Table 2 Influence of miscarriage on past/present illness

	No miscarriage		Sporadic miscarriage			RSA			*P* for
	Yes/no	OR	Yes/no	OR	(95%CI)	Yes/no	OR	(95%CI)	trend
Gastric ulcer	132/1402	Reference	95/760	1.31	(0.99–1.74)	13/90	1.50	(0.82–2.77)	0.035
Duodenal ulcer	82/1455	Reference	64/787	1.41	(1.01–1.99)	5/100	0.88	(0.35–2.21)	0.194
Gastritis	135/1391	Reference	124/719	1.71	(1.31–2.22)	28/76	3.70	(2.31–5.92)	<0.0001
Colon polyp	82/1448	Reference	65/781	1.29	(0.91–1.81)	6/98	0.97	(0.41–2.29)	0.317
Hepatitis B	10/1518	Reference	10/834	1.81	(0.74–4.39)	2/101	2.99	(0.65–13.86)	0.090
Hepatitis C	17/1513	Reference	9/835	0.91	(0.40–2.05)	1/104	0.82	(0.11–6.23)	0.774
Cirrhosis	6/1532	Reference	4/848	1.20	(0.33–4.32)	0/105	<0.001	<0.001-	0.882
Fatty liver	104/1415	Reference	67/768	1.14	(0.83–1.57)	15/89	2.23	(1.24–3.99)	0.031
Tuberculosis	35/1501	Reference	24/827	1.01	(0.59–1.72)	4/100	1.45	(0.50–4.22)	0.671
Asthma	103/1437	Reference	62/789	1.17	(0.84–1.63)	10/94	1.55	(0.78–3.06)	0.163
Bronchitis	46/1493	Reference	28/819	1.09	(0.68–1.77)	5/99	1.62	(0.63–4.18)	0.398
Diabetes	77/1462	Reference	48/803	0.94	(0.65–1.37)	3/102	0.48	(0.15–1.54)	0.336
Hyperlipidemia	399/1134	Reference	244/592	0.97	(0.79–1.18)	28/73	0.95	(0.59–1.52)	0.716
Hypertension	302/1234	Reference	201/648	1.06	(0.86–1.31)	29/76	1.38	(0.87–2.21)	0.243
Myocardial infarction	30/1510	Reference	32/818	1.49	(0.89–2.50)	5/99	2.05	(0.76–5.55)	0.065
Stroke	20/1521	Reference	19/832	1.53	(0.81–2.91)	3/102	2.03	(0.59–6.98)	0.121
Atopic dermatitis	102/1429	Reference	71/777	1.44	(1.05–1.99)	9/95	1.42	(0.69–2.92)	0.031
Urinary calculus	80/1457	Reference	62/787	1.35	(0.95–1.90)	7/97	1.26	(0.56–2.80)	0.125
Mastopathy	257/1265	Reference	160/679	1.16	(0.93–1.45)	18/87	1.02	(0.60–1.72)	0.312

CI, confidence interval; OR, odds ratio; RSA, recurrent spontaneous abortion.

seven women with a BMI of 30 or greater in the present study. Thus, there was no influence of BMI on the risk of miscarriage in the Japanese population.

Fatty liver is common in women with the polycystic ovary syndrome (PCOS).[10] PCOS is a well-known causative factor of RSA in Caucasian women.[11] It is unclear whether PCOS may also cause RSA in Japanese women, because our previous study, which was conducted to investigate this issue, failed to demonstrate any association; furthermore, the frequency of PCOS is relatively low in Japanese women.[12] In the present study, a direct association was noted between fatty liver and RSA.

Luteal phase defect, defined by the criterion of a mid-luteal serum progesterone level of <10 ng/mL, was seen in 23.4% of the patients with RSA.[13] The progesterone levels did not have a predictive value for further miscarriages. There is no evidence to support the routine use of progestogens to prevent miscarriages.[14] However, there seems to be some evidence of the beneficial effect of progestogen use in women with a history of RM.[14] Further study is warranted to confirm whether progesterone treatment might prevent further miscarriages in women with a history of irregular menstruation.

The present study is the first to provide evidence of the existence of an association between miscarriage and atopic dermatitis/gastric disease. Atopic dermatitis is a major public health problem worldwide, with a lifetime prevalence in children of 10–20% and a prevalence of 1–3% in adults.[15] The disease prevalence has increased by two- to threefold during the past 3 decades in industrialized countries, whereas it remains much lower in agricultural regions, such as China, Eastern Europe, and rural Africa. The risk factors include a small family size, increased income and education, migration from rural to urban environments, and increased use of antibiotics, all of which represent the so-called Western lifestyle.[16] Several genes have been identified that may explain the occurrence of the disease in some cases.[17] While allergens, such as house dust mites and foods, may be important in some cases, non-allergic factors, such as rough clothing, *Staphylococcus aureus* infections, exposure to microbes during infancy, exposure to excessive heat, and exposure to irritants that disrupt the function of the skin barrier, may also be important.[16]

Two hypotheses concerning the mechanism of atopic dermatitis have been proposed. One suggests that the primary defect resides in an immunologic disturbance that causes IgE-mediated sensitization, with epithelial-barrier dysfunction occurring as a consequence of the local inflammation. The other proposes that an intrinsic defect in the epithelial cells leads to dysfunction of the skin barrier; the immunologic aspects are considered to be epiphenomena.[18] A high seroprevalence of

autoantibodies, such as antiphospholipid antibody and anti-laminin 1 antibody, has been found in patients with RSA.[8,19] An immunologic disturbance of the semi-allograft at the feto-maternal interface is one of the proposed hypotheses to explain the mechanism underlying unexplained RSA.

Helicobacter pylori infection-related diseases are known to include gastritis, gastric and duodenal ulcer, gastric cancer, idiopathic thrombocytopenic purpura, iron-deficient anemia, urticaria, reflux esophagitis, and some lifestyle-related diseases.[20] Existence of an association between *Helicobacter pylori* infection, reduced cobalamin absorption and the cobalamin status, and consequently, elevated homocysteine levels, has been suggested.[21] It has been shown that homocysteine involved in the pathogenesis of arteriosclerosis induces lifestyle-related diseases. Elevated levels of homocysteine may be among the causative factors of miscarriage.[22] Further study concerning the relation with *Helicobacter pylori* infection is needed, as there is no evidence in the published reports yet, to the best of our knowledge, of *Helicobacter pylori* infection as a causative factor of miscarriage.

A previous study reported that women who experienced miscarriages (hazard ratio: 1.22 [95%CI: 1.08–1.38]; $P = 0.001$) or stillbirths (hazard ratio: 1.40 [95%CI: 1.10–1.79]; $P = 0.007$) were at a significantly greater risk of their relationships ending, as compared with women whose pregnancies ended in live births.[1] In the present study, a higher risk of divorce was seen in women with a history of miscarriages (hazard ratio: 1.596 [95%CI: 1.036–2.460]; $P = 0.043$) and RSA (hazard ratio: 3.103 [95%CI: 1.474–6.53]; $P = 0.0043$). Miscarriage and its recurrence were found to have a more severe adverse influence on marital relationships in Japan than in the USA. The emotional impact of miscarriage is higher in women than in men, and this discrepancy might alter the spousal relationship.[23]

The most important limitation of this study was that the history of illness was not confirmed by medical records but by self-declaration, and stillbirth was not distinguished from miscarriage. However, it was found that at least 89.5% of the women with a history of RSA had subsequent live births, although our previous study reported that the cumulative live birth rate in women with RSA was 85.5%.[4] Therefore, in order to prevent decline of marital relationships related to this issue, it is necessary to inform women that miscarriage is a very common complication during pregnancy and that about 90% of women with RSA may be expected to have live births.

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A merged presentation of clinical and radiographic data using probability plots in a clinical trial, the JESMR study

In terms of the relationship between synovial inflammation and radiographic changes, including both joint damage repair and progression,[1] in rheumatoid arthritis (RA), pre-existing joint damage and persistent synovitis may promote joint destruction, while in the absence of synovitis, damaged joints may heal.[2 3] Although presentation of radiographic results using cumulative probability plots has substantially improved understanding of clinical trial data,[4] the effects of treatments on radiographic progression and improvement (regression) in individual RA patients has not yet been fully explained.

In the JESMR study,[5 6] 151 active RA patients unresponsive to treatment with methotrexate (MTX) were randomised into 1 of 2 treatment groups: etanercept (ETN) 50 mg/week with 6–8 mg/week of MTX (the E+M group), or ETN alone (the E group). Radiographs of the hands and feet before ETN (baseline) and during the first year of treatment were available from 53 (72%) and 68 (88%) patients in the E and E+M groups, respectively. Baseline characteristics of patients were comparable between those with and without available radiographic data in each treatment group (data not shown). However, most patients without data did not complete the study up to Week 52 as per protocol, chiefly due to lack of efficacy in the E group.[6] The mean baseline total Sharp-van der Heijde score (TSS)[7] was 114.5 in the E group and 113.1 in the E+M group (disease duration: 10.0 years and 8.4 years, respectively), and the smallest detectable change (SDC) in TSS over 52 weeks was 1.9.

Cumulative probability plots provided by the American College of Rheumatology (ACR)-N[8] clearly demonstrated a superior response (figure 1A,B) and a significantly greater ACR50 response rate in the E+M group at week 52 (76.5% vs 50.9%, p=0.0041, Fisher's exact test). Merged probability plots of individual radiographic change over 52 weeks (ΔTSS) suggested preferential existence of aggressive radiographic progressors among ACR50 non-responders in the E group. The relationship among treatment, clinical disease activity, and radiographic change was further addressed using time-averaged disease activity score of 28 joints (DAS28) over 52 weeks in place of ACR-N at Week 52 (figure 1C,D). Significant correlation between time-averaged DAS28 and ΔTSS was observed in the E (r^2=0.097, p=0.023) but not the E+M group (r^2=0.019, p=0.26). Aggressive radiographic progression was preferentially observed among patients with moderate or high activity on average in the E group (figure 1C), while in the E+M group, radiographic progression among these patients seemed to be balanced by radiographic regression among those in remission or with low disease activity (figures 1D–F).

