033	8.5	−1.19	20.83	0.154
9	1	113.18	0.033	9	−1.16	22.12	0.160
9.5	1	115.32	0.034	9.5	−1.04	23.52	0.165
10	1	117.53	0.034	10	−0.84	25.08	0.170
10.5	1	119.89	0.035	10.5	−0.60	26.76	0.176
11	1	122.35	0.035	11	−0.40	28.51	0.182
11.5	1	124.76	0.036	11.5	−0.28	30.26	0.187
12	1	127.03	0.036	12	−0.25	31.94	0.189
12.5	1	129.14	0.037	12.5	−0.26	33.50	0.190
13	1	131.03	0.037	13	−0.26	34.93	0.191
13.5	1	132.69	0.037	13.5	−0.23	36.23	0.191
14	1	134.14	0.038	14	−0.17	37.40	0.191
14.5	1	135.37	0.038	14.5	−0.10	38.43	0.191
15	1	136.38	0.038	15	−0.01	39.33	0.192
15.5	1	137.24	0.038	15.5	0.08	40.11	0.192
16	1	137.96	0.039	16	0.16	40.79	0.192
16.5	1	138.56	0.039	16.5	0.23	41.39	0.192
17	1	139.07	0.039	17	0.28	41.93	0.192
17.5	1	139.49	0.039	17.5	0.32	42.43	0.192
18	1	139.87	0.039	18	0.37	42.91	0.192
18.5	1	140.23	0.039	18.5	0.40	43.36	0.191
19	1	140.58	0.039	19	0.44	43.81	0.191
19.5	1	140.91	0.039	19.5	0.47	44.24	0.191
20	1	141.24	0.039	20	0.51	44.67	0.190

plateau.[16] Table 2 summarizes the number of subjects grouped by karyotype. There was no significant difference in height between 45,X and non-45,X subjects (regression coefficient: 0.19+/−0.14 cm, $P = 0.17$).

Centile curves were fitted to the data of all subjects together using the LMS method. For height, the distribution was assumed to be normal, while for weight there was appreciable skewness, to which the age-varying power transformation was adjusted. Table 3 provides values for L, M and S of height and weight by age. Clinical growth references for height and weight are shown in Figures 1 and 2, respectively. References for height and weight expressed as SDS are superimposed on those that are currently used in Figures 3 and 4.

Discussions

We produced new clinical reference growth charts for Japanese girls with TS who did not present with puberty. The charts were constructed using the LMS method, which we believe is one of the most widely applied approaches.[17] The LMS method is often used to construct age-related references not only of normal populations[18] but also of Down syndrome[19] and Williams syndrome[20] disease-specific populations. The number of subjects analyzed in this study was sufficient, being comparable to numbers analyzed in the construction of other TS-specific charts. All subjects were confirmed by chromosomal analyses to meet the definition of TS and were properly selected, excluding subjects who had undergone pubertal development or previous growth-promoting treatment or both. Although these charts were not derived from a totally unbiased TS population, they can be presumed to represent growth in girls with TS who are ordinarily seen in clinical practice, because the charts were constructed using adjacent data before GH treatment. We believe that these charts have been adequately and successfully produced taking these points into consideration.

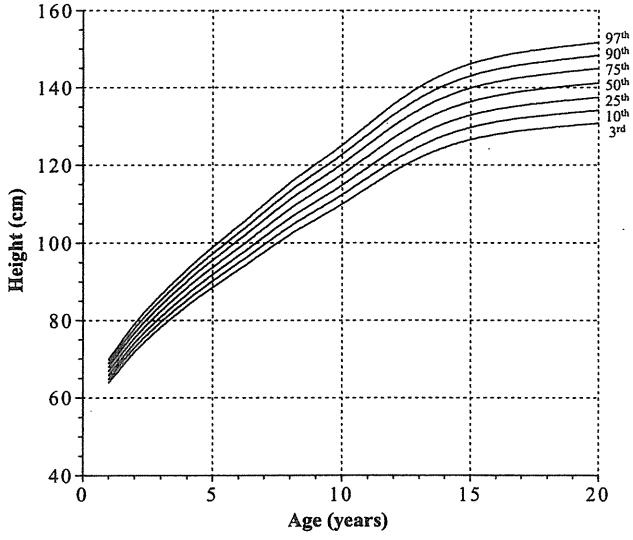

Fig. 1 Height chart for Japanese girls with Turner syndrome without puberty.

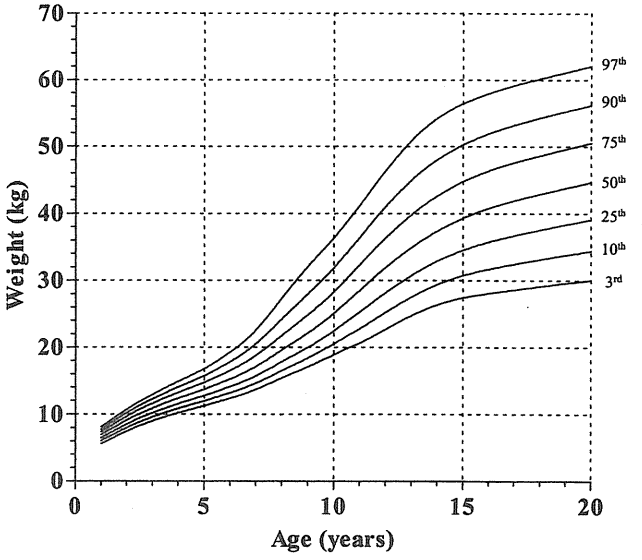

Fig. 2 Weight chart for Japanese girls with Turner syndrome without puberty.

Differences can be detected between the two charts in both height and weight growth (Figs 3 and 4). For example, the adult height from the new chart is 141.2 cm, which is 3.0 cm taller than the previous height when it is defined as the mean height at the age of 20 years. In the previous study, birth years ranged from 1955 to 1989 (median unknown). Given the year of publication, the adult height in the study had to be derived from subjects born before 1972. The standard adult heights of Japanese women in 1970, 1975, 1980, 1985, 1990, 1995, 2000 and 2005 were 155.6 cm, 156.3 cm, 157.0 cm, 157.6 cm, 157.9 cm, 158.0 cm, 158.1 cm and 158.0 cm, respectively.[21] This indicates that the secular trend in adult height has reached a plateau since approximately 1990 in Japan. Judging from the birth-year distribution, we know therefore that the old Japanese charts for TS were constructed with data from subjects the majority of whom were born before growth in height reached a plateau. On the other hand, the birth years in the present study ranged from 1970 to 2002 and 85.2% of the subjects were born after 1980. The new TS-specific growth charts therefore differ because they were constructed with data from a generation in which appreciable advances in secular height growth had disappeared. Secular growth trends in TS subjects have also been noted and studied in other countries.[9,22] In countries where secular trends

