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Original Contribution

Epidemiology of Down Syndrome: New Insight Into the Multidimensional Interactions Among Genetic and Environmental Risk Factors in the Oocyte

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Down syndrome birth is attributable to multiple maternal risk factors that include both genetic and environmental challenges, but there is limited understanding of the complicated interactions among these factors. In the present study, a case-control analysis of approximately 400 infants with or without suspected Down syndrome reported between 2003 and 2009 and their parents in and around Kolkata, India, was conducted. Maternal exposure to 2 environmental risk factors (smokeless chewing tobacco and oral contraceptive pills) was recorded, and families were genotyped with microsatellite markers to establish the origin of nondisjunction errors as well as recombination patterns of nondisjoined chromosome 21. With logistic regression models, the possible interactions among all of these risk factors, as well as with maternal age, were explored. Smokeless chewing tobacco was associated with significant risk for meiosis II nondisjunction and achiasmate (nonexchange) meiosis I error among young mothers. By contrast, the risk due to oral contraceptive pills was associated with older mothers. Study results suggest that the chewing tobacco risk factor operates independently of the maternal age effect, whereas contraceptive pill-related risk may interact with or exacerbate age-related risk. Moreover, both risk factors, when present together, exhibited a strong age-dependent effect.

contraceptives, oral; Down syndrome; maternal age; nondisjunction, genetic; recombination, genetic; tobacco, smokeless

Abbreviations: CI, confidence interval; OR, odd ratio.

The risk factors associated with the birth of a child with Down syndrome are enigmatic. Free trisomy due to nondisjunction of chromosome 21 at oogenesis accounts for ~90% of total incidence (1–3). This much greater risk in the female parent is probably due to the way the oocytes develop and progress toward maturity. The protracted phase of meiotic arrest probably allows the risk factors to accumulate in the milieu of the ovarian environment.

The overall maternal risk for Down syndrome births is clearly multifactorial and includes both genetic and environmental factors (1–3) that impart adverse effects in either an age-dependent manner or a stochastic age-unrelated fashion (4, 5). Among these risk factors, reduction in meiotic recombination frequency and altered chiasma positions are 2 well-recognized ''genetic culprits'' that imperil normal chromosome segregation (6). In addition to these genetic correlates, some prospective candidates for environmental risk factors have been associated with Down syndrome births in several epidemiologic studies (7–12). Within this list of proximate risk agents, the practice of periconceptional smoking and oral contraceptive use are of particular interest as these are more common habits than the others. The genotoxic effects of smoking and tobacco use on reproductive health and fertility have been clearly established in the human and the mouse (13–16). The associations of Down syndrome birth with a maternal periconceptional smoking habit (17–19) and with maternal periconceptional oral contraceptive use (11, 20) were reported but contradicted in other studies (21–24). The study

of Yang et al. (10) inferred that the habit of periconceptional smoking is associated with meiosis II nondisjunction among women aged <35 years and, when modeled together with oral contraceptive use, the risk of periconceptional smoking increases with age. That study, however, did not suggest any interactions between oral contraceptive use and the genetic risk factors like stage of nondisjunction and pattern of recombination.

In the present study, we attempt for the first time to create and test joint models of how recombination, environmental exposures, and maternal age interact to predict Down syndrome risk. Our study is a case-control analysis of Down syndrome birth in the region surrounding Kolkata (formerly known as Calcutta), India. The epidemiology of risk exposure in this cohort is somewhat different from that seen in previous reports. Specifically, in this Indian Down syndrome cohort, the women are usually unlikely to be smokers; rather, they usually use smokeless chewing tobacco, a crude form of chopped tobacco leaves, from the very early days of their adolescence. In addition, a considerable number of women in this culture start irregular oral contraceptive use without physician consultation immediately after commencement of their sexual activity and continue this practice irregularly even after they conceive. Because of the heavy exposure to these 2 important environmental risk factors, we believe that this population is an excellent one in which to study the important question of risk factor interaction.

MATERIALS AND METHODS

The study was conducted following the principles outlined in the Declaration of Helsinki. The experiments and analyses were reviewed and approved by the institutional ethics committee.