The absence of radiographic regressors (>SDC) among clinical responders in the E group (figure 1A,C,E) was surprising, although 18.2% of those patients showed regression within the SDC. This may be partly explained by the limitations of the study due to the small number of patients involved. Another limitation was much lower MTX dose at study enrolment than the current global standard dosage: 7.0±1.4 (the mean±SD) and 7.4±1.1 in the E and E+M groups, respectively.

In summary, we first demonstrated the relationship between individual clinical responses and radiographic changes by merging cumulative probability plots of ACR-N or time-averaged DAS28 and ΔTSS. These presentations clearly show the relationships between two parameters as a whole, facilitating further post hoc analyses of clinical trials. Further, merged presentation of probability plots is useful in comparing a single parameter (eg, health assessment questionnaire-disability index: HAQ-DI) before and after treatments (figure 2). However, merged presentation of probability plots must be followed by statistical analyses after being classified into binary or ternary categories, as we showed here.

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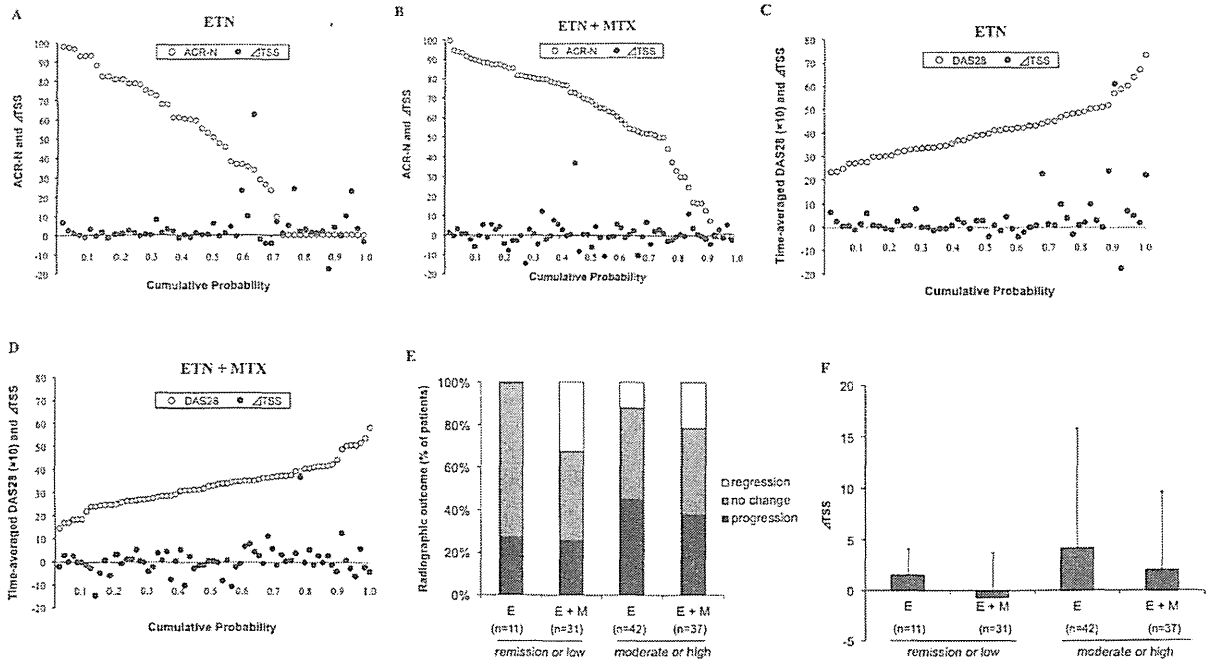
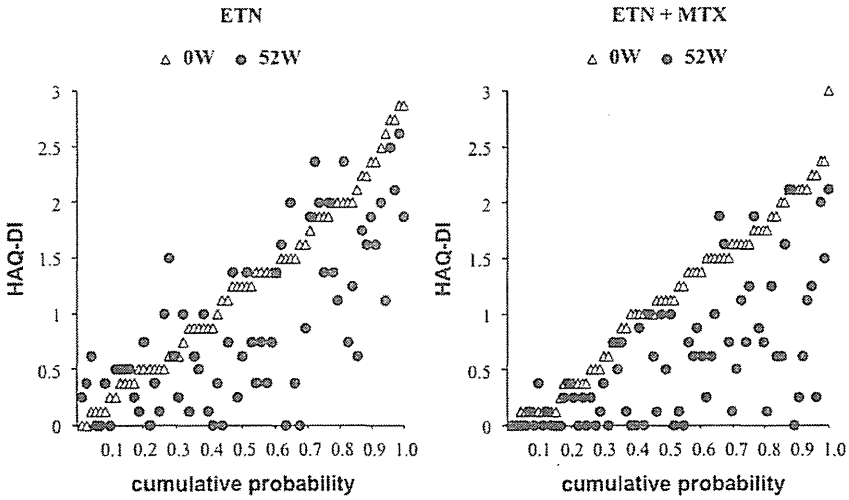


Cumulative probability plot analysis of ACR-N (A,B) or time-averaged DAS28 (C,D) and radiographic changes in the E (A,C) and E+M groups (B,D), merged to keep same patients on the vertical line, followed by the radiographic outcomes (E) and changes (F) stratified by the treatment and time-averaged disease activity state. Time-averaged DAS28 was calculated by the area under the curve of DAS28 at weeks 0, 2, 4, 8, 12, 24 and 52, divided by 52. No significant differences were observed between groups using Pearson's test (E) and Kruskal-Wallis test (F). ACR, American College of Rheumatology; DAS28, disease activity score of 28 joints; ETN, etanercept; MTX, methotrexate; TSS, total Sharp-van der Heijde score.



Merged probability plots of individual health assessment questionnaire-disability index (HAQ-DI) scores at baseline (open triangle) and Week 52 (closed circle) in the E (left) and E+M groups (right). Subsequent analyses included comparison of the rate of HAQ-DI≤0.5 at 52 weeks in patients with baseline HAQ-DI>1.5. None of 15 patients (0.0%) in the E group and 6 of 23 patients (26.1%) in the E+M group, respectively; p=0.037 by Fisher's exact test (one-sided). ETN, etanercept; MTX, methotrexate.

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Serum hepcidin level is not an independent surrogate biomarker of disease activity or of radiographic progression in rheumatoid arthritis: results from the ESPOIR cohort

Hepcidin is an interleukin-6 induced peptide hormone involved in iron metabolism and inflammation.[1] Serum hepcidin level may distinguish anaemia due to chronic inflammation and/or iron deficiency in rheumatoid arthritis (RA) patients.[2] Furthermore, some studies have suggested that serum hepcidin could reflect disease activity raising its measurement as a new surrogate biomarker of RA.[3–6] These studies have several drawbacks (unreliable pro-hormone quantification, small number of patients).[7 8] Therefore, we assessed the serum level of the mature form of hepcidin by ELISA, (Bachem, St Helens, Merseyside, UK) in 791 individuals from the French cohort of early arthritis (ESPOIR) including 632 patients with RA fulfilling the American College of Rheumatology (ACR) - European League Against Rheumatism (EULAR) criteria at inclusion and 159 with undifferentiated arthritis in order to address whether hepcidin accurately reflects RA features, disease activity or radiographic disease progression.[9 10]

Beyond expected differences between RA and undifferentiated arthritis, serum hepcidin level was higher in RA (table 1).

Baseline characteristics of 791 patients with early rheumatoid arthritis or undifferentiated arthritis

	Undifferentiated arthritis (n=159)	Early RA (n=632)	p Value
Age	47.2±13.8	48.5±12.2	0.46
Women, n (%)	117 (74)	492 (78)	0.25
First symptom (months)	6.6±7.7	6.9±8.5	0.72
DAS28 value	4.04±1.03	5.40±1.23	<0.0001
CRP level (mg/l)	17.15±29.3	21.10±33.14	0.0028
ESR (mm)	25.3±22.4	30.6±24.9	0.0014
Positive anti-CCP antibodies, n (%)	2 (1.26)	313 (49.5)	<0.0001
Positive RF, n (%)	5 (3.)	365 (57.75)	<0.0001
Swollen joint count	3.5±2.4	8.2±5.2	<0.0001
Tender joint count	3.2±2.6	9.9±7.2	<0.0001
HAQ	0.69±0.58	1.05±0.69	<0.0001
VAS fatigue	42.8±31.1	49.2±27.2	0.0118
x-ray erosion at inclusion, n (%)	0 (0)	108 (17.1)	<0.0001
Haemoglobin (g/dl)	13.0±1.21	13.0±1.3	0.9582
Ferritinemia (µg/l)	151.4±164.7	149.2±157.5	06 802
MCV (μ^3)	88.41±4.55	88.75±5.1	0.2141
Serum hepcidin level	39.6±39.9	53.0±48.5	p<0.0001

Data are mean±SD unless indicated. Baseline characteristics of RA and undifferentiated arthritis patients were compared by χ^2 or Fisher's exact tests for discrete variables and unpaired t tests, Wilcoxon signed rank tests for continuous variables.
Anti-CCP, anticyclic citrullinated protein peptide antibodies; CRP, C reactive protein; DAS28, Disease Activity Score in 28 joints; ESR, erythrocyte sedimentation rate; HAQ, Health Assessment Questionnaire; MCV, mean cell volume; RA, rheumatoid arthritis; RF, rheumatoid factor; VAS, visual analogue scale.