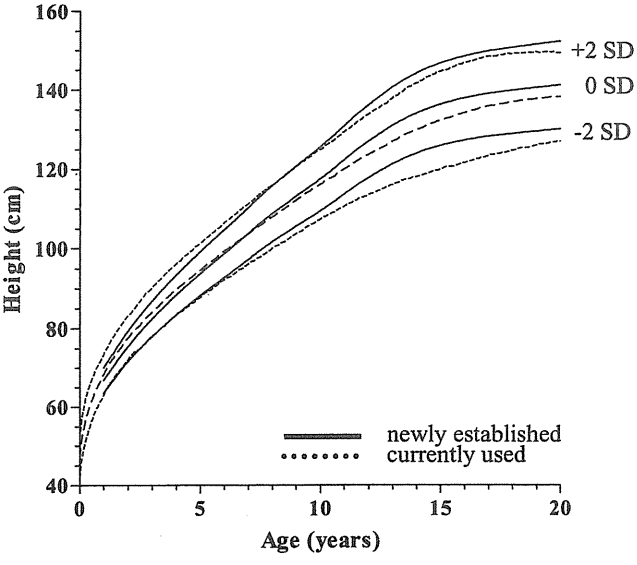

Fig. 3 Height chart for Japanese girls with Turner syndrome without puberty in comparison with the currently used one.[12] Solid line, newly established; dotted line, currently used.

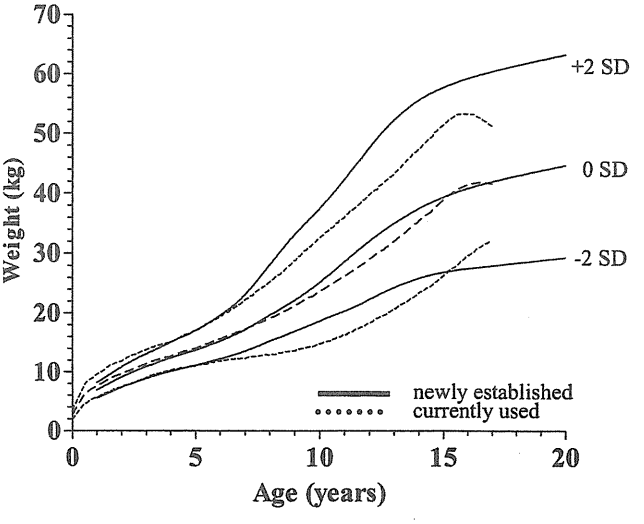

Fig. 4 Weight chart for Japanese girls with Turner syndrome without puberty in comparison with the currently used one.[12] Solid line, newly established; dotted line, currently used.

have reached a plateau, there is little need to construct newer charts. Taken together, we conclude that the new charts can be used hereafter as the more adequate reference for the evaluation of the growth of girls with TS born after approximately 1970.

There are three limitations to the present study. The first is a selection bias. This retrospective cohort consists of those diagnosed with TS in medical centers, a subpopulation from which subjects who are not significantly smaller than the normal population are more easily omitted. More specifically, physicians do not usually register girls with TS if they are taller than −2 SDS of the female standard, because the registry is designed primarily to designate candidates for GH treatment. It is of note that in Japan the indication of GH for TS is limited to subjects shorter than −2 SDS. The heights of the majority of girls with TS usually drop below the fifth percentile of the normal girl growth curve only after the subjects are from two to five years old.[1] This implies that this kind of bias more severely affects subjects younger than approximately three years of age. Before general application of these reference charts for TS, further validation is rewarding, especially in younger ages. The second limitation is the number of study samples. The numbers of infants and older children are small, so at these ages (especially under three years and over sixteen years) the charts may not be sufficiently reliable. This limitation is shared by the other reference charts, including currently used Japanese charts. With regard to older girls, it has become more difficult to obtain height data from subjects without previous growth-promoting treatment, because GH treatment for girls with TS has become very common in Japan and, what's more, the starting age has been decreasing. Despite this limitation, the adult height in this study is −3.3 SDS of the normal population[23] and is considered to be valid by comparison to adult heights in other countries (−4.2 to −2.5 SDS).[2] The third limitation is derived from the fact that this study is a cross-sectional study. We do not know whether subjects without puberty at the time of registration will or will not develop spontaneous puberty later. It is reported that those with spontaneous puberty are significantly taller than those without puberty from 12 years of age onward, although pubertal development and growth spurt do not seem to affect final adult height.[7] Theoretically, two types of growth charts may be needed during the peripubertal period, but we produced one specific for girls without pubertal signs because of the limited number of pubertal subjects. However, when we plotted the data from all 107 subjects with pubertal development on the new chart, with only one exception they were distributed within +/−2 SDS of the other subjects' data (data not shown). Accordingly, the presence of puberty does not influence the major difference in the pubertal height, though further investigation is necessary.

Conclusions

We have constructed new clinical reference growth charts for Japanese girls with TS using data from 1447 subjects who did not present with puberty. As they are assumed to belong to the generation beyond the secular trend in Japan, these charts are expected to be widely used in various clinical settings with all the limitations in mind and await further validation.

