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Sister chromatid exchanges, chromosome aberrations and micronuclei in female lymphocytes: correlations with biological rhythms, miscarriages and contraceptive pill use

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Our study looked at the variation in peripheral blood lymphocytes, during the menstrual cycle, of frequencies of sister chromatid exchanges (SCE) and micronuclei (MN) in 819 women and cells with aberrant chromosomes (CA) in a selected sample of 136 volunteers. We observed significant fluctuations in SCE and CA frequencies: SCEs reached a maximum value at the end of menstruation and a low at the time of ovulation, whereas CAs showed a continuous increase from the beginning of the menstrual cycle up to the time of ovulation and a progressive decrease thereafter. MN frequency did not fluctuate in a statistically significant way. No statistically significant differences in SCE, CA and MN frequencies were observed when fertile women were compared with women taking the contraceptive pill or those in menopause and no difference was found between women who had undergone physiological or surgically induced menopause. Moreover, no difference was found between women with a history of miscarriages and matched controls. These data together suggest that the natural variations in sexual hormone levels, but not those due to the contraceptive pill or their reduction at menopause, can contribute in modulating the baseline frequencies of SCEs and CAs. Moreover, these data suggest that the increased risks either of producing a chromosome imbalance in the progeny (eliciting miscarriages) or of occurrence of gynaecological diseases is not predictable by evaluating cytogenetic end-points in peripheral blood lymphocytes.

Introduction

Fluctuations in spontaneous sister chromatid exchanges (SCEs) and chromosomal aberrations (CAs) in peripheral lymphocytes were found to be associated with physiological variations in concentrations of testosterone, estradiol, follicle stimulating hormone (FSH) and progesterone (Tucker *et al*., 1987; Furuya *et al*., 1991; Joseph-Lerner *et al*., 1993). D'Suoza *et al.* (1988) described low level SCE frequencies for the progestogenic phase of the menstrual cycle, intermediate for the estrogenic phase and the highest levels for the ovulatory phase. *In vitro* treatment of lymphocytes with progesterone and estradiol, at physiological concentrations, produced a dose-related reduction in proliferation rate (Herrera *et al*., 1992). An increase in SCE levels has been observed in females who have undergone gonadotropin administration and in pregnant women (Joseph-Lerner *et al*., 1993). Moreover, SCE fluctuations have been found to be correlated with the levels of chorionic gonadotropin (Sharma and Das, 1986). Increased CA frequencies have been reported for women taking the contraceptive pill (Littlefield and Mailhes, 1975; Littlefield *et al*., 1975). However, the increase in chromosome breakage in lymphocytes from women who take oral contraceptives could not be correlated with length of time on the pill or stage of the pill cycle. In general, these studies ruled out the conclusion that synthetic hormones do not directly damage lymphocyte chromosomes, rather, since estrogens and progestogens are known to affect many metabolic and biochemical systems, it is possible that these hormones may induce a slightly altered *in vivo* condition in which increased chromosome breakage may be expressed. Finally, the variation in spontaneous micronucleus (MN) frequency has been poorly studied with respect to the menstrual cycle in women, although is well known that women have a higher background level than men and that MN frequency increases with age in females (Catalan *et al*., 1998). Such an age-related increase was not observed in males over 40 (Barale *et al*., 1998b). Therefore, to better understand the role of these factors in determining the spontaneous level of chromosome abnormalities, here we have analysed the possible association between SCE, CA and MN frequencies and the menstrual cycle, use of the contraceptive pill, occurrence of miscarriages and menopause/fertile status in a sample of 819 volunteer females.

Materials and methods

Donors

The studied subjects belong to the same general population already described (Barale *et al*., 1998a,b) from which only healthy women not taking drugs were selected. Out of the 819 volunteer females enrolled in the study, 350 were fertile, while 397 were in the menopause phase. Seventy-two women, out of the 350 who were fertile, had been regularly taking the contraceptive pill by oral administration for at least 1 year. The entire group had baseline SCE and MN frequencies recorded. Due to financial limitations, CA scoring was limited to 136 partially randomly selected subjects. The random selection was guided in such a way as to increase the share of fertile women in this subsample. To do this we randomly selected the first 100 subjects from the whole sample, obtaining 47 menopause women and 53 fertile. A further 36 women were randomly chosen among only the fertile ones. For MNs and SCEs ~50% fertile and 50% menopause women were selected.