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GYNAECOLOGY

Possible improvement of depression after systematic examination and explanation of live birth rates among women with recurrent miscarriage

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We conducted a prospective study to determine whether systematic examinations and provision of explanation regarding the successful birth rates might improve mood or anxiety disorders among childless women with recurrent miscarriages. A total of 305 first-visit patients with a history of 2–12 miscarriages completed a first questionnaire battery, including: 'K6', a new screening instrument for mood and anxiety disorders, the 'Symptom Checklist-90 Revised' (SCL-90-R) and the 'Emotional Impact' questionnaire. Of these, 170 patients who underwent routine examinations and received an explanation about successful live birth rates responded to the second questionnaire. A total of 15.4% of the patients were estimated to suffer from diagnosable depression or anxiety disorders. Patients with high scores on K6 also showed elevated scores on all the subscales of SCL-90-R, including depression and anxiety. The K6 of patients with translocation was significantly higher than that of patients with antiphospholipid antibodies. The K6 and depression scores in the 2nd questionnaire survey were significantly lower than those in the 1st survey in the 170 patients. Improvement in depression was found in patients who underwent routine examination and received an explanation.

Keywords: Depression, K6, mental distress, recurrent miscarriage, Symptom Checklist 90-Revised

Introduction

Miscarriage (spontaneous abortion) can induce depression, anxiety, denial, anger and a sense of loss and inadequacy. In one series, 10.9% of women with sporadic miscarriages experienced at least one episode of major depression (Neugebauer et al. 1997). Craig et al. (2002) reported psychiatric disorders in 33% of patients with recurrent miscarriages. However, this morbidity estimate was based on questionnaire scores and not on diagnoses. The true morbidity is therefore unknown, and there is no established treatment method for mood and anxiety disorders in patients with recurrent miscarriage.

Established causes of recurrent miscarriages include abnormal chromosomes in either partner, particularly translocations, as well as presence of antiphospholipid antibodies (aPLs) in the serum and uterine anomalies (Farquharson et al. 1984; Sugiura-Ogasawara et al. 2004; Sugiura-Ogasawara et al. 2010a). An abnormal embryonic karyotype is also a well-known cause of recurrent miscarriages and has been reported in about 25–50% of aborted conceptuses (Ogasawara et al. 2000; Carp et al. 2001; Sullivan et al. 2004). The relatively wide range may reflect differences in the maternal mean age and the mean number of previous miscarriages, since these could conceivably exert an influence.

Embryonic aneuploidy is the most important cause of miscarriage before the completion of 10 weeks' gestation, and our previous study showed that about 70% of sporadic miscarriages are caused by an abnormal embryonic karyotype (Ogasawara et al. 2000). A more recent comparative genomic hybridisation microarray analysis also indicated a rate of about 80% (Shimokawa et al. 2006). Thus, the incidence of recurrent miscarriages caused by an abnormal embryonic karyotype can be calculated as $(0.8)^n$ in n consecutive miscarriages; using this formula; that of patients with three miscarriages can be calculated as 51%. This rate is in line with the previous finding that about 50% of karyotypes were abnormal in the aborted conceptuses of a group of women with recurrent miscarriages (Ogasawara et al. 2000). An abnormal embryonic karyotype is also well-known as a predictor of subsequent successful birth (Ogasawara et al. 2000; Carp et al. 2001; Sullivan et al. 2004). In fact, about 85% of patients suffering unexplained recurrent miscarriages were found cumulatively to have successful live births (Sugiura-Ogasawara et al. 2009; 2010a; Franssen et al. 2006). We reported that the live birth rate (p) can be calculated as follows: $\text{logit}(p) = 3.964 - 0.0652 \times (\text{age}) - 0.408 \times (\text{previous number of miscarriages})$.

However, conventional examination of couples with recurrent miscarriages yields no putative cause in $> 50\%$ of cases, because the aborted conceptuses are seldom karyotyped clinically (Clifford et al. 1994). It is speculated that such patients might give up becoming pregnant because of the fear of further miscarriage.

We therefore conducted the present prospective study to examine the morbidity arising from mental disorders in childless women with recurrent miscarriages and to determine whether systematic examination and provision of an explanation concerning the expected success rates may improve the psychological distress levels in these subjects, using K6, a new screening scale for psychological distress.

Materials and methods

Patients

This subject sample consisted of 305 patients with a history of two or more (2–12) consecutive miscarriages and no child

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at the first visit, for discussion about further attempts to have a baby between January 2008 and November 2010. The mean (SD) age was 33.0 years (4.8). The first questionnaire was administered at the first visit during the patients' waiting time. Systematic examination included: hysterosalpingography; chromosome analysis for both partners; determination of serum aPL; including lupus anticoagulant and β2-glycoprotein I dependent anticardiolipin antibodies (Ogasawara et al. 1996); and blood tests for hypothyroidism, diabetes mellitus before a subsequent pregnancy.

Once all of these examinations were completed, a designated obstetrician explained the results and the expected live birth rate. We offered combined low-dose aspirin and heparin therapy for patients with the antiphospholipid syndrome and predicted a 70–80% success rate. We provided genetic counselling with regard to the mean probability of a live birth of 50% by natural pregnancy and the pre-implantation genetic diagnosis for translocation carriers (Sugiura-Ogasawara et al. 2004; Sugiura-Ogasawara et al. 2008; Kyu Lim et al. 2004), and also counselling to explain the live birth rate of 60% in the absence of surgery for congenital uterine anomalies (Sugiura-Ogasawara et al. 2010a). We provided information about the live birth rate at the first pregnancy after the examination to unexplained patients, based on the women's age and number of previous miscarriages from our database, which is 76% in patients with two miscarriages; 70% in patients with three miscarriages and 60% in patients with four miscarriages (Sugiura-Ogasawara et al. 2009). At this time, we asked the participants to reply to a second questionnaire at home 2 weeks later and provided the questionnaire and a stamped addressed envelope.

The study was conducted with the approval of the Research Ethics Committee. Each participant provided written informed consent after being provided with a full explanation about the purpose of the study and the methods to be employed.

The first questionnaire survey

The first questionnaire included K6, the Symptom Checklist-90-Revised (SCL-90-R), knowledge of miscarriage and anxiety about miscarriage before the first pregnancy and the emotional impact (EI) for each miscarriage. The frequency of depression or anxiety; the influence on depression of knowledge about miscarriage; the association between EI and the first, second, third and subsequent miscarriages or clinical, chemical or stillbirths were recorded. Knowledge regarding the frequency of miscarriage of about 15% in the first pregnancy was recorded using a four-category scale (not at all, a little bit, quite a bit, extremely).

The items in K6 covered how frequently the respondents experienced symptoms of psychological distress (e.g. feeling so sad that nothing can cheer you up) during the previous 30 days. Responses were recorded using a five-category scale (4 = all of the time; 3 = most of the time; 2 = a little of the time; 1 = none of the time), producing, therefore, a scale range 0–24 (Kessler et al. 2002). According to the Japanese validation study of K6 in the general population, the positive predictive value of K6 scores of 10 or more was 0.49 (Furukawa et al. 2008).

SCL-90-R is a self-reported questionnaire containing 90 short questions, to which the answers are classified on a five-category scale (0 = not at all, 1 = a little bit, 2 = moderately, 3 = quite a bit, 4 = extremely). It covers the whole spectrum of psychiatric symptoms, including: depression; somatisation; anxiety; obsessive-compulsive behaviour; interpersonal sensitivity; hostility (aggression and irritability); phobic anxiety; paranoid ideation (mistrust and persecutory feelings) and psychoticism.

The second questionnaire survey

The second questionnaire survey also included K6 and SCL-90-R.

Statistical analyses

Data were analysed by *t*-tests using SPSS for Windows Version 10.0. Values with $p < 0.05$ were considered to be statistically significant.

First, we examined the prevalence of mood or anxiety disorders using the cut-off of 10/9 and the associated positive predictive value discussed above with the Japanese version of K6. We then compared the psychopathological characteristics of high versus low K6 scores in terms of the SCL-90-R subscales. The association between K6 scores and the EI of miscarriages; the EI of the number of previous miscarriage; and the EI of stillbirths, early abortions and chemical abortions were also examined.

In addition, we examined the changes in the K6 scores and scores for the SCL-90-R subscales in the first to second questionnaire surveys.

Results

In the first questionnaire survey, 96 of the 305 patients (31.5%) were found to have K6 scores of ≥ 10. Given the positive predictive value of such K6 scores (Furukawa et al. 2008), it was estimated that 15.4% of the patients with recurrent miscarriages would be suffering from mood or anxiety disorders. This prevalence is significantly higher than the 1.9% reported for the Japanese general population (Kawakami et al. 2005).

The mean (SD) scores for the SCL-90-R subscales in the subjects with K6 ≥ 10 and K6 < 10 are shown in Table I. The means (SDs) for all subscales were significantly higher for K6 ≥ 10. The first K6 scores (scores in the first K6 survey) were correlated with the scores for all the subscales of the first SCL-90-R (scores in the first SCL-90-R survey), but did not differ depending on the age or number of previous miscarriages.

The K6 score was correlated with the EI of the first miscarriage (1st EI) ($r = -0.130$, $p = 0.027$) and third miscarriage ($r = -0.192$, $p = 0.03$). The EI of the second miscarriage was significantly higher than that of the first miscarriage in patients with a history of three miscarriages.

As the level of knowledge of miscarriage decreased, the negative 1st EI increased (extremely: -53.1 ± 29.3; quite a bit: -74.5 ± 33.8; a little bit: -77.7 ± 50.3; not at all: -82.4 ± 24.8, $p = 0.02$). However, the score for the subscale of depression in the SCL-90-R increased as the level of knowledge increased (extremely: 0.99 ± 0.64; quite a bit: 0.99 ± 0.66; a little bit: 0.77 ± 0.63; not at all: 0.72 ± 0.60, $p = 0.006$).