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References

1 Saenger P. Turner's syndrome. *N. Engl. J. Med.* 1996; **335**: 1749–54.
2 Ranke MB, Grauer ML. Adult height in Turner syndrome: Results of multinational survey 1993. *Horm. Res.* 1994; **42**: 90–4.
3 Baxter L, Bryant J, Cave CB, Milne R. Recombinant growth hormone for children and adolescents with Turner syndrome. *Cochrane Database Syst. Rev.* 2007; Jan 24; (**1**): CD003887.
4 Bernasconi S, Larizza D, Benso L *et al.* Turner's syndrome in Italy: Familial characteristics, neonatal data, standards for birth weight and for height and weight from infancy to adulthood. *Acta Paediatr.* 1994; **83**: 292–8.
5 Haeusler G, Schemper M, Frisch H, Blümel P, Schmitt K, Plöchl E. Spontaneous growth in Turner syndrome: Evidence for a minor pubertal growth spurt. *Eur. J. Pediatr.* 1992; **151**: 283–7.
6 Lyon AJ, Preece MA, Grant DB. Growth curve for girls with Turner syndrome. *Arch. Dis. Child.* 1985; **60**: 932–5.
7 Massa G, Vanderschueren-Lodeweyckx M, Malvaux P. Linear growth in patients with Turner syndrome: Influence of spontaneous puberty and parental height. *Eur. J. Pediatr.* 1990; **149**: 240–50.
8 Naeraa RW, Nielsen J. Standards for growth and final height in Turner's syndrome. *Acta Paediatr. Scand.* 1990; **79**: 182–90.
9 Ranke MB, Pflüger H, Rosendahl W *et al.* Turner syndrome: Spontaneous growth in 150 cases and review of the literature. *Eur. J. Pediatr.* 1983; **141**: 81–8.
10 Rongen-Westerlaken C, Corel L, Broeck JVD *et al.* Reference values for height, height velocity and weight in Turner's syndrome. *Acta Paediatr.* 1997; **86**: 937–42.
11 Sempé M, Hansson BC, Limoni C. Growth curves in untreated Ullrich-Turner syndrome: French reference standards 1–22 years. *Eur. J. Pediatr.* 1996; **155**: 862–9.
12 Suwa S. Standards for growth and growth velocity in Turner's syndrome. *Acta Paediatr. Jpn.* 1992; 206–21.
13 Murata M, Hibi I. Nutrition and the secular trend of growth. *Horm. Res.* 1992; **38** (Suppl. 1): 89–96.
14 Tanaka T, Takano K, Hanew K *et al.* Registration system for growth hormone (GH) treatment with standardized immunoreactive GH values in Japan. *Endocr. J.* 1998; **45**: 459–65.
15 Cole TJ, Green PJ. Smoothing reference centile curves: The LMS method and penalized likelihood. *Stat. Med.* 1992; **11**: 1305–19.
16 Ogata T, Matsuo N, Tamai S, Osano M, Tango T. Target height and target range for the Japanese (in Japanese). *Jpn. J. Pediatr.* 1990; **94**: 1535–40.
17 Wright EM, Royston P. A comparison of statistical method for age-related reference intervals. *J. R. Stat. Soc. Ser. A* 1997; **160**: 47–69.
18 Cole TJ, Freeman JV, Preece MA. British 1990 growth reference centiles for weight, height, body mass index and head circumstance fitted by maximum penalized likelihood. *Stat. Med.* 1998; **17**: 407–29.
19 Styles ME, Cole TJ, Dennis J, Preece MA. New cross sectional stature, weight, and head circumference references for Down's

syndrome in the UK and Republic of Ireland. *Arch. Dis. Child.* 2002; **87**: 104–8.
20 Martin NDT, Smith WR, Cole TJ, Preece MA. New height, weight and head circumference charts for British children with Williams syndrome. *Arch. Dis. Child.* 2007; **92**: 598–601.
21 Ministry of Education. *Annual Report of School Health Statistics.* The Printing Office, The Ministry of Finance, Tokyo, 2005 (in Japanese).
22 Ranke MB, Stubbe P, Majewski F, Bierich JR. Spontaneous growth in Turner's syndrome. *Acta Paediatr. Scand.* 1988; **343** (Suppl.): 22–30.
23 Suwa S, Tachibana K. Standard growth charts for height and weight of Japanese children from birth to 17 years based on a cross-sectional survey of national data. *Clin. Pediatr. Endocrinol.* 1993; **2**: 87–97.

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Prediction of pre-eclampsia by an analysis of placenta-derived cellular mRNA in the blood of pregnant women at 15–20 weeks of gestation

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Objective A panel of cellular mRNA markers was used to predict the occurrence of pre-eclampsia in pregnant women at 15–20 weeks of gestation.

Design Prospective cohort study.

Setting The Department of Obstetrics and Gynaecology, University of Indonesia, Cipto Mangunkusumo National Hospital, Indonesia.

Sample Peripheral blood samples from asymptomatic pregnant women.

Methods Among 660 women, 62 developed pre-eclampsia at later gestation (pre-eclampsia group) and each case was matched with five controls. Therefore, the RNA expression levels in the cellular component of maternal blood in 62 women with pre-eclampsia were compared with those in 310 controls.

Main outcome measures The cellular RNA expression levels of genes related to angiogenesis and oxidative stress were compared between pre-eclampsia and control groups. A receiver operating characteristic (ROC) curve was used to analyse the sensitivity of each available marker. A logistic regression analysis was performed to calculate the odds for each woman to be classified as a case.

Results The univariate ROC analysis identified soluble vascular endothelial growth factor receptor-1 (Flt-1) and endoglin (ENG) as the markers with the highest sensitivity. The best multivariate model was obtained by combining Flt-1, ENG, placental growth factor (PlGF) and parity. The relative ROC curve yielded a sensitivity of 66% at a 10% 1 − specificity rate with an area under the curve of 0.884 ($P < 0.001$).

Conclusion A panel of cellular mRNA markers in maternal blood can predict the development of pre-eclampsia long before clinical onset.

Keywords Cellular RNA, endoglin, prediction, pre-eclampsia, vascular endothelial growth factor receptor-1.

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Introduction

Despite advances in perinatal care, pre-eclampsia (PE) is the most common cause of maternal and perinatal mortality and morbidity worldwide.[1] Recently, anti-angiogenic factors, such as soluble vascular endothelial growth factor receptor-1 (sFlt-1) and soluble endoglin (sENG), which are both produced in the placenta, have been shown to play important roles in the pathogenesis of PE.[2–5]

Vascular endothelial growth factor (VEGF) is a pro-angiogenic factor and causes vasodilatation through the production of nitric oxide and prostacyclin.[6–8] As Flt-1 combines with VEGF and placental growth factor (PlGF), and the serum level of soluble Flt-1 increases in pregnant women who develop PE,[3] free PlGF and free VEGF in maternal serum decline prior to the development of PE.[4] Another anti-angiogenic factor is endoglin (ENG), which regulates the endothelial nitric oxide synthase activity and local vascular tone.[9] Venkatesha *et al.*[5] have reported that the placenta is a major source of soluble ENG during pregnancy, and that ENG is up-regulated in the pre-eclamptic placenta, releasing soluble ENG into the

maternal circulation, which correlates with the severity of PE.