Variables analysed

Volunteers filled out a detailed questionnaire providing information about their age, smoking habits, job position, fertility status, the number of days after the onset of menstruation at the moment of blood collection, use of the contraceptive pill, possible gynaecological surgical intervention and the rate of miscarriage (ROM), calculated as the number of miscarriages of the number of recognized pregnancies. The causes of miscarriages were also taken into account and classified as obstetrical, gynaecological, lung-related diseases and 'unknown'. Job type was ranked as follows: students, pensioners, housewives, blue and white collar workers. Smoking habits were classified by grouping people as smokers or non-smokers. Ex-smokers were considered together with never smokers when more than 8 years had elapsed since they had stopped smoking. Actually, previous observations (Barale *et al*., 1998b) showed that after 8 years cessation of smoking the spontaneous level of SCEs in ex-smokers falls to the level of non-smokers. In this study red and white blood cell counts, hematocrit and lymphocyte percentages were also recorded to check their possible contribution (data not shown, from the Hematology Department of S. Chiara Hospital, Pisa, Italy).

Lymphocyte culture

Blood samples were obtained from the volunteers by venipuncture, stored at room temperature and processed within 24 h. Two blood cultures were

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Table I. Averages and standard deviations of cytogenetic end-points according to days after the start of the menstrual cycle

Days after onset of menstruation	SCEs		CAs gaps ($\times 10^{-2}$)		MNs ($\times 10^{-3}$)	
	Count	Average ± SD	Count	Average ± SD	Count	Average ± SD
1–5	85	7.62 ± 1.41	21	1.33 ± 1.17	84	3.75 ± 3.39
6–10	59	8.20 ± 1.78	14	1.98 ± 2.32	59	3.34 ± 2.62
11–15	48	7.67 ± 1.05	11	2.36 ± 1.34	46	4.19 ± 3.74
16–20	44	7.47 ± 1.53	5	3.97 ± 1.38	43	3.20 ± 2.61
21–25	57	7.72 ± 1.11	16	1.95 ± 1.60	56	2.81 ± 3.28
26–30	40	7.71 ± 1.35	6	1.21 ± 0.88	40	3.82 ± 4.12
30–35	15	7.38 ± 0.95			15	2.37 ± 2.74
Total	350	7.72 ± 1.40	74	1.94 ± 1.66	343	3.47 ± 3.29

Table II. Analysis of variance (data adjusted for experimental variability) for cytogenetic end-points: age (co-variate), smoking status (smokers, non smokers) and job category (student, pensioner, housewives, blue and white collar worker)

	d.f.	SCEs			CAs gaps			MNs		
		Sum of squares	*F*	*P*	Sum of squares	*F*	*P*	Sum of squares	*F*	*P*
Co-variate										
Age	1	1.66	0.87	0.35	58.2	5.34	0.023	28.5	13.7	0.02
Factors										
Smoking status	1	51.6	27.1	<0.001	0.81	0.07	0.78	7.88	3.79	0.05
Job	4	3.83	2.01	0.0751	11.24	1.03	0.40	5.13	2.47	0.03

established for each genetic end-point (CAs, SCEs and MNs). A heparinized whole blood sample (0.3 ml) was added to 4.7 ml of culture medium composed of 4.025 ml Ham's F10 medium (ICN, Irvine, CA) supplemented with 0.5 ml (10%) fetal calf serum (ICN), 0.075 ml (1.5%) phytohemagglutinin (PHA) (Wellcome, Pomezia, Italy) and antibiotics (100 IU penicillin and 100 μg/ml streptomycin; Sigma, St Louis, MO). 5-Bromo-2′-deoxyuridine (BrdU) (Sigma), used for SCE analysis, was added to the cultures (9 μg/ml) for the entire incubation period of 72 h. In the analysis of CA we employed the same conditions specified above, but harvesting was at 48 h of growth and BrdU was not added to the cultures. MN were processed according to the standard technique (Fenech and Morley, 1985), as described in detail elsewhere (Barale *et al*., 1998a). Briefly, cells were treated at 44 h with 3 μg/ml cytochalasin B (Sigma) and MN were counted in binucleated cells, scoring a total of 1000 binucleated cells from each donor.

Materials were purchased in large amounts with the aim of reducing possible variations that could arise from different batches during the study. All batches were recorded. Materials and media were prepared by the same technician throughout the study. Large amounts of culture media were dispensed in culture tubes and stored at –80°C until needed.