The EI (-97.86 ± 5.79) of stillbirth (more than 10 weeks' gestation) was significantly higher than of early abortion (-82.43 ± 25.02) or chemical abortion (-70.43 ± 27.23, $p = 0.002$).

Table I. The association between K6 and SCL-90-R (mean ± SD).

SCL-90-R	K6 ≥ 10 ($n = 96$)	K6 < 10 ($n = 209$)	*p* value
Depression	1.47 ± 0.64	0.55 ± 0.41	< 0.001
Somatisation	0.76 ± 0.55	0.37 ± 0.34	< 0.001
Anxiety	0.91 ± 0.66	0.26 ± 0.30	< 0.001
Obsessive-compulsive behaviour	1.10 ± 0.68	0.50 ± 0.42	< 0.001
Interpersonal sensitivity	1.16 ± 0.63	0.45 ± 0.40	< 0.001
Hostility	0.93 ± 0.68	0.31 ± 0.35	< 0.001
Phobic anxiety	0.55 ± 0.56	0.15 ± 0.28	< 0.001
Paranoid ideation	0.45 ± 0.45	0.16 ± 0.27	< 0.001
Psychoticism	0.56 ± 0.43	0.16 ± 0.22	< 0.001

A total of 170 (81.7%) of the 208 patients who had completed the systematic examinations, sent in their responses to the second questionnaire. No patients received medication to prevent miscarriages at the first or second questionnaire. No patients with translocation wished for PGD. No patients with uterine anomaly received surgery. The 2nd K6 of patients with translocation (8.0 ± 3.85) was significantly higher than that of patients with aPLs (3.44 ± 3.43, $p = 0.03$).

On average, the 2nd K6 (5.17 ± 4.28) and score for the depression subscale of SCL-90-R in the second survey (0.79 ± 0.69) were significantly lower than the 1st K6 (7.59 ± 5.22) and depression score in the first survey (0.89 ± 0.67) ($p < 0.0001$). In all, 27 of the 170 patients (15.9%) had a 2nd K6 ≥ 10, indicating that 7.8% of patients suffered from mood or anxiety disorders after undergoing routine examinations and receiving the 'explanation'. At the individual level, a significantly higher number of patients showed improved scores after the conventional examinations and the 'explanation' ($p < 0.0001$).

Discussion

Patients with K6 ≥ 10 were found to have higher scores on all the subscales of SCL-90-R, namely: depression; somatisation; anxiety; obsessive-compulsive behaviour; interpersonal sensitivity; hostility; paranoid ideation; phobic anxiety and psychoticism, than those with K6 < 10. SCL-90-R is one of the most widely used symptom questionnaires in the field of psychiatry, whose primary purpose is to depict the symptomatological profile of the respondents and not to screen for the presence/absence of certain disorders. Our results demonstrate that not anxiety but depression could improve after routine examination and provision of information concerning expected live birth rates in patients with recurrent miscarriages. K6 was found to be a useful instrument for screening for mental disorders in patients with recurrent miscarriages.

Kessler et al. (2002) developed the K6, a 6-item very short screening instrument using modern items response theory methods to select questions that maximally discriminate respondents in the 90th to 99th percentile range of the population distribution, because it is known that between 5–10% of the general population suffer from serious mental illness at any point in time. K6 was found to strongly discriminate between community cases and non-cases of the Diagnostic and Statistical Manual Disorders, 4th edn (DSM-IV). Furukawa et al. (2008) developed the Japanese version according to the standard back-translation procedure, and the screening performance was shown to be essentially equivalent to that reported for the original English version, indicating the success in producing a cross-culturally applicable screening scale. K6 was, in fact, incorporated into the World Health Organization (WHO) World Mental Health Survey undertaken in multiple countries, including Japan (Demyttenaere et al. 2004).

In a very recent epidemiological study, the morbidity associated with DSM-IV mood disorders (depression, dysthymia) or anxiety disorders (panic disorder, agoraphobia, social phobia, generalized anxiety disorder, post-traumatic stress disorder), during 1 month in the Japanese general population, was found to be 1.9% (Kawakami et al. 2005). In clear contrast, 15.4% of patients with recurrent miscarriages were speculated to suffer from mood or anxiety disorders, because theoretically, 49.0% of cases with K6 ≥ 10 can be estimated to suffer from one or more of these disorders (Furukawa et al. 2008).

In the present study, the patients undergoing systematic examinations and receiving the explanation exhibited significant improvements of the K6 scores and scores for the depression subscale of SCL-90-R. There was an interval of about 2 months between the two questionnaire surveys, and this interval itself might have alleviated the mental distress, to a certain extent. We could not conduct a case–control study because it was difficult to assess an appropriate control group. However, receiving conventional examination and information about the expected success rate might help patients' mood and improve their mental distress.

The fact that the EI of a second miscarriage was significantly higher than that of a first, in patients with a history of three miscarriages, is consistent with the results of our previous study (Aoki et al. 1998). Negative EI increased as the level of knowledge about miscarriage decreased. Japanese women have limited knowledge of reproduction (Sugiura-Ogasawara et al. 2010b). Education concerning miscarriages is needed for preventing mental disorders among women of the reproductive age.

The mental disorders in patients with translocation were found to be significantly more severe than those in patients with aPLs. Patients with aPLs received information about established treatments for a subsequent pregnancy, which could have led to desirable psychological effects.

Sugiura-Ogasawara et al. (2002) proved that depression influences the likelihood of further miscarriage in recurrent cases. It is unclear whether improvement of the K6 score might lead to improved live birth rate. Since there are no established treatment methods for preventing miscarriages in cases with unexplained recurrent miscarriages (Rai and Regan 2006), the possibility that psychosocial support might be able to prevent the patients giving up on a subsequent pregnancy, clearly warrants further attention.

We usually explain the treatment methods to patients with aPLs; provide genetic counselling for translocation carriers and provide information on the expected live birth rates for patients with unexplained miscarriages after systematic examinations. Both maternal age and reproductive history are independent predictors of further pregnancy outcomes (Sugiura-Ogasawara et al. 2009; Stephenson et al. 2002). Several couples in our experience give up trying to conceive after recurrent miscarriages, because they have the misconception that it would be impossible for them to have a live baby. Psychological tender loving care might be the most important requirement to continue trying to conceive until it results in a live birth (Liddell et al. 1991). Some patients remained depressed even after the examination, and cognitive behavioural therapy may be useful to obtain improvement in such cases.

In this study, 15.4% of patients were found to have mood or anxiety disorders. This is the first report of possible improvement of depression after conventional examinations and provision of information concerning the expected success rates in patients with recurrent miscarriages. Since our sample size was relatively small, further study with a larger sample in multiple settings would be needed to examine and confirm our conclusions.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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human reproduction

ORIGINAL ARTICLE *Early pregnancy*

Abnormal embryonic karyotype is the most frequent cause of recurrent miscarriage

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BACKGROUND: We previously found that a normal karyotype in a previous miscarriage is a predictor of subsequent miscarriage. However, the prevalence of recurrent miscarriage caused by an abnormal embryonic karyotype has not yet been reported, since embryonic karyotype is not typically analyzed during conventional examinations.

METHODS: A total of 482 patients who underwent both embryonic karyotype determination and conventional examinations for recurrent miscarriage were enrolled in this study. The distribution of the causes and the live birth rate for each cause were examined.

RESULTS: The total percentage of subjects in whom conventional causes of recurrent miscarriage could be detected was 29.5%. The prevalence of the abnormal embryonic karyotype was 41.1% in the subjects in whom no conventional causes of miscarriage could be identified. The prevalence of recurrent miscarriage of truly unexplained cause, that is, of subjects without conventional causes in whom the embryonic karyotype was ascertained to be normal, was 24.5%. Among the patients in whom the first determination revealed an abnormal embryonic karyotype, 76.2% (32/42) showed an abnormal embryonic karyotype in the repeat determination as well. The cumulative live birth rate (71.9%) in women with recurrent miscarriages caused by the abnormal embryonic karyotype was significantly higher than that (44.7%) in women with recurrent miscarriages associated with the embryonal euploidy.

CONCLUSION: An abnormal embryonic karyotype was found to represent the commonest cause of recurrent miscarriage, and the percentage of cases with recurrent miscarriage of truly unexplained cause was limited to 24.5%.The two groups should be distinguished for both clinical and research purposes.

Key words: embryonic karyotype / live birth rate / prevalence / recurrent miscarriage

Introduction

Established causes of recurrent miscarriage include the presence of antiphospholipid antibodies (aPLs), uterine anomalies and abnormal chromosomes, particularly translocations, in either partner, (Farquharson *et al.*, 1984; Sugiura-Ogasawara *et al.*, 2004; Sugiura-Ogasawara et al., 2010). According to previous reports, in about half of the cases seen at research centers, the cause of recurrent miscarriage remains unexplained despite conventional examinations conducted to identify the cause (Clifford et al., 1994; Stephenson, 1996).

A majority of miscarriages that occur before 10 weeks of gestation are due to chromosomal aneuploidies arising from new non-disjunctional events, such events being more frequent in very early miscarriages (Sierra and Stephenson, 2006). We found that the abnormal embryonic karyotype rate was as high as 51% in subjects with recurrent miscarriages, even though it was significantly lower than that of 76.3% in patients with sporadic miscarriages (Ogasawara *et al.*, 2000). Many reports have suggested that the abnormal embryonic karyotype contributes to not only sporadic, but also to recurrent miscarriage (Stern *et al.*, 1996; Carp *et al.*, 2001; Stephenson *et al.*, 2002; Sullivan *et al.*, 2004). A recent review recommended chromosomal analysis of the products of conception in addition to the conventional tests in the evaluation of women with recurrent miscarriage (Branch *et al.*, 2010). However, to the best of our knowledge, there are no reports of the precise distribution of all causes of recurrent miscarriage, because embryonic karyotype analysis cannot be performed in all centers, including research centers. Patients wishing to undergo investigation

for recurrent miscarriage usually visit hospitals while they are not pregnant.