Although the molecular mechanism regulating the production of Flt-1 and ENG in the placenta is unknown, it has been suggested that hypoxia or oxidative stress of trophoblasts is associated with the production of these factors. Li *et al.*[10] reported the up-regulation of Flt-1 to be associated with increased oxidative stress as a consequence of hypoxia in placental trophoblasts. Haem oxygenase-1 (HO-1) is known to have antioxidant, anti-inflammatory and cytoprotective functions. HO-1 is an oxygen sensor and its expression is inducible under hypoxic conditions.[11] Although low HO-1 levels in the placenta result in an abortion,[12] the up-regulation of HO-1 by adenoviral administration works protectively during pregnancy.[13] Furthermore, PE is associated with diminished placental HO-1 levels.[14] Moreover, the adenoviral overexpression of HO-1 inhibits soluble ENG release in placental villous explants, whilst also inhibiting Flt-1 production in endothelial cells.[15]

Therefore, anti-angiogenic and anti-oxidative factors are considered to play a crucial role in the pathogenesis of PE. These placental alterations in women who develop PE in later gestation are thought to begin during the first trimester, when extravillous trophoblasts remodel into the endothelial cells of the spiral arteries. The *in vivo* alteration of gene expression has been observed in the first-trimester trophoblasts from pregnant women destined to develop PE later, confirming this hypothesis.[16] In this study, tissue samples of villous trophoblasts at the time of fetal karyotype analysis were collected prospectively through chorionic villous sampling (CVS), and the mRNA expression of these genes was assessed. The results revealed that the expression levels of Flt-1, ENG, VEGF and transforming growth factor-β1 (TGFβ1) were significantly higher in the CVS tissues from pregnant women who later developed PE, whereas the levels of PlGF, HO-1 and superoxide dismutase (SOD) were lower.[16] These findings suggest that the genes associated with angiogenesis and reduced anti-oxidant stress play crucial roles in the pathogenesis of PE, and that the measurement of the expression of these factors in maternal blood may enable the prediction of the onset of PE.

Fetal/placental RNA circulates in the maternal plasma and has enabled the development of several promising approaches for the noninvasive evaluation of placental function.[17,18] Subsequently, cell-free RNA concentrations of VEGF, Flt-1 and ENG were assessed in the plasma of women with and without PE.[19] These transcripts increased in the plasma of pre-eclamptic women and correlated positively with disease severity. An additional study of postpartum samples found the mRNA transcripts to decrease rapidly after delivery, thus suggesting that the majority of the transcripts were derived from the placenta/fetus.[19] To demonstrate the possibility of the prediction of PE by cell-free RNA, the expression of seven genes, including Flt-1, ENG and VEGF, was assessed in the plasma of pregnant women between 15 and 20 weeks of gestation. It was found that this panel allowed an 84% prediction rate for PE with a 5% false positive rate at 15–20 weeks of gestation by means of a discriminant analysis model. This finding indicates that the analysis of cell-free RNA is a highly promising method for the evaluation of alterations in placental function.[20]

The expression of placenta-specific genes, such as human placental lactogen (hPL) and human chorionic gonadotrophin (hCG), has also been shown to be detectable in the cellular component of maternal blood, and the mRNA concentrations of hPL and hCG correlate with the protein assay.[17] Furthermore, the cellular mRNA concentration is approximately ten times greater than that of maternal plasma RNA.[17] These findings indicate that some trophoblasts and placental debris circulate in the blood of normal pregnant women, and that the analysis of the cellular component of maternal blood may be more suitable than maternal plasma analysis for the evaluation of alterations in placental function. Therefore, because the gene expression of anti-angiogenic factors and anti-oxidant enzymes is associated with the pathogenesis of PE, the cellular RNA expression in the blood from asymptomatic pregnant women during the early second trimester was analysed to compare the mRNA levels with the clinical outcomes.

Materials and methods

Subjects

This investigation was performed as part of a series together with previously reported studies.[19–22] The investigation was designed as a prospective cohort study in early pregnant women (gestational weeks 15–20) who visited the Department of Obstetrics and Gynaecology, University of Indonesia, Cipto Mangunkusumo National Hospital, Indonesia from mid-2005 to 2006. Singleton pregnant women without any pre-existing medical diseases at screening or antenatal complications at the time of blood drawing were invited to participate in the cohort. The pregnancies were dated by ultrasound, which was performed during the first trimester. All women provided informed consent to participate in the study, which was approved by the Research Ethics Committee of both the University of Indonesia and Showa University.

Of the 683 women enrolled, 23 with incomplete information on outcome, whose pregnancy ended before 20 weeks or who experienced stillbirth were excluded. Among the remaining 660 women, 62 developed PE. Each case was matched with five controls of the same gestational age at the time of blood testing (within 1 week and ranging

from 15 to 20 weeks), maternal weight and fetal gender. Therefore, 62 women who developed PE and 310 controls with a normal course of pregnancy were enrolled in the study. In the control group, women with fetal growth restriction (below −1.5 SD) based on the Japanese fetal growth curve (http://www.jsum.or.jp/committee/diagnostic/diagnostic) were excluded. No special management or treatment other than antenatal care was provided before the clinical signs of PE presented. If abnormalities of blood pressure and/or proteinuria were found, women were recommended to admit themselves to hospital.

Mild and severe PE and haemolysis, elevated liver enzymes and low platelets (HELLP) syndrome was defined as described previously.[22,23] In brief, PE was defined as gestational hypertension (systolic pressure of ≥140 mmHg or diastolic blood pressure of ≥90 mmHg on two or more occasions after gestational week 20) with proteinuria (≥0.3 g/day). Severe PE was defined by the presence of one or more of the following: (i) severe gestational hypertension (systolic pressure of ≥160 mmHg or diastolic blood pressure of ≥110 mmHg on two or more occasions after gestational week 20); or (ii) severe proteinuria (≥5 g protein in a 24-hour urine specimen or ≥3 g in two random urine samples collected ≥4 hours apart).

Processing of blood samples

Peripheral blood samples (2.5 ml) were collected in PAXgene blood RNA tubes (PreAnalytic, Hombrechtikon, Switzerland) and kept at room temperature for 3 hours, and then stored at −20°C until transport to Japan at −20°C. Molecular analysis was performed at the Department of Obstetrics and Gynecology, Showa University School of Medicine, Tokyo, Japan. RNA extraction was performed according to protocols described elsewhere.[24] In brief, cellular component samples were centrifuged twice at 4000 *g* for 10 minutes at room temperature in order to remove the entire supernatant and any mRNA present in residual plasma. The pellet was then washed, resuspended and incubated in optimised buffer solution containing proteinase K to digest protein. A second round of centrifugation was performed to remove any residual cell debris, and the resulting supernatant was transferred to a fresh microcentrifuge tube. Thereafter, 100% ethanol was added to the supernatant to adjust the binding conditions, and the resultant lysate was then applied to a PAXgene spin column (PreAnalytiX; PreAnalytic), resulting in selective binding of RNA to the silica-gel membrane of the spin column. After the column had been washed three times, pure RNA was eluted in 80 μl of RNase-free water.