Slide preparation and scoring

For SCE analysis, 50 metaphases/subject were analysed by five readers who each scored 10 metaphases on different slides from two different replicate cultures (at least 2 slides/culture). Slide staining for SCE detection was conducted according to the fluorescence plus Giemsa procedure (Perry and Wolf, 1974). For chromosome aberration analysis, only one operator scored all of the 200 metaphases on two slides from two different cultures. CAs were recorded by distinguishing between chromosome or chromatid aberration type, excluding gaps. The statistical analysis was conducted considering the relative frequency of total aberrant cells (CA), which were defined as those metaphases showing one or more chromosome/chromatid aberrations.

Statistical analysis

CA and MN frequency distributions were tested for normality and a satisfactory similarity to the Gaussian distribution was obtained by arc sine (√relative frequency) transformation. SCE frequency did not require any transformation (data not shown). Transformation of the data and removal of undesired sources of variation were done using a procedure reported in detail elsewhere (Barale *et al*., 1998a,b). Briefly, all of the experimental variables, but for the day of menstrual cycle, were entered into a multifactor analysis of variance (MANOVA) and their effects were then removed by calculation of the residuals. These residuals were then regressed on time (days of cycle) by means of polynomial regression using a stepwise increase in the polynomial degree in order to model fluctuations throughout the fertile cycle. Data was processed by the software STATGRAPHICS Plus 2.1 for Windows (Plus Ware, Rockville, MD). High frequency cells for SCE (HFC) were calculated as suggested by Moore and Carrano (1982).

Results

The observed SCE, CA and MN mean frequencies are reported in Table I. Fertile females were grouped according to the amount of time elapsed after the onset of menstruation by blocks of 5 days. SCE means ranged from 7.38 (for women declaring the onset of last menstruation over 30 days before) to 8.20 (6–10 days after the onset of last menstruation), CAs from 1.21 (26–30 days) to 3.97% (16–20 days) and MN from 0.0237 (>30 days) to 0.0419‰ (11–15 days). The multiple range test was applied in order to check if the observed differences were statistically significantly different. All the possible couples of values were checked and we can, as a preliminary observation, affirm that the cytogenetic frequencies are not steady (analysis not shown), but fluctuate with time. In contrast to the analysis of the SCE means, the analysis of HFC did not reveal any statistically significant difference among the classes described above using the χ^2 test (not shown). This was quite unexpected because HFC are considered a more sensitive tool in detecting differences in SCEs among subjects (Tates *et al*., 1994).

In order to properly assess the observed fluctuations, other factors, such as age, smoking habit and job position, were evaluated by MANOVA and their effects are reported in Table II. Increased age was found to be significantly associated with increases in CAs ($P = 0.023$) and MNs ($P = 0.020$). Smokers showed a significant ($P < 0.001$) increase in SCEs and a borderline ($P = 0.05$) decrease in MNs. In addition, MN frequencies were significantly higher ($P = 0.03$) in blue and white collar workers as compared with the other occupation groups. The best models that fit the SCE, CA and MN frequency fluctuations throughout the menstrual cycle are represented by the following equations:

$$SCEs = -0.713 + 0.468\times x - 0.0640\times x^2 + 0.0030\times x^3 - 0.000046\times x^4$$

(model, $P = 0.01$; coefficients, $P < 0.001$ each; constant, $P = 0.01$)

$$CAs\ (-gaps) = -3.06 + 1.94\times x - 0.41\times x^2 + 0.0394\times x^3 - 0.0016\times x^4 + 0.000024\times x^5$$

(model, $P = 0.03$; coefficients 5th degree, $P = 0.048$, 1st–4th degrees, $0.05 < P < 0.09$; constant, $P = 0.03$)

$$MNs = 0.2025 - 0.03214\times x + 0.00063\times x^2$$

(model, $P = 0.18$; coefficients, $P > 0.05$; constant, $P = 0.22$),

where x is the time (days) elapsed after the onset of menstruation.

Polynomial fittings of SCE and CA fluctuations were statistically significant and are reported in Figure 1a and b. On the other hand, after taking into account biological and lifestyle factors, no polynomial fit was obtained for MN daily variations (Figure 1c), in contrast to the preliminary analysis performed in the multiple range test.