Information about the prevalence of an abnormal embryonic karyotype in women with a history of recurrent miscarriage and their future prognosis is still limited. Therefore, the present study was conducted to assess the subsequent live birth rate due to various causes, in women presenting with a history of recurrent miscarriage.

Methods

Patients

We studied 482 patients with a history of two or more (2–21) consecutive miscarriages who completed a systematic conventional examination and whose embryonic karyotype was ascertained at least once and documented in our medical records. Patients wishing for a second opinion after undergoing an examination at another hospital or who requested specific treatment were excluded to avoid a selection bias in the present study. The mean [standard deviation (SD)] age and number of previous miscarriages were 32.4 (4.45) and 3.04 (1.38), respectively.

Investigations for maternal causes of recurrent miscarriage

All patients completed conventional examinations, such as hysterosalpingography, chromosomal analysis of both partners, determination of aPL, including lupus anticoagulant, by diluted activated partial thromboplastin time, diluted dilute Russel viper venom time and β2glycoprotein I-dependent anticardiolipin antibody methods (Ogasawara *et al.*, 1996) and blood tests for hypothyroidism and diabetes mellitus (DM), before a subsequent pregnancy. Transvaginal ultrasonography was performed to examine the morphology of any polycystic ovaries.

Conventional causes included antiphospholipid antibody syndrome (APS), occasional aPLs, abnormal chromosomes in either partner and uterine anomalies, excluding arcuate uterine and endocrine abnormalities. Hypothyroidism, DM and polycystic ovary syndrome (PCOS) were included as endocrine abnormalities.

APS was diagnosed according to the criteria of the International Congress on Antiphospholipid Antibodies (Miyakis *et al.*, 2006). Patients with APS were treated with low-dose aspirin plus heparin (after 1995, Cowchock *et al.*, 1992) or low-dose aspirin plus prednisolone (before 1995, Farquharson *et al.*, 1984). Occasional aPL-positive cases were included as a separate group, because it was found that the live birth rate in these patients could be improved by treatment with low-dose aspirin alone (Sugiura-Ogasawara *et al.*, 2008).

Karyotyping the aborted conceptus

Karyotyping of the conceptus was performed routinely and not in response to some indication in the Nagoya City University hospital. Gestational age was calculated from basal body temperature charts. Ultrasonography was performed once or twice a week from 4 to 8 weeks of gestation. Dilation and curettage was performed on all patients diagnosed as having miscarriage. Part of the villi was cultured, and the cells were harvested after 6–22 days of cultivation to analyze the chromosomes. A total of 635 aborted conceptuses could be karyotyped using a standard G-banding technique. The 234 aborted conceptuses and 18 patients reported in our previous study were included in the present analyses (Ogasawara *et al.*, 2000; Mizutani *et al.*, 2011). In this paper, abnormal embryonic (fetal) karyotype patient refers to patients without conventional causes of recurrent miscarriage whose embryonic (fetal) karyotype was abnormal. Mixed patient refers to patients without conventional causes who had miscarried both normal embryos/fetuses and embryos/fetuses with an abnormal karyotype. Unexplained patient refers to patients without conventional causes whose embryonic (fetal) karyotype was normal.

The study was conducted with the approval of the Research Ethics Committee at the Nagoya City University Medical School.

Statistics

In the present study, we examined the distribution of the causes of recurrent miscarriage, including the abnormal embryonic karyotype, the cumulative live birth rate for each cause of recurrent miscarriage and the distribution of causes in cases with secondary versus primary recurrent miscarriage and in women over 40 years old versus those under 40 years of age.

The analysis was carried out using the SPSS for Windows, Version 19.0. $P < 0.05$ was considered to denote statistical significance.

Results

Of the 635 aborted products, normal and abnormal karyotypes were 44.9% ($n = 285$; euploid 280, translocation 5) and 55.1% ($n = 350$; trisomy 199, double trisomy 22, monosomy 25, polyploidy 38, tetraploidy 9, derivative of translocation 34, others 23), respectively.

The distribution of the causes and the characteristics of the subjects are shown in Table I and Figure 1. The total percentage of cases in which conventional causes of miscarriage could be detected was 29.5%. The prevalence of the abnormal embryonic karyotype was 41.1% in the subjects in whom no conventional causes of miscarriage could be identified. The prevalence of recurrent miscarriage of truly unexplained cause, in women without conventional causes whose embryonic karyotype was ascertained to be normal, was limited to 24.5%.

Details of abnormal embryonic abnormalities were included in Table I. The frequency of derivative was 47.9% (34/71) in aborted conceptus of patients with translocation.

The cumulative live birth rates in patients with abnormal embryonic karyotype, abnormal chromosome in either partner, uterine anomalies and miscarriages of truly unexplained cause were 71.2, 58.0, 65.2 and 52.5%, respectively. The mean ages at pregnancy of the patients with abnormal chromosomes in either partner, uterine anomaly and unexplained causes were significantly younger than that of the patients with an abnormal embryonic karyotype. The mean total numbers of losses of patients with occasional aPL, abnormal chromosomes in either partner, and unexplained and mixed causes were significantly higher than that of the patients with an abnormal embryonic karyotype. These results suggest that the prognosis of patients with abnormal chromosomes in either partner and unexplained causes is poor.

The embryonic karyotypes in 95 of 482 women could be analyzed at least twice (Table II). The abnormal embryonic karyotype was the cause of miscarriages in 32 women with repeated miscarriages, while 38 women with repeated miscarriages showed embryonic euploidy. In all, 73.7% (70/95) of the women were found to show repeated miscarriages of the same cause (Table II). In 76.2% of (32/42) the patients in whom the first determination revealed an embryonic karyotype was abnormal, the second determination also revealed an abnormal embryonic karyotype.

Table I Distribution of the causes, the subject characteristics and the cumulative live birth rate

	Conventional causes					No conventional causes		
	APS	**Occasional aPL**	**Abnormal chromosome in either partner**	**Major uterine anomaly**	**Hypothyroidism, DM, PCOS**	**Unexplained**	**Mixed**	**Abnormal embryonic karyotype**
Prevalence [% (*n*)]	2.5 (12)	6.2 (30)	10.4 (50)	4.8 (23)	5.6 (27)	24.5 (118)	4.1 (20)	41.1 (198)
Mean (SD) age at pregnancy	33.2 (4.7), NS	32.9 (4.6), NS	30.4 (4.5)[a], $P < 0.0001$	31.2 (4.4)[a], $P = 0.04$	33.7 (3.6), NS	31.3 (4.3)[a], $P = 0.0002$	32.1 (3.9), NS	33.2 (4.4)
Mean (SD) number of losses	3.7 (1.2), NS	4.8 (2.0)[a], $P = 0.0006$	4.9 (2.0)[a], $P < 0.0001$	5.0 (2.6), $P = 0.056$	4.5 (1.6), NS	4.9 (3.1)[a], $P = 0.001$	5.8 (2.7)[a], $P = 0.005$	3.9 (1.2)
The prevalence [% (*n*)] of women with at least one stillbirth	25.0 (3)	6.7 (2)	4.0 (2)	4.3 (1)	3.7 (1)	8.5(10)	15.0 (3)	4.0 (8)
The prevalence [% (*n*)] of women with at least one previous live birth	8.3 (1)	13.3 (4)	18.0 (9)	4.3 (1)	11.1 (3)	16.9 (20)	10.0 (2)	18.7 (37)
% of embryos with abnormal karyotype[b]	46.7 (7/15)	41.9 (18/43)	73.2 (52/71)	13.8 (4/29)	44.4 (16/36)	0 (0/158)	44.9 (23/49)	100 (229/229)
The prevalence [% (*n*)] of women with at least one abnormal karyotype	50.0 (6)	53.3(16)	86.0 (43)	17.4 (4)	48.1(13)	0 (0)	100 (20)	100 (198)
Karyotype (*n*)								
	Trisomy 4 Double trisomy 1 Triploidy 1 Tetraploidy 1	Trisomy 10 Double trisomy 1 Monosomy 4 Triploidy 2 Other 1	Derivative 34[c] Trisomy 10 Double trisomy 3 Monosomy 1 Triploidy 2 tetraploidy 1 Other 1	Monosomy 2 Triploidy 1 Tetraploidy 1	Trisomy 11 Double trisomy 2 Tetraploidy 1 Other 1		Trisomy 14 Triploidy 7 Other 2	Trisomy 149 Double trisomy 1 Monosomy 16 Triploidy 25 Tetraploidy 5 Other 18
Cumulative live birth rate [% (*n*)]	50.0 (6)	60.0 (18)	58.0 (29)	65.2 (15)	70.4 (19)	52.5 (62)	65.0 (13)	71.2 (141)

Conventional causes: APS, occasional aPL, abnormal chromosome in either partner, major uterine anomaly and endocrine abnormality including hypothyroidism, DM and PCOS. Three patients with CAM and one patient with protein C deficiency were excluded.

[a]The comparison was performed between patients with an abnormal embryonic karyotpe and the others.

[b]The number of abnormal karyotypes/the number of conceptuses karyotyped.

[c]The frequency of derivative was 47.9% in aborted conceptus of patients with translocation.