Real-time quantitative reverse transcription polymerase chain reaction (RT-PCR)

Reverse transcription of mRNA was performed using an Omniscript RT Kit (Qiagen, Hilden, Germany). Real-time quantitative PCR was then performed using a QuantiTect Probe PCR Kit (Qiagen). RT-PCR was performed according to the manufacturer's instructions. cDNA products were amplified by real-time quantitative PCR according to the manufacturer's instructions (QuantiTect Probe PCR kit, Qiagen) using a 2-μl aliquot of cDNA and the kit's components in a reaction volume of 20 μl. TaqMan PCR analyses for VEGF, Flt-1, ENG, PlGF, TGFβ1, P-selectin, placenta specific-1 (PLAC1), HO-1 and SOD were performed using predeveloped and commercially available primers and probe sets (Cat # Hs00900054_m1 for VEGF, Cat # Hs01052936_m1 for Flt-1, Cat # Hs00923997_g1 for ENG, Cat # Hs00182176_m1 for PlGF, Cat # Hs0000171257_m1 for TGFβ1, Cat # Hs00174583_m1 for P-selectin, TaqMan Probes for PLAC1 gene have been described previously,[21] Cat # Hs00157965_m1 for HO-1 and Cat# Hs00166575_m1 for SOD; Applied Biosystems, Foster City, CA, USA). The thermal cycling protocol used for PCR was as follows: initial denaturation at 95°C for 15 minutes, 40 cycles of denaturation at 94°C for 15 seconds and annealing at 60°C for 1 minute. Initially, each PCR assay was confirmed to be specific to mRNA and not to genomic DNA. Amplification data were collected and analysed with an ABI Prism 7900T Sequence Detector (Applied Biosystems). Each sample was analysed in duplicate, and multiple negative water blanks were included in every analysis. Quantification of gene expression was performed by investigators blind to the outcome of the pregnancy. The amounts of mRNA samples were expressed in term of copies per millilitre by the method reported elsewhere.[22]

Statistical analysis

The distributions of the demographic characteristics and logarithmic mRNA concentrations were analysed after conversion to multiples of the median (MoMs). MoMs were also adjusted for the body mass index (BMI). Median values were stratified retrospectively according to the severity of PE and the development of HELLP syndrome. The non-parametric analysis of variance (Kruskal–Wallis test) and Dunn *post hoc* test were used for comparisons among and between groups. The sensitivity and 1 − specificity rate (false positive rate) were calculated for each available marker using a univariate receiver operating characteristic (ROC) curve. A multivariate analysis was performed using logistic regression to calculate the odds for each woman for classification as a case. The logistic output was adjusted for the incidence of PE in the general population (2%) by calculation of the sampling fraction, as described by Collett.[25] Finally, a ROC curve for the calculation of multivariate sensitivity was built using, as the test variable, the calculated odds for PE by a logistic regression analysis for each woman in the series.

Results

The demographics of the pregnant women from the control and PE groups are shown in Table 1. Although no differences were observed in the maternal age, BMI, gestational age at blood drawing and blood pressure at the time of blood drawing between groups, significant differences were noted in birth weight and gestational age at delivery, and these findings were consistent with those reported in the existing literature.

The values of the available markers are reported in Figure 1A–G. The mRNA expression levels of Flt-1, ENG, P-selectin and PLAC1 were higher in the PE group than in the controls, but those of PlGF and HO-1 were lower. TGFβ1, VEGF and SOD did not reach a significant value in the comparison of PE cases versus controls (Kruskal–Wallis test), and were therefore excluded from any further analysis. For all the other markers, a significant difference was found in the comparisons of HELLP cases versus controls and, for some, including Flt-1, ENG and PlGF, a significant difference was found for any generated subgroup (mild PE, severe PE, HELLP) according with the severity (Dunn *post hoc* test). A univariate analysis identified Flt-1 and ENG as markers with the highest sensitivity. Table 2 reports the output of the ROC curve analysis for those markers with a significant difference in PE cases versus controls, together with the actual MoMs for each marker associated with the given sensitivity. The best multivariate model was obtained by combining Flt-1, ENG, PlGF and parity (Table 3). Only the final model is presented, which was obtained by stepwise logistic regression. After adjusting for the incidence of PE (2% or 1:50), the mean quoted odds (obtained by solving the logistic equation having the odds as a dependent variable for each of the subjects in the study) for PE was 1:50 (0.02) for controls and 1:7 (0.14) for women who developed PE. A ROC curve obtained using the estimated score for PE as the test variable yielded a sensitivity of 66% at a 10% 1 − specificity rate with an area under the curve of 0.884 (0.844–0.922, 95% CI; $P < 0.001$; Figure 2), and therefore with a reasonable discrimination to identify cases. Again, sensitivity and the odds for each woman for classification as PE were correlated with the severity (sensitivity of 50% for mild PE, 70% for severe PE and 75% for HELLP; odds of 1:12 for mild PE and 1:5 for both severe PE and HELLP).

Discussion

The present study examined prospectively mRNA expression in the cellular component of maternal blood samples from

Table 1. Demographic characteristics. Data are expressed as the median (minimum–maximum)

	Controls (n = 310)	Mild PE (n = 26)	Severe PE (n = 24)	HELLP (n = 12)	P value*,**
% Nulliparous	59.8	65.4	87.5	91.7	0.008***
Maternal age (years)	28 (15–43)	32 (20–40)	27.5 (19–42)	24.5 (20–35)	0.065
GA at blood test (weeks)	17 (15–20)	18 (15–20)	16 (15–18)	17.5 (15–20)	0.479
BMI at blood test	22.94 (13.79–34.22)	24.93 (19.31–33.33)	21.93 (17.42–29.48)	21.71 (18.39–30.30)	0.150
SBP (mmHg) at blood test	110 (90–130)	110 (90–125)	100 (90–120)	100 (90–130)	0.388
DBP (mmHg) at blood test	70 (60–85)	70 (60–85)	70 (60–80)	70 (60–85)	0.625
Birth weight (g)	3195 (2600–4080)	2775 (1900–3920)	2500 (2100–3900)	2400 (1800–3130)	<0.001***
Birth weight centiles	50 (14–94)	22 (1.5–92)	16 (1.5–55)	10 (1–90)	<0.001
GA at delivery (weeks)	38 (37–41)	37 (33–40)	37 (34–40)	36 (32–40)	<0.001***

*Chi-squared test.
**Kruskall–Wallis and Dunn test.
Significant comparisons: ***control versus severe PE and HELLP.
BMI, body mass index; DBP, diastolic blood pressure; GA, gestational age; HELLP, haemolysis, elevated liver enzymes and low platelets; PE, pre-eclampsia; SBP, systolic blood pressure.

Figure 1. (A–I) Box-and-whisker plots of the distribution of vascular endothelial growth factor receptor-1 (Flt-1) (A), endoglin (B), vascular endothelial growth factor (VEGF) (C), transforming growth factor-β1 (TGFβ1) (D), haem oxygenase-1 (HO-1) (E), placental growth factor (PlGF) (F), superoxide dismutase (SOD) (G), P -selectin (H) and placenta specific-1 (PLAC1) (I) mRNA levels in controls and women with pre-eclampsia, stratified in accordance with the severity of pre-eclampsia [mild and severe pre-eclampsia, and haemolysis, elevated liver enzymes, low platelet (HELLP) syndrome]. The medians are indicated by a line inside each box, and the 75th and 25th percentiles by the box limits; the upper and lower bars represent the 10th and 90th percentiles, respectively. The y-axes represent multiples of the median (MoMs) of each gene expression. Asterisks (*) indicate all specimens above or below the 90th or 10th percentile specimens. CT, control; HELLP, HELLP syndrome; mild, mild pre-eclampsia; severe, severe pre-eclampsia.