Trisomic sample

Families with an infant with suspected Down syndrome were referred to our laboratory randomly after initial phenotypic screening by a birth defect surveillance group consisting of pediatricians. These families were reported between the years 2003 and 2009 and, from those, we selected 183 families as eligible to participate in the study. Eligibility criteria included the following: availability of a complete set of DNA samples from the father, mother, and Down syndrome infant; free trisomy 21 and liveborn Down syndrome as determined by classical karyotyping at our laboratory; and completion of a lifestyle questionnaire that included information on periconceptional smokeless chewing tobacco and oral contraceptive use. Interviews of mothers were conducted very privately, in person, after all consents were obtained. A preprinted, extensive set of questions was used for each family to collect detailed family history, information about lifestyle, birth control preference, and other relevant epidemiologic details. The confidentiality of all information was maintained very carefully at our laboratory. Almost all risk-positive women remained exposed to smokeless chewing tobacco and/or oral contraceptives until 10–15 weeks after they conceived, and their mothers (grandmothers) were also smokeless chewing tobacco positive. The participating cohort sample consisted chiefly of Bengali-speaking families from West Bengal, the majority of whom were Hindus and Muslims.

Normally disjoining samples

We recruited 195 families as controls, each having a healthy (euploid) infant as determined by classical karyotyping at our laboratory. The controls were identified and selected randomly from healthy newborns without any birth defect from the enrolled patient databases and birth registers of the hospitals that provided the cases. We chose those hospitals for control selection to ensure maximum possible similarity in demographic distribution of the cases and controls. Care was also taken in control selection to maintain maximum similarity in ethnicity, language, religions, maternal age, and socioeconomic status between cases and controls (Table 1). Controls were also chosen to be approximately age matched with cases. The minimum requirement for enrollment of a control family was the completion of the maternal questionnaire and availability of at least maternal and child DNA samples.

Laboratory methods

Each participating family was genotyped with a battery of 11 highly polymorphic short tandem repeat markers spanning from the pericentromeric region to the telomere of 21q. The order of markers was centromere-D21S369, D21S215, D21S258, D21S120, D21S1432, D21S11, D21S1437, D21S210, D21S1270, D21S167, D21S1412, D21S2055, D21S1260, D21S1411, D21S1446-qter. The maternal origin of nondisjunction was determined by establishing the contribution of maternal alleles to the Down syndrome child. The first 4 markers are used to determine the stage of meiotic origin of nondisjunction, that is, meiosis I and meiosis II error. We inferred a meiosis I error when the parental heterozygosity of these markers was retained in the trisomic child (i.e., the marker was ''nonreduced'') and meiosis II error when parental heterozygosity was ''reduced'' to homozygosity. There is evidence (2) suggesting that some proportion of so-called meiosis II errors actually originate at meiosis I. Despite this fact, we took the conventional approach and treated apparent ''meiosis I'' and ''meiosis II'' errors separately in many of our analyses. The determination of meiosis I or meiosis II was done while blinded to the risk exposure of cases.

Recombination in our case families was scored by using standard methods for trisomic data (25). This recombination scoring is possible even though only one child is genotyped, because the trisomic child has 2 copies of chromosome 21 and thus is essentially a self-contained sibling pair for that chromosome. Because conventional recombination scoring requires either grandparents or at least 2 siblings, we were not able to estimate recombination in our controls. Thus, recombination was considered only in the case-only phase of our analysis (refer to text below). Briefly, the recombination event was scored as a transition of 2 successive markers from nonreduction to reduction or vice versa in the ordered panel of makers along the 21q. We scored recombination frequency in interval-wise fashion. For example, the interval between the last centromeric marker D21S120 and the next marker D21S1432 was designated as interval 1, between D21S1432

Table 1. Demographic Particulars of Study Participants, Kolkata and Adjoining Area, India, 2003–2009

Particulars	Cases		Controls	
	No.	Mean (SD)	No.	Mean (SD)
Sample size	183		195	
Maternal age				
Group distribution				
Young (≤28 years)	76		59	
Middle (29–34 years)	59		72	
Old (≥35 years)	48		64	
Mean age by group, years				
Young (≤28 years)		22.76 (3.55)		22.93 (3.27)
Middle (29–34 years)		31.30 (1.63)		31.51 (1.77)
Old (≥35 years)		37.91 (2.49)		38.39 (2.17)
Locality				
Kolkata metropolitan area	105		110	
Suburbs	71		79	
Rural	7		6	
Religions				
Hinduism	146		152	
Islam	32		37	
Others	5		6	
Socioeconomic status[a]				
High income (≥20,000 rupees)	22		25	
Middle income (>5,000–19,999 rupees)	95		110	
Low income (≤5,000 rupees)	66		60	

Abbreviation: SD, standard deviation.
[a] One Indian rupee = ~0.02 US dollar.

and D21S11 as interval 2, and so on. In the cases in which a recombination could not be assigned to a specific interval because of uninformative markers, the position was scored at the midpoint of the 2 intervals; for example, a recombination localized to interval 1 or 2 would be scored as 1.5. Genotyping was also done for controls with the same set of markers to eliminate cryptic mosaicism.