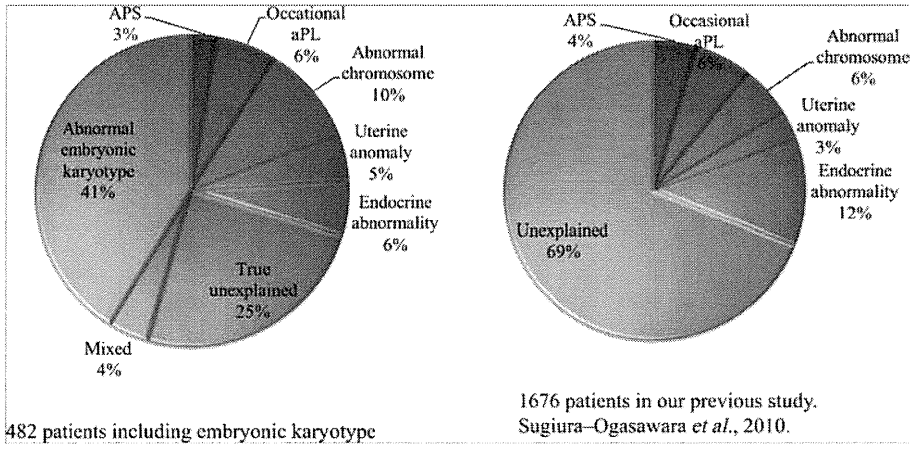

Figure 1 Comparison of the distribution of causes between our 482 subjects including those with the abnormal embryonic karyotype and the 1676 subjects of a previous study.

Table II The live birth rate and characteristics of the 95 patients in whom the embryonic karyotype could be analyzed at least twice.

Embryonic karyotype	Abnormal	Mixed	Normal	*P*-value[a], odds ratio (95% CI)
No. of patients	32	25	38	
Mean (SD) age at pregnancy	34.0 (4.8)	32.2 (3.9)	30.4 (3.9)	0.001
Mean (SD) number of losses	5.0 (1.5)	5.8 (2.6)	7.2 (4.4)	0.007
The prevalence [% (*n*)] of women with at least one stillbirth	12.5 (4)	12.0 (3)	15.8 (6)	NS
The prevalence [% (*n*)] of women with at least one previous live birth	25.0 (8)	8.0 (2)	10.5 (4)	NS
Live birth rate (%)	71.9 (23)	64.0 (16)	44.7 (17)	0.02, 1.6 (1.072–2.532)

[a]Comparison was performed between patients with abnormal and normal embryonic karyotypes.

The mean age and previous number of losses in the women with recurrent miscarriages associated with abnormal embryonic karyotype were significantly higher and fewer, respectively, than those in the women with recurrent miscarriages associated with the embryonic euploidy,($P = 0.001$ and 0.007). The cumulative live birth rate (71.9%) in women with recurrent miscarriages caused by the abnormal embryonic karyotype was significantly higher than that (44.7%) in women with recurrent miscarriages associated with the embryonal euploidy ($P = 0.02$, odds ratio 3.2, Table II).

Comparison between the 404 patients with primary and 78 patients with secondary recurrent miscarriages revealed that compared with primary (primary versus secondary; Fishers' exact probability test, $P = 0.044$, Fig. 2a) fewer secondary patients exhibited APS (2.7, versus 1.3%) or uterine anomalies (5.4 versus 1.3%) but more exhibited abnormal embryonic karyotype (39.8 versus 47.4%) or unexplained cause (24.3 versus 25.6%). Comparison of the 455 subjects who were under 40 years old and 27 subjects who were over 40 years old (<40 versus ≥ 40; Fishers' exact probability test, NS, Fig. 2b) revealed that older patients appeared less likely to exhibit APS (2.6 versus 0%) or uterine anomalies (5.1 versus 0%) but more likely to exhibit abnormal embryonic karyotype (40.2 versus 55.6%) or unexplained cause (24.8 versus 18.5%), although these differences were not statistically significant. APS or uterine anomalies were seldom found in patients with secondary recurrent miscarriages or who were over 40 years old. In all 5 patients with secondary recurrent miscarriages who were >40 years old, the cause was abnormal embryonic karyotype.

Discussion

This is the first study to show the prevalence of the abnormal embryonic karyotype as a cause of recurrent miscarriage. In the present study, the prevalence was 41.1%. An abnormal embryonic karyotype was detected as the commonest cause of recurrent miscarriage. The subjects in the present study can be regarded as representative Japanese patients with recurrent miscarriage because patients wishing for a second opinion after undergoing an examination at another hospital or who requested specific treatment were excluded; thus, a selection bias in the distribution of each cause was avoided.

The prevalence of abnormal embryonic karyotypes was reported to be 57% (Stern *et al.*, 1996), 29% (Carp *et al.*, 2001), 46% (Stephenson *et al.*, 2002), 25.4% (Sullivan *et al.*, 2004) and 55.1% (present study). The difference depends on women's age and the number of miscarriages. The abnormal rate increases as the women's age increases

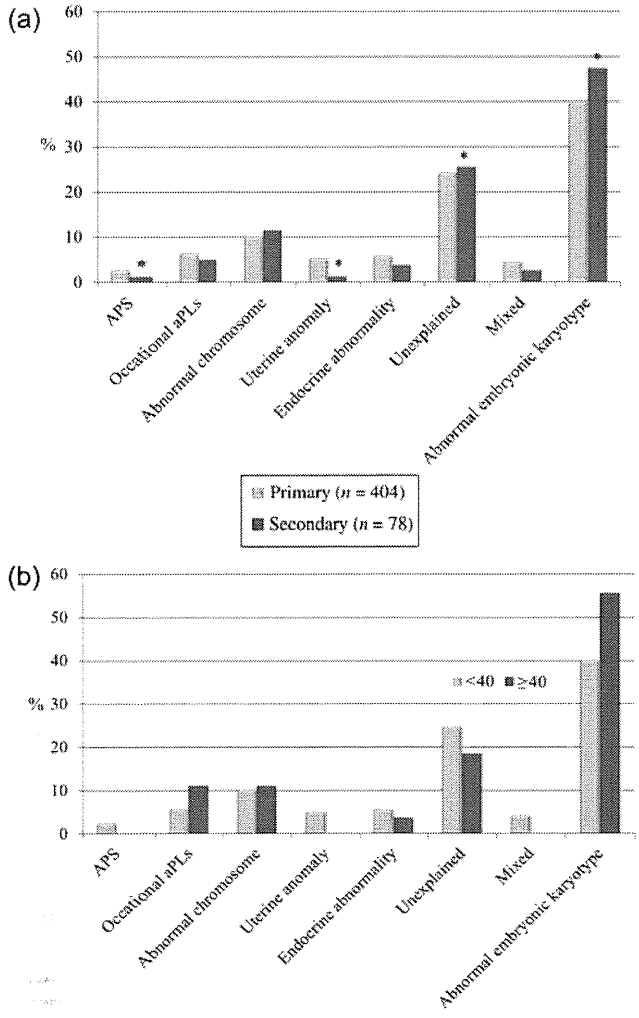

Figure 2 (**a**) Comparison of the distribution of causes of recurrent pregnancy loss between women with primary versus secondary recurrent miscarriage. *Significantly fewer secondary patients than primary exhibited APS or uterine anomaly but more exhibited abnormal embryonic karyotype or unexplained cause ($P = 0.044$). (**b**) Comparison of the distribution of causes of recurrent pregnancy loss between women <40 versus those ≥40 years old.

and the previous number of miscarriages decreases (Ogasawara *et al.*, 2000).

Several reports have suggested that the abnormal embryonic karyotype predicts subsequent live birth (Ogasawara *et al.*, 2000; Carp *et al.*, 2001). The cumulative live birth rate (71.2%) in women with miscarriage caused by an abnormal embryonic karyotype was found to be higher than that (52.5%) in women with recurrent miscarriage of truly unexplained cause. Patients with abnormal and normal chromosomes were found to have repeat miscarriages with abnormal and normal chromosomes, respectively. This was consistent with the results of our previous study conducted with a small sample size (Mizutani *et al.*, 2011). Karyotype analysis should be performed at least once, because the abnormal embryonic karyotype tends to repeat itself and is also a predictor of good prognosis.

Patients with recurrent miscarriage caused by the abnormal embryonic karyotype might have gene mutations associated with aneuploidy such as SYCP3. Unexplained reasons may cause the miscarriage of a euploid embryo and may continue to cause further miscarriages. SYCP3 mutations in women were found to generate an aberrant synaptonemal complex in a dominant-negative manner and to contribute to abnormal chromosomal behavior that could potentially lead to recurrent miscarriage (Bolor *et al.*, 2009). We found no clinical significance in the routine examination of the SYCP3 mutation because only one benign mutation was ascertained in 101 patients (Mizutani *et al.*, 2011). However, some gene mutations, such as MLH1, may influence aneuploidy because a double trisomy can be detected in recurrent but not in sporadic, miscarriage patients (Edelmann *et al.*, 1996; Ogasawara *et al.*, 2000). Preimplantation screening (PGS) is performed worldwide, though it is unclear whether PGS can improve the live birth rate in patients with recurrent miscarriage (Harper *et al.*, 2010). The cumulative live birth rate in patients with an abnormal embryonic karyotype was higher because such patients could have a euploid embryo. PGS may be useful in specific patients affected by a candidate gene mutation.

According to previous reports, about half of the women at research centers are determined as having recurrent miscarriage of unexplained cause despite receiving conventional examinations to determine the cause. There have been some reports on the distribution of the causes at individual centers. The prevalences of aPL, abnormal chromosome, uterine anomaly, endocrine abnormality (DM, both PCO morphology and mid-follicular serum LH level > 10 IU/L) and unexplained causes were 14, 3.6, 1.8, 6.8 and 73.8%, respectively, in Clifford's study (mean age, 32.9 years; median miscarriages, 4; Clifford *et al.*, 1994). The prevalences of aPL, abnormal chromosome, uterine anomaly, endocrine abnormality and unexplained causes were 20, 3.5, 16, 20 and 42.6%, respectively, in Stephenson's study (Stephenson, 1996). These previous studies did not distinguish between truly unexplained causes and an abnormal embryonic karyotype. The prevalence of unexplained causes was 69% among our patients (Fig. 1, Sugiura-Ogasawara *et al.*, 2010). The differences in the distribution of causes at each center may depend on age, number of previous miscarriages and methods of diagnosis for each factor. We compared the distribution of the causes between the 482 couples in the present study and 1676 couples of a previous study among whom the aborted conceptuses of all the patients were not analyzed (Sugiura-Ogasawara *et al.*, 2010, Fig. 1). The true percentage of subjects with recurrent miscarriage of unexplained cause was limited to 24.5%. Moreover, 482 patients in our present study experienced further miscarriages after undergoing a systematic examination. The patients with APS miscarried despite anticoagulant therapy. The prevalences of abnormal chromosomes in either partner and uterine anomaly were higher than the prevalences in previous study. This finding suggests that the prognosis of abnormal chromosomes and uterine anomaly is poor.