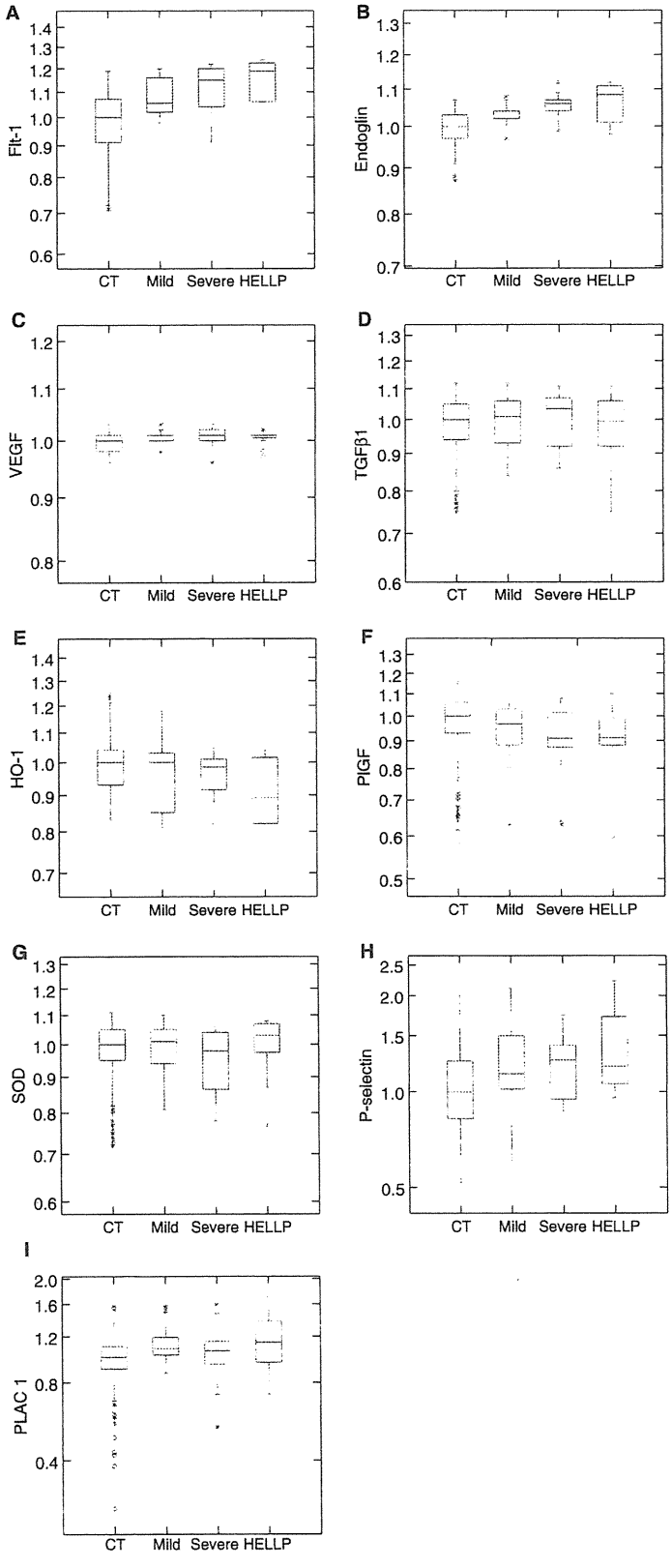

Table 2. Receiver operating characteristic (ROC) curve for each marker

mRNA	AUC	*P* value	Lower 95% bound	Upper 95% bound	Sensitivity (%) at 5% 1 – specificity	Sensitivity (%) at 10% 1 – specificity	MoM cut-off at 5% 1 – specificity	MoM cut-off at 10% 1 – specificity
P-selectin	0.665	0.039	0.588	0.742	18.2	29.1	1.61	1.45
PLAC1	0.631	0.044	0.545	0.717	20	20	1.31	1.26
Flt1	0.806	0.032	0.744	0.868	43.6	52.7	1.11	1.13
Endoglin	0.840	0.029	0.773	0.879	47.3	50.9	1.13	1.15
HO-1	0.588	0.042	0.502	0.675	8.1	14.5	0.84	0.87
PlGF	0.627	0.003	0.547	0.708	24.2	25.8	0.69	0.84

Sensitivity is shown at different cut-off values of 5% and 10% 1 – specificity.
AUC, area under the curve; HO-1, haem oxygenase-1; PLAC1, placenta specific-1; PlGF, placental growth factor.

Table 3. Logistic regression output for plasma cellular RNA levels

Variable	Odds ratio	95% CI		*P* value
		lower	upper	
Flt-1	2.760	1.910	3.988	<0.001
Endoglin	3.214	2.190	4.718	<0.001
PlGF	0.611	0.455	0.821	0.001
Parity (primi or pluri versus nulli)	2.822	1.316	6.052	0.008
Constant (ln)	–7.583			<0.001

The variables were expressed as multiples of the median (MoMs) and categorised at four levels (<25th, 25th–50th, 50th–75th and >75th percentiles).
Flt-1, vascular endothelial growth factor receptor-1; PlGF, placental growth factor.

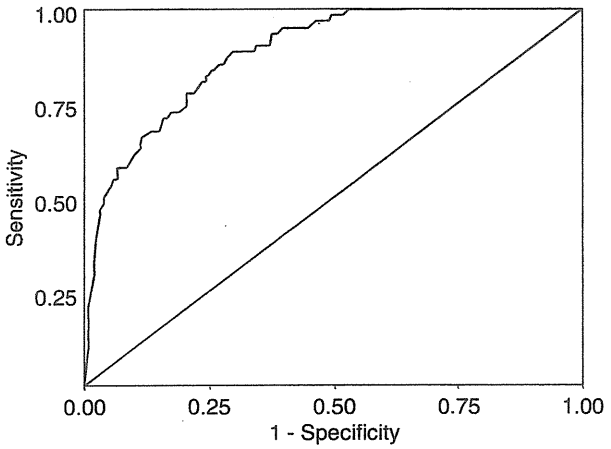

Figure 2. Receiver operating characteristic (ROC) curve obtained by plotting the values of the estimated odds for pre-eclampsia. A multivariate model consisting of endoglin (ENG), vascular endothelial growth factor receptor-1 (Flt-1), placental growth factor (PlGF) and parity was used to generate the ROC curve.

women at gestational weeks 15–20. The study assessed nine species of gene expression associated with angiogenesis and oxidative stress, which coded for factors thought to be important in the pathogenesis of PE. The mRNA levels were compared with the clinical outcomes. As a result, the mRNA expression levels of ENG, Flt-1, P-selectin and PLAC1 were found to be significantly higher in the PE group than in the controls, whereas PlGF and HO-1 levels were lower in the PE group. The blood samples were obtained at an average of 17 weeks, which is almost 20 weeks before the onset of PE. At this gestational age, the mRNA expression of anti-angiogenic factors and anti-oxidants is already altered in pregnant women who subsequently develop PE. These factors play a crucial role in the pathogenesis of PE, and the analysis of cellular components of maternal blood for these transcripts may allow for the prediction of PE.