SCE frequencies showed two peaks, the highest peak at days 6–8 and the second peak just before menstruation. The minimum point was observed around the presumed ovulation time. It should be considered that, although statistically significant, the extent of variation was small ($r^2 = 2.7\%$ of the total variance).

CA frequencies showed a completely different trend. CA frequency increased from the beginning of the cycle up to day 15, corresponding to the time of ovulation. Then a downward turn was observed with a low at the end of the menstrual cycle.

When the proliferation index (PI) was analysed by polynomial regression, no statistically significant fluctuations were observed and PIs remained constant over time during the menstrual cycle (analysis not shown). The same observation held true for red cell concentration, hematocrit and white cell count.

SCE, CA and MN frequencies were also analysed for their possible association with use of the contraceptive pill and menopause stage. When SCEs, CAs and MNs were adjusted for age, no significant differences among fertile women, menopause women and women taking the pill were detected by MANOVA. No effects attributable to hormone modification, either due to pill use or to menopause were observed (Table III). In addition, no statistically significant differences in SCE, CA and MN frequencies were found when taking into account the type of menopause, i.e. physiological or surgically induced (Table IV).

The possible association of SCEs, CAs and MNs with ROM was analysed only in women who had had miscarriages not attributable to any evident pathological causes ($n = 30$). We are aware that ROM is not a very precise index of the occurrence of miscarriages: women in our sample experienced pregnancy three times as a maximum (two subjects), making stochastic fluctuation in miscarriages important. The regression analysis showed that SCE and CA baseline levels were not associated with ROM, whereas a borderline statistically significant association ($P = 0.053$) was observed for MN frequencies. Surprisingly, this regression line had a negative slope ($b = -0.05$), indicating that women with higher spontaneous MN frequencies showed lower ROM. However, this relationship appeared to be strongly affected by the presence of two women with ROM = 1 (they had only one pregnancy ending as a miscarriage) and a low MN frequency. If these two women were excluded from the analysis, the regression slope approached 0 and it was no longer significant ($P = 0.32$). An alternative approach to the use of a ROM index was