The reported live birth rate in women with APS treated with low-dose aspirin plus heparin is 70–80% (Cowchock *et al.*, 1992; Rai *et al.*, 1997). The cumulative live birth rate in this group in the present study was relatively low, because women with APS gave up after the first treatment and failure. The reported prevalence range, in review articles, of APS is 5–15% (Branch *et al.*, 2010). Several reports have indicated that about 10–15% of women with recurrent

miscarriage are diagnosed with APS (Clifford *et al.*, 1994; Rai *et al.*, 1995; Yetman and Kutteh, 1996). However, it is unclear whether the aPLs persisted according to International Criteria (Miyakis *et al.*, 2006). The prevalence of APS according to International Criteria was found to be 2.5% in the present study, presumably because the figure represents the prevalence after one treatment failure. The single positive rate of aPLs was 10.7% and the recurrent positive rate was 4.5% in our previous study (Sugiura-Ogasawara *et al.*, 2008). There are many methods used for the detection of aPLs. However, there are limited reports on which method might be most suitable for prediction of recurrent miscarriage or intrauterine fetal death. The positive rate might be large if methods with a large false-positive rate were used. The prevalence of 'true' APS might be <5%, although it depends on the age of the women comprising the study population and the method used for the detection of APS.

The frequency of major congenital uterine anomalies has been reported to be between 3.2 and 6.9% in women with a history of recurrent miscarriage, the variation largely depending on the method of selection and the criteria used for the diagnosis (Sugiura-Ogasawara *et al.*, 2011). Uterine anomalies are encountered in miscarriages associated with euploidy (Sugiura-Ogasawara *et al.*, 2010). However, the prognosis in these cases is better than that in patients presenting with recurrent miscarriage of unexplained cause.

Recently, the mean age of the population has been increasing year by year, and the proportion of women in their 40s has been increasing in Japan. APS and uterine anomalies were found to be rare in subjects with secondary recurrent miscarriage and women over 40 years old in the present study. This should be borne in mind before evaluation of the screening tests.

The prevalence of recurrent miscarriage of truly unexplained cause, in whom the embryonic karyotype was ascertained to be normal, was found to be 24.5% in this study. Kaandorp *et al.* (2010) demonstrated, based on the results of an RCT, that aspirin plus heparin therapy has no beneficial effect in patients with two or more miscarriages. Further studies are needed in women with recurrent miscarriage of truly unexplained cause, after excluding cases with an abnormal embryonic karyotype, to confirm the conclusion.

Limitation

The prevalence of each cause differs among centers, depending on the background of the patients, such as the mean age and previous number of miscarriages of the women in the studied population and the selected method and criteria for the diagnosis.

Subjects with thrombophilia, infection, fibroid or deficiency of progesterone were excluded from the analysis, because the contribution of these factors to recurrent miscarriage has not yet been established (Branch *et al.*, 2010).

Although the standard G-band technique is the gold standard for evaluating chromosomal abnormalities, it has several limitations, including the need for tissue culture and the possibility of maternal cell contamination. Additional analysis, such as by comparative genomic hybridization (CGH), could not be performed in the present study. Further abnormalities could be detectable and also contamination with maternal tissue could be distinguishable by CGH. A recent microarray CGH indicated that about 80% of sporadic spontaneous abortions were caused by an abnormal embryonic karyotype (Shimokawa *et al.*, 2006). Thus, if the prevalence is similar in women with recurrent miscarring and they have no predisposing factors, the incidence of the abnormal embryonic karyotype as a cause of recurrent miscarriage can be estimated to be $(0.8)^n$ in patients with n consecutive miscarriages.About 51% of patients caused by an abnormal embryonic karyotype can be expected to exist in patients with three miscarriages occasionally. The incidence might increase if the candidate gene such as SYCP3 or MLH affect.

Among subjects with abnormal chromosomes in the present study, 26.8% (19/71) carried embryos that were normal or balanced (Table I). It is possible that CGH would have revealed lack of balance, because 40% of 42 balanced translocation carriers as assessed by cytogenetic analysis were found to show a loss of balance as assessed by CGH (De Gregori *et al.*, 2007).

Conclusion

The prevalence of the abnormal embryonic karyotype in subjects with recurrent miscarriage was found to be 41.1%. The abnormal embryonic karyotype was found to be the commonest cause of miscarriage and often recurred as the cause of subsequent miscarriages. The prevalence of recurrent miscarriage of truly unexplained cause was found to be 24.5% in the present study. This should be a target of an RCT to determine the effectiveness of a treatment. These two groups should be distinguished for both clinical and research purposes. Embryonic analysis should be added to the list of evaluation items in women with recurrent miscarriages, for both clinical and research purposes.

Authors' roles

M.S.-O. has contributed to design the study, perform analysis and draft the article. Y.O., K.K., N.S., T.K. and E.M. have contributed to acquisition and analysis of data.

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Conflict of interest

None declared.

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Increase in the plasma levels of protein Z-dependent protease inhibitor in normal pregnancies but not in non-pregnant patients with unexplained recurrent miscarriage

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Summary

Protein Z (PZ)-dependent protease inhibitor (ZPI) is a serine protease inhibitor which efficiently inactivates activated factor X, when ZPI is complexed with PZ in plasma. Reduced plasma levels of ZPI and PZ have been reported in association with thrombosis. It has also been reported that PZ increases during pregnancy and that its partial deficiency is related to early pregnancy loss or recurrent miscarriage (RM). However, until now there has been no report on ZPI in pregnancy. To explore the possible role(s) of ZPI in the maintenance of pregnancy, we studied 42 non-pregnant normal women, 32 women with normal pregnancies, and 134 cases of unexplained RM in Japan, as well as 64 non-pregnant normal German females. Plasma ZPI was measured by in-house ELISA. There were significantly higher concentrations of plasma ZPI in normal pregnancies compared to non-pregnant women. The present study also confirmed that both factor X, the major target of ZPI, and protein Z increased during normal pregnancies. This increased ZPI and PZ may counteract the increased activated factor X, which may in turn contribute to the maintenance of normal placental circulation. Plasma ZPI levels were unchanged in non-pregnant RM women, while the plasma PZ level was slightly reduced, a finding consistent with existing reports. The exact relationship between RM and this unaltered ZPI with mild PZ reduction relative to normal pregnancies warrants further investigation.

Keywords

Anti-coagulation system, protein Z-dependent protease inhibitor, protein Z, pregnancy, recurrent miscarriage

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Introduction

Protein Z (PZ) is one of seven vitamin K-dependent coagulation proteins (VKDPs) with structural homology to coagulation factors VII, IX (FIX), and X (FX), and protein C (1). It serves as a cofactor for the inactivation of activated FX (FXa) by a serine protease inhibitor called PZ-dependent protease inhibitor (ZPI) (2). Since normal plasma contains excess ZPI relative to the amount of PZ, all the PZ is bound in a complex with ZPI (3); thus there is some free ZPI in normal plasma. While the ZPI-PZ complex efficiently inhibits FXa (4–6), free ZPI inactivates activated factor XI (FXIa) (5) as well as activated FIX (FIXa) to some extent (7).

PZ is considered to be synthesised mainly by the liver since its plasma levels were reduced in patients with chronic liver disorders (8). In addition, a liver-enriched transcription factor plays a crucial role in human *PZ* gene expression (9). The liver is also a major source of ZPI, the mRNA of which is most abundant in this organ (10). To our knowledge, the regulation of human *ZPI* gene expression has not been reported as yet.

Although the ZPI-PZ system seems to play a role(s) in anti-coagulation via the inactivation of FXa (and FXIa and FIXa) *in vitro*, the clinical significance of PZ for venous and arterial thrombosis, *in vivo*, remained controversial (11,12). Recently, a meta-analysis showed that low PZ levels were associated with an increased risk of thrombosis such as arterial vascular disease and venous thromboembolic disease, as well as with pregnancy complications (13).

The first patient with severe congenital PZ deficiency developed not only deep-vein thrombosis but also miscarriage (14). Recently, a relative/partial deficiency of PZ (PZ levels below 1.0–1.2 μg/ml=16–19 nM, in general) has been linked to serious complications with pregnancy (15, 16), such as early fetal loss, preterm delivery, etc.

In contrast to the numerous reports on PZ levels, there are only three existing publications describing plasma ZPI levels: one recent

report cited a significant relation between low ZPI levels and peripheral arterial disease (17), while another failed to find an association between low ZPI levels and venous thrombosis (18); still another found no difference in ZPI levels between normal controls and patients with anti-phospholipid antibodies (19).

In the present study, we have explored the possible contribution of ZPI (and PZ) to the stages of gestation in humans, as well as to recurrent miscarriage (RM).