Although many tests have been proposed for the prediction of PE, the results have been inconsistent and contradictory.[26–28] The present study has demonstrated that a panel of cellular RNA markers quantified long before clinical onset predicts PE occurrence with a degree of accuracy comparable with previous reports, including uterine artery Doppler velocimetry with or without demographic and biochemical parameters.[26] The univariate analysis showed Flt-1 and ENG to be the markers with the highest sensitivity. The best multivariate model was obtained by combining Flt-1, ENG, PlGF and parity. The ROC curve yielded a sensitivity of 66% at a 10% 1 – specificity rate with an area under the curve of 0.884. These results indicate that cellular RNA in maternal blood can be used to assess the pathophysiological alterations which occur in pregnant women who later develop PE.

The cell-free RNA in maternal plasma was assessed in a previous study. The expression of seven transcripts was assessed in the plasma of pregnant women at gestational weeks 15–20.[20] The target genes were Flt-1, ENG, VEGF, plasminogen activator inhibitor-1 (PAI-1), tissue-type

plasminogen activator (tPA), PLAC1 and P-selectin. In the statistical univariate analysis, Flt-1 showed the highest degree of discrimination, followed by ENG, PAI-1, P-selectin, tPA, VEGF and PLAC1. The best multivariate model was obtained by the combination of all markers. A ROC curve yielded a sensitivity of 84% (95% CI, 71.8–91.5) at a 5% 1 − specificity rate with an area under the curve of 0.927 ($P < 0.001$).[20] It has been suggested previously that cellular RNA analysis is not as useful as plasma RNA analysis.[17] In our previous study of the hPL gene, the coefficient of variation of our cellular RNA analysis was approximately 20%; it was not calculated in the current study and this is a significant limitation of this study.[17] However, as cellular RNA can be preserved in the PAXgene blood RNA tube, we have confirmed that the RNA is stable below −20°C at least for 1 year. Moreover, the blood processing of cellular RNA is much easier than that of plasma RNA. In addition, the expression levels of hPL and hCG are approximately ten times higher than those in plasma RNA.[17] Therefore, we suggest that the analysis of plasma cellular RNA is a promising method for the evaluation of the pathophysiological alterations occurring in pregnant women who later develop PE. In both the previous cell-free RNA study and the present cellular RNA study, the levels of Flt-1 and ENG were two of the best predictors for PE. This indicates that these anti-angiogenic factors play a crucial role in the pathogenesis of PE.

The origin of the cellular RNA seen in maternal blood has not been resolved. In our previous study, hPL and hCG expression levels in the cellular components of maternal blood correlated with the corresponding protein levels.[17,24] This finding suggests that some trophoblasts or placental debris circulate in maternal blood. The half-times of hPL expression in cellular and plasma RNA were 203.8 and 32.2 minutes, respectively. Therefore, the half-time of cellular RNA is much longer than that of plasma RNA, and cellular RNA is not removed from the maternal circulation rapidly after delivery. The RNA originating from circulating trophoblasts in maternal blood could be detected for several months, as fetal nucleated erythrocytes reportedly circulate in maternal blood for 3 months after delivery.[29] These findings suggest that the levels of trophoblast-derived RNA in the cellular RNA in maternal blood could reflect the pathophysiological alterations of the placenta. However, real-time evaluation of placental function through cellular RNA may be inferior to the evaluation of cell-free RNA.

Another study assessed the mRNA expression of trophoblasts obtained from CVS at week 11. The expression levels of Flt-1, ENG and VEGF in CVS tissue obtained from pregnant women who later developed PE were higher, and those of PlGF and HO-1 were lower, than those of normal pregnancies.[16] These findings indicate that the up-regulation of anti-angiogenic factors and the down-regulation of anti-oxidant factors have already occurred in first-trimester trophoblasts,[16] and that the alterations could be evaluated by the analysis of cellular RNA. All of these findings support the hypothesis that some mRNA expression of Flt-1, ENG, PlGF, VEGF and HO-1 is derived from circulating trophoblasts, and that the alteration of these mRNA levels may reflect mRNA alterations associated with the pathogenesis of PE in the placenta. Therefore, it is suggested that the evaluation of cellular mRNA may allow for the indirect monitoring of placental function.

In this study, PE occurred in 9% of pregnant women, a higher frequency than seen in the published literature. No previous large studies have clarified the prevalence of PE in Indonesian populations, and this higher prevalence could confound the result. Furthermore, because the case number of gestational hypertension or early-onset-type PE was not sufficient for statistical analysis, women with hypertension in pregnancy were excluded and those with early-onset PE were not analysed separately. Further study in more developed countries is needed to confirm the predictive efficiency of cellular RNA in maternal blood.

In conclusion, the current study has demonstrated that Flt-1 and ENG expression increases in the cellular RNA in the blood from pregnant women who develop PE, whereas HO-1 and PlGF expression decreases. These alterations increase with the severity of the clinical symptoms of PE at later gestation. Furthermore, an analysis of the expression of these transcripts allows the accurate detection of high-risk pregnant women who are likely to develop PE in populations at low risk for the development of PE.

Disclosure of interest

None.

Contribution to authorship

AS, TO, YP and AF designed the research and approved the final, submitted version. AS, MN, HS, NW and YP collected, analysed and interpreted the data, and drafted the manuscript. AF and NR performed the statistical analysis.

Details of ethics approval

Approved by the ethics committee of Showa University, #86, and by the University of Indonesia, #92a/PT02.FK/2006.