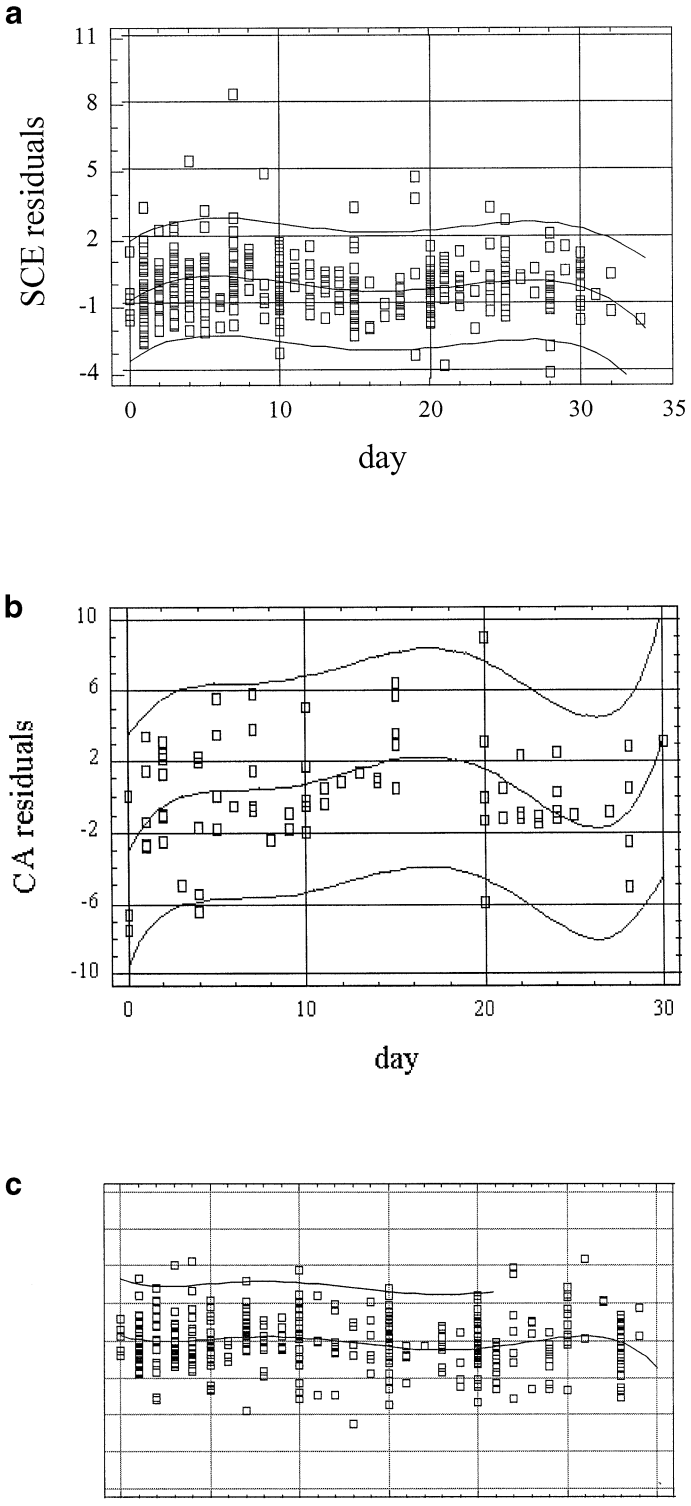

Fig. 1. Time-dependent fluctuation of SCEs (**a**), CAs (**b**) and MNs (**c**) through the menstrual cycle. Polynomial trend and prediction limits are shown.

then adopted. The 30 women were pair matched with control women extracted from the sample having similar age, smoking habits, period of blood collection, job and contraceptive method. Student's *t*-test for paired and unpaired data was used. No statistically significant difference appeared between controls and cases, although the data confirmed a tendency of 'control' women to have higher average MN frequencies than

Table III. SCE, CA and MN (means ± SE) in fertile women, women taking the pill and women undergoing menopause

	SCEs (count)	CAs gaps ($\times 10^{-2}$) (count)	MNs ($\times 10^{-3}$) (count)
Fertile	7.78 ± 0.093 (350)	1.99 ± 0.19 (74)	3.52 ± 0.18 (343)
Contraceptive	8.04 ± 0.210 (72)	1.42 ± 0.41 (15)	3.41 ± 0.40 (71)
Menopause	8.00 ± 0.090 (397)	2.57 ± 0.24 (47)	4.52 ± 0.17 (390)

Table IV. SCE, CA and MN (means ± SD) for women undergoing menopause, classified as physiological or surgical menopause

Type	SCEs		CAs gaps ($\times 10^{-2}$)		MNs ($\times 10^{-3}$)	
	Count	Average ± SD	Count	Average ± SD	Count	Average ± SD
Physiological	329	8.00 ± 0.099	44	2.67 ± 0.25	324	4.46 ± 0.18
Surgical	68	8.06 ± 0.220	3	2.44 ± 0.96	66	5.11 ± 0.41

Table V. SCE, CA and MN (means ± SD) for 30 women who had at least one miscarriage and for their matched controls

	Count	SCEs	MN ($\times 10^{-3}$)	Count	CA-gaps ($\times 10^{-2}$)
Case	30	8.05 ± 1.56	3.52 ± 3.82	6	2.10 ± 4.06
Controls	30	7.99 ± 1.15	4.71 ± 2.81	8	1.27 ± 1.88

the 'miscarriage' women (Table V). In addition, no significant effect of smoking on miscarriage rate was observed, the ratio being 0.36 between smokers and non-smokers among the miscarriage group and 0.26 among the control group (χ^2 test with Yates correction, $P = 0.54$).

Since miscarriages might have a paternal origin as well, we analysed the SCE, CA and MN baseline frequency in husbands of 'control' and 'miscarriage' women. Student's unpaired *t*-test did not reveal any significant difference between the two groups of husbands for the three genetic end-points considered. This finding was not surprising because no significant correlation has been observed between somatic and germline genetic abnormalities in men (Brandriff *et al*., 1985; Neel *et al*., 1990; Satoh *et al*., 1996).

Discussion

To our knowledge, only two cytogenetic studies have so far been carried out on women repeatedly sampled over time during the menstrual cycle. This approach limits the sample size because of the difficulty of persuading a large number of healthy donors to be repeatedly sampled without any substantial reason other than research purposes. In the present case, within the framework of a large human population study, we had the opportunity to randomly sample 350 healthy women devoid of any drug use, other than the contaceptive pill, during the menstrual cycle. The large amount of data obtained allowed us to apply appropriate statistical models to evaluate the possible effects of several variables, including biological and lifestyle factors, which can act as modulators or confounders. Therefore, the final data, including these factors as 'residuals', have been regressed on time, thus allowing a reliable estimation of the possible effects of menstrual cycle on SCE, CA and MN frequencies.