Methods

Subjects

This study was performed with the approval of our Institutional Review Board. The work was conducted entirely in accordance with the Declaration of Helsinki. Informed consent was obtained from all individuals. One-hundred thirty-four non-pregnant Japanese women who had experienced RM, defined as two or more consecutive miscarriages (less than 22 weeks of gestation), participated in the present study. None of these patients had any readily identifiable causes of RM, such as uterine anomaly or chromosomal abnormality in either partner; however, there were five cases with anti-phospholipid syndrome. None had a history of thrombosis. We also studied 32 normal pregnant Japanese women without pregnancy complications or a history of RM, as well as 42 non-pregnant Japanese women with a history of normal pregnancy and without a history of apparent pregnancy losses, as controls, and 64 non-pregnant normal German females. None of these subjects was using contraceptives or hormone-containing drugs including oestrogen or progesterone. Demographical data of individuals involved in this study are shown in Table 1.

Blood

Blood samples were collected and anticoagulated with 3.8% sodium citrate in a ratio of anticoagulant to blood of 1:9, and plasma samples were obtained by centrifugation at 1,000g for 10 minutes (min) at 4°C, and stored at –80°C until analysis.

ELISA

An immunoassay for ZPI was performed using an in-house ELISA system. A rabbit anti-human ZPI polyclonal antibody was coated in a microtitre plate. Wells were washed with Tris-buffer, and plasma samples diluted 1/250 and 1/500 were applied and incubated for 120 min. After washing, a biotin-conjugated rabbit anti-human ZPI polyclonal antibody was added and incubated for 90 min. After washing, streptavidin-horseradish peroxidase (GE Healthcare, Little Chalfont, UK) was added and incubated for 60 min. After final washing, 3, 3', 5, 5'-tetramethylbenzidine (Bio-Rad, Hercules, CA, USA) was added, and the reaction was stopped after 10 min by adding 1 M H_2SO_4. Absorbance at 450 nm was recorded by a microtitre plate reader Biolumin 960 (Molecular dynamics, San Diego, CA, USA) and compared to a standard curve, using recombinant ZPI expressed in baby hamster kidney cells transfected with a ZPI cDNA. Recombinant ZPI was purified as reported by Han et al. (4), and its amount was determined by both biuret reaction using a bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL, USA) and densitometry of Coomassie brilliant blue staining of a sodium dodecyl sulfate-polyacrylamide gel after electrophoresis using bovine serum albumin as a standard protein. The ZPI concentration of a pooled normal plasma (obtained from seven healthy individuals) was determined using recombinant ZPI, and a standard curve was made every time using the pooled plasma as a reference for this assay (the range of 9–36 fmol, precision; ± 7%, lower limit of detection; 7 fmol, co-efficient of variation; 13.6%).

PZ antigen levels in plasma were measured by commercial sandwich ELISA methods, ZYMUTEST RK031A (Hyphen Biomed, Eragny, France), according to the manufacturer's instructions.

FX assay

FX in plasma was determined using a Thrombocheck FACTOR X kit (Sysmex, Kobe, Japan), according to the manufacturer's instructions.

Statistical analysis

Results are presented as medians and inter-quartile ranges in nM of three assays, and were analysed using the software program JMP ver. 6.0.3 (SAS Institute, Cary, NC, USA) by non-parametric (Wilcoxin/Kruskal-Wallis or Mann-Whitney) tests. A p-value less than 0.05 was considered statistically significant.

Results

Plasma concentrations of ZPI and PZ among non-pregnant normal Japanese females

Since what constitutes normal concentrations of both ZPI and PZ in plasma has not been determined among Japanese, we first developed an ELISA system for ZPI, as described in *Methods*. Medians (interquartile ranges, IQR) of plasma ZPI and PZ levels were 51.8 (45.1–59.6) and 29.9 (23.3–41.0) nM, respectively (Fig. 1A, B), among 42 healthy non-pregnant Japanese females aged 34.3 ± 4.3 years (mean ± SD).

Sixty-four non-pregnant healthy German females aged 28.1 ± 6.7 showed ZPI and PZ levels as 59.5 (54.4–63.3) and 35.8 (27.1–49.7) nM, respectively. Accordingly, the plasma ZPI and PZ levels in normal Japanese females were lower than those in German females ($p<0.001$ for ZPI and 0.058 for PZ, respectively; Fig. 1 A, B), among those individuals whose data we examined. No effect of age was seen on either ZPI or PZ levels in both normal Japanese females ($R^2=0.05$, $p=0.67$; $R^2=0.08$, $p=0.076$, respectively; see Suppl. Fig. 1A, available online at www.thrombosis-online.com) and normal German ($R^2=0.004$, $p=0.62$; $R^2=0.005$, $p=0.58$, respectively; Suppl. Fig. 1B, available online at www.thrombosis-online.com).

Since all the PZ is bound in a complex with ZPI in the circulation (2, 3), the concurrent change in concentrations of plasma ZPI and PZ suggest that levels of these two proteins are related (17–19). As expected, regression analysis demonstrated a linear relationship between plasma ZPI levels and PZ levels in normal non-pregnant Japanese controls ($R^2=0.28$, $p<0.001$; Fig. 2A). This is also true in normal non-pregnant German females ($R^2=0.23$, $p<0.0001$; Fig. 2B).

Plasma levels of ZPI and PZ during normal pregnancy

Blood samples were obtained from 32 women with normal pregnancy (aged 32.8 ± 4.9 years) during the 1st (9.1 ± 1.4 weeks of gestation, WG), the 2nd (22.3 ± 1.4 WG), and the 3rd trimesters (32.8 ± 1.8 WG), as well as during the puerperal period (Puerp.; 5.3 ± 6.0 weeks; 3.8 ± 1.5 weeks, when two individuals are excluded). To the best of our knowledge, there has been no report on plasma ZPI levels during pregnancy.

Plasma ZPI levels significantly increased in the 1st trimester when compared with 42 non-pregnant normal controls (Fig. 3A). When these pregnant individuals were recruited in the longitudinal study, blood samples of all 32 participants were available for all study time points, i.e. the 1st, 2nd and 3rd trimesters, and the puerperal period. The median ZPI value increased significantly from the 1st to 2nd trimesters (Fig. 3A). Following delivery (Puerp.), ZPI concentrations fell significantly ($p<0.01$), although the median value of this period did not return to that of non-pregnant controls, most likely because blood samples of the participants were collected too soon after delivery (3.8 weeks for 30 individuals).

When each individual's plasma ZPI level was consecutively measured, 29 among 32 women demonstrated a steady increase with gestational age (Suppl. Fig. 2A, available online at www.thrombosis-online.com). However, the remaining three showed reduced ZPI levels, i.e. the average of ZPI values of the 2nd and 3rd trimesters was lower than that of the 1st trimester for these three subjects.

Plasma concentrations of PZ significantly increased from the 2nd trimester and continued rising throughout the rest of the pregnancy (Fig. 3B). When individuals' plasma PZ levels were consecutively measured, 31 among 32 women demonstrated a steady increase in these levels in relation to their gestational age (see Suppl. Fig. 2B, available online at www.thrombosis-online.com).

Table 1: Demographical data of individuals involved in the present study.

	Controls	Normal pregnancies	RM cases
No. of participants	42	32	134
Age (mean ± SD)	34.2 ± 4.3	32.8 ± 4.9	32.1 ± 4.1
Age (range)	24–42	22–41	24–42
Overall No. of pregnancies	--	38	369
No. of pregnancy losses	0	9	327
No. of pregnancy loss per participant	0	0.28	2.4
Partus per participant	1.7	0.56	0.18

No., number.

Maternal age did not influence ZPI and PZ levels ($R^2=0.023$, $p=0.09$; $R^2=0.0001$, p=0. 90, respectively; data not shown), as in non-pregnant normal controls (see Suppl. Fig. 1A, B, available online at www.thrombosis-online.com).

A linear regression analysis demonstrated a significant relationship between levels of plasma ZPI and plasma PZ during normal pregnancy ($R^2=0.27$, $p<0.001$; see Fig. 3A, available online at www.thrombosis-online.com), which is similar to the non-pregnant normal women as described above. When stratified by gestation stages, there was also a significant correlation during the 2nd trimester and Puerp. ($R^2=0.23$, $p=0.005$ and $R^2=0.31$, $p=0.001$, respectively; see Suppl. Fig. 3A, available online at www.thrombosis-online.com), while a trend toward a positive correlation was observed during the 1st and 3rd trimesters ($R^2=0.12$, $p=0.052$ and $R^2=0.11$, $p=0.059$, respectively; Suppl. Fig. 3A, available online at www.thrombosis-online.com).

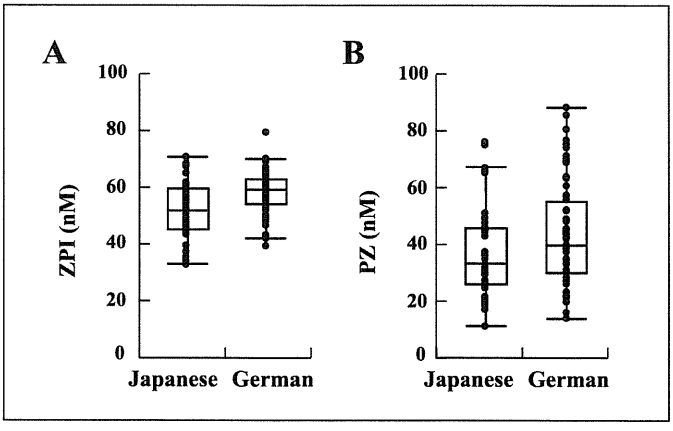

Figure 1: Plasma ZPI (A) and PZ (B) levels in non-pregnant normal Japanese and German female controls. Plasma concentrations of ZPI and PZ were measured by ELISA methods. Data are presented as box-plots of medians with ranges of 25–75% and whiskers for ranges of 10–90%, and outliners are also included. A statistically significant difference was observed for ZPI between normal Japanese (n=42) and German (n=64) controls ($p<0.001$), while a trend toward a PZ lower than German was found in Japanese ($p=0.058$).