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References

1 Redman CW, Sargent IL. Latest advances in understanding preeclampsia. *Science* 2005;308:1592–4.
2 Levine RJ, Lam C, Qian C, Yu KF, Maynard SE, Sachs BP, *et al.* Soluble endoglin and other circulating antiangiogenic factors in preeclampsia. *N Engl J Med* 2006;355:992–1005.
3 Levine RJ, Maynard SE, Qian C, Lim KH, England LJ, Yu KF, *et al.* Circulating angiogenic factors and the risk of preeclampsia. *N Engl J Med* 2004;350:672–83.
4 Maynard SE, Min JY, Merchan J, Lim KH, Li J, Mondal S, *et al.* Excess placental soluble fms-like tyrosine kinase 1 (sFlt1) may contribute to endothelial dysfunction, hypertension, and proteinuria in preeclampsia. *J Clin Invest* 2003;111:649–58.
5 Venkatesha S, Toporsian M, Lam C, Hanai J, Mammoto T, Kim YM, *et al.* Soluble endoglin contributes to the pathogenesis of preeclampsia. *Nat Med* 2006;12: 642–9.
6 Ferrara N. Role of vascular endothelial growth factor in regulation of physiological angiogenesis. *Am J Physiol Cell Physiol* 2001;280: C1358–66.
7 He H, Venema VJ, Gu X, Venema RC, Marrero MB, Caldwell RB. Vascular endothelial growth factor signals endothelial cell production of nitric oxide and prostacyclin through flk-1/KDR activation of c-Src. *J Biol Chem* 1999;274:25130–5.
8 He Y, Smith SK, Day KA, Clark DE, Licence DR, Charnock-Jones DS. Alternative splicing of vascular endothelial growth factor (VEGF)-R1 (FLT-1) pre-mRNA is important for the regulation of VEGF activity. *Mol Endocrinol* 1999;13:537–45.
9 Toporsian M, Gros R, Kabir MG, Vera S, Govindaraju K, Eidelman DH, *et al.* A role for endoglin in coupling eNOS activity and regulating vascular tone revealed in hereditary hemorrhagic telangiectasia. *Circ Res* 2005;96:684–92.
10 Li H, Gu B, Zhang Y, Lewis DF, Wang Y. Hypoxia-induced increase in soluble Flt-1 production correlates with enhanced oxidative stress in trophoblast cells from the human placenta. *Placenta* 2005;26:210–7.
11 De Marco CS, Caniggia I. Mechanisms of oxygen sensing in human trophoblast cells. *Placenta* 2002;23(Suppl. A):S58–68.
12 Zenclussen AC, Sollwedel A, Bertoja AZ, Gerlof K, Zenclussen ML, Woiciechowsky C, *et al.* Heme oxygenase as a therapeutic target in immunological pregnancy complications. *Int Immunopharmacol* 2005;5:41–51.
13 Zenclussen ML, Anegon I, Bertoja AZ, Chauveau C, Vogt K, Gerlof K, *et al.* Over-expression of heme oxygenase-1 by adenoviral gene transfer improves pregnancy outcome in a murine model of abortion. *J Reprod Immunol* 2006;69:35–52.
14 Ahmed A, Dunk C, Ahmad S, Khaliq A. Regulation of placental vascular endothelial growth factor (VEGF) and placenta growth factor (PlGF) and soluble Flt-1 by oxygen – a review. *Placenta* 2000;21(Suppl. A):S16–24.
15 Cudmore M, Ahmad S, Al-Ani B, Fujisawa T, Coxall H, Chudasama K, *et al.* Negative regulation of soluble Flt-1 and soluble endoglin release by heme oxygenase-1. *Circulation* 2007;115:1789–97.
16 Farina A, Sekizawa A, DeSanctis P, Purwosunu Y, Okai T, Cha DH, *et al.* Gene expression in chorionic villous samples at 11 weeks' gestation from women destined to develop preeclampsia. *Prenat Diagn* 2008;28:956–61.
17 Okazaki S, Sekizawa A, Purwosunu Y, Iwasaki M, Farina A, Okai T. Measurement of mRNA of trophoblast-specific genes in cellular and plasma components of maternal blood. *J Med Genet* 2006;43:e47.
18 Poon LL, Leung TN, Lau TK, Lo YM. Presence of fetal RNA in maternal plasma. *Clin Chem* 2000;46:1832–4.
19 Purwosunu Y, Sekizawa A, Farina A, Wibowo N, Koide K, Okazaki S, *et al.* Evaluation of physiological alterations of the placenta through analysis of cell-free messenger ribonucleic acid concentrations of angiogenic factors. *Am J Obstet Gynecol* 2008;198:124 e1–7.
20 Purwosunu Y, Sekizawa A, Okazaki S, Farina A, Wibowo N, Nakamura M, *et al.* Prediction of preeclampsia by analysis of cell-free messenger RNA in maternal plasma. *Am J Obstet Gynecol* 2009;200:386 e1–7.
21 Purwosunu Y, Sekizawa A, Farina A, Wibowo N, Okazaki S, Nakamura M, *et al.* Cell-free mRNA concentrations of CRH, PLAC1, and selectin-P are increased in the plasma of pregnant women with preeclampsia. *Prenat Diagn* 2007;27:772–7.
22 Purwosunu Y, Sekizawa A, Koide K, Farina A, Wibowo N, Wiknjosastro GH, *et al.* Cell-free mRNA concentrations of plasminogen activator inhibitor-1 and tissue-type plasminogen activator are increased in the plasma of pregnant women with preeclampsia. *Clin Chem* 2007;53:399–404.
23 ACOG Practice Bulletin. Diagnosis and management of preeclampsia and eclampsia. Number 33, January 2002. American College of Obstetricians and Gynecologists. *Int J Gynaecol Obstet* 2002;77:67–75.
24 Okazaki S, Sekizawa A, Purwosunu Y, Farina A, Wibowo N, Okai T. Placenta-derived, cellular messenger RNA expression in the maternal blood of preeclamptic women. *Obstet Gynecol* 2007;110:1130–6.
25 Collett D, editor. *Modelling Binary Data*, 2nd edn. Boca Raton, FL: Chapman & Hall/CRC; 2003.
26 Conde-Agudelo A, Villar J, Lindheimer M. World Health Organization systematic review of screening tests for preeclampsia. *Obstet Gynecol* 2004;104:1367–91.
27 Espinoza J, Romero R, Nien JK, Gomez R, Kusanovic JP, Gontalves LF, *et al.* Identification of patients at risk for early onset and/or severe preeclampsia with the use of uterine artery Doppler velocimetry and placental growth factor. *Am J Obstet Gynecol* 2007;196:326 e1–13.
28 Farag K, Hassan I, Ledger WL. Prediction of preeclampsia: can it be achieved? *Obstet Gynecol Surv* 2004;59:464–82; quiz 485..
29 Sekizawa A, Purwosunu Y, Matsuoka R, Koide K, Okazaki S, Farina A, *et al.* Recent advances in non-invasive prenatal DNA diagnosis through analysis of maternal blood. *J Obstet Gynaecol Res* 2007;33:747–64.