The data show that in our sample of fertile women, CA and SCE (but not MN) frequencies significantly fluctuate as a function of the menstrual cycle. The trend of SCE frequencies described in this present study does not totally parallel previous studies which showed an additional SCE peak at the time of ovulation (Tucker *et al*., 1987; D'Souza *et al*., 1988). This disagreement might be due to the large difference in the number of women involved in these three studies and/or to the high daily variability in SCE when few donors were sampled.

The menstrual cycle explained 2.7% of total SCE variance. This value might appear small, but it assumes some relevance if compared with the effect of smoking, which accounted for 5.5% of variance and represented the most important non-methodological factor acting on SCE in our population (Barale *et al*., 1998a,b).

SCE and CA fluctuations are probably not attributable to differential lymphocyte growth rates, because the PI did not show any statistically significant fluctuations. Also SCE and CA fluctuations were not associated with any variation in red cell concentration, hematocrit or white cell count. Nevertheless, the mechanisms involved in the observed SCE and CA fluctuations remain unknown. It has been suggested that steroids may induce the enzyme topoisomerare II, involved in DNA double-strand breakage and rejoining (D'Souza *et al*., 1988). On the other hand, it is known that sex hormones may modulate the activity of cytochromes P450 (Balogh *et al*., 1990; Henderson *et al*., 1992), which are involved in the metabolism of endogenous and exogenous mutagens. Another hypothesis concerns the mutagenicity of the hormones themselves, but as of now there is no supporting evidence for this (Liehr *et al*., 1986). It appears difficult to demonstrate a direct role of hormones in mutagenesis. On the basis of these considerations we suggest an alternative hypothesis: during the menstrual cycle, subpopulations of lymphocytes, characterized by differential spontaneous rates of SCEs and CAs, might be differentially responsive to PHA mitogenic stimulation.

Our data also showed that use of the contraceptive pill is not associated with the frequency of SCEs, CAs and MNs in comparison with fertile women and menopause women. Previous research had shown that use of the contraceptive pill is associated with a significant increase in SCE and CA values (Littlefield *et al*., 1975; Murthy and Prema, 1983; Pinto, 1986). Probably, the reduction in hormone dosage in pills that has occurred in recent decades has had a lot to do with this difference.

Menopause did not seem to influence the spontaneous levels of SCEs, MNs and CAs when the effect of age is accounted for. The hormone rhythms occurring during the fertile period

seem to determine SCE and CA fluctuations over spontaneous levels, which increase constantly with age. Moreover, our results indicate that surgical intervention and gynaecological illness are not associated with any detectable difference in the basal levels of cytogenetic indicators.

The correlation study between cytogenetic abnormalities in somatic cells and miscarriages was possibly affected by at least three biases. The variable ROM was probably a weak indicator of susceptibility to miscarriage due to the low number of pregnancies that occurred in each woman. Secondly, we did not perform the cytogenetic analysis at the same time after miscarriage, i.e. in some cases several years elapsed between the two end-points and the possibility of revealing an increase in damage attributable to a transient genotoxic factor was missed. Thirdly, the cause of miscarriage was not reported in depth by the volunteers and no cytogenetic analyses were performed on the fetuses. These biases could be responsible for the observed lack of correlations between SCE, CA, MN and ROM. However, in spite of these possible biases, paired *t*-test analysis on 'miscarriage' women and controls confirmed our previous findings. Our results on use of the contraceptive pill and on the rate of miscarriage in women can be evaluated together with the data from previous studies showing a reduced risk of miscarriage and of chromosomal abnormalities in women with a longer history of pill use. In this last case the authors propose that long-term use of the oral contraceptive pill protects against abortion due to aneuploidy by preserving the number of follicles (Sackoff *et al*., 1994; Ford and MacCormac, 1995). These data together further suggest that cytogenetic analysis carried out on somatic cells might be a poor indicator of the possible germline damage in the absence of any genotoxic exposure able to significantly affect both types of cells.

By studying the present population, we found that methodological, biological and lifestyle factors accounted for about 30% of the total variance (Barale *et al*., 1998a,b; present paper). A large part of the total variation still remains undetermined and will be studied further in the hope of accounting for the remaining variance by taking into account the genetic make-up of donors.

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