Statistical methods

All the cases with maternal origin whose meiotic stage of nondisjunction was determined unambiguously were recruited for further analyses. For most analyses, the participating case mothers were divided into 3 groups on the basis of the age of the mother at the time of conception following our previous definition (5): ≤28 years or ''younger,'' 29–34 years or ''middle,'' and ≥35 years or ''older.'' For all the analyses, the maternal age of conception was considered as proxy for oocyte age, as direct estimation of the latter was beyond the scope of the present study. In our primary analyses, we used logistic regression models implemented in the software package STATA (StataCorp LP, College Station, Texas) to study a variety of questions about risk factors (smokeless chewing tobacco and oral contraceptives) and their interaction with maternal age. We used this approach to address 4 principal questions as mentioned in the Results.

RESULTS

We designed all the analyses to address 4 principal questions: 1) Does any difference exist in oral contraceptive or smokeless chewing tobacco use between cases and controls, and does this depend on maternal age? 2) Among cases, is there any difference between meiosis I and meiosis II in oral contraceptive or smokeless chewing tobacco use, and does this depend on maternal age? 3) Considering meiosis I and meiosis II cases separately, is there any relation between oral contraceptive and smokeless chewing tobacco use and the amount of meiotic recombination? 4) Again considering meiosis I and meiosis II cases separately, is there any relation between oral contraceptive and smokeless chewing tobacco use and the location of meiotic recombination? Note that, because our controls were chosen to approximately match the ages of cases, our case-control comparison does not test questions about maternal age per se as a risk factor, only its interaction with smokeless chewing tobacco and oral contraceptive use.

The interpretation of these models is quite complex, because so many potentially interacting risk factors are involved. However, one outcome of particular interest is when 2 given risk factors are either negatively or positively correlated with each other. Negatively correlated risk factors (within a group of individuals who all have a particular condition) suggest that the 2 risk factors are independent causes of the condition, so that affected individuals tend to have either one risk factor or the other (as long as the risk factors are uncorrelated in controls). By contrast, positive correlation suggests either a casual relation between the factors or a biologic interaction in which the risk factors augment each other's effect (again assuming independence between the factors in controls). We have previously discussed this general principle in the context of Down syndrome (4, 5), pointing out that a factor should be considered maternal age dependent when its prevalence in the population increases gradually with advancing maternal age. This principle is also well-recognized in the general epidemiologic literature (26). In contrast, when the prevalence of a risk factor is highest among young mothers and diminishes with age, the factor should be recognized as maternal age independent.

Model 1: cases versus controls

The cases and controls who participated in the study were very demographically comparable (Table 1). Because our cases and controls were group matched on age, we fit separate logistic regression models in each age group to predict the odds of Down syndrome birth as a function of smokeless chewing tobacco and oral contraceptive use. These analyses showed both smokeless chewing tobacco and oral contraceptive use to be risk factors for Down syndrome birth. Moreover, both risk factors showed striking, but different, interactions with maternal age (Web Table 1, the first of 4 supplementary tables posted on the *Journal*'s Web site (http://aje.oupjournals.org/)). In older mothers, oral contraceptive use increased the odds of Down syndrome birth by a factor of approximately 5 (odd ratio (OR) = 5.53, 95% confidence interval (CI): 2.30, 13.27). In the middle age group, oral contraceptive use increased risk by a smaller amount (OR = 2.50, 95% CI: 1.10, 5.71), and in the young age group, oral contraceptive use had no detectable effect on risk (OR = 0.86, 95% CI: 0.33, 2.24). The pattern for smokeless chewing tobacco use was the opposite, with no statistically significant effect in older mothers (OR = 2.10, 95% CI: 0.92, 4.80) or in the middle age group (OR = 1.24, 95% CI: 0.61, 2.52) but a large effect in the younger mothers (OR = 4.17, 95% CI: 2.01, 8.64). In a joint model across age groups, the *P* value for interaction between age and smokeless chewing tobacco use was 0.074 and between age and oral contraceptive use was 0.023.

Model 2: meiosis I versus meiosis II

Our second model was a case-only analysis to look at whether smokeless chewing tobacco and oral contraceptive use affect the relative risk of meiosis I and meiosis II errors. The rationale for this case-only analysis is 3 fold. First, unlike the case-control analysis above, this can be viewed as a prospective analysis, because meiosis I/meiosis II status was not determined until after subjects were enrolled in the study. Thus, any risk factors that show different effects on meiosis I and meiosis II nondisjunction are strongly implicated without fear of any selection bias contaminating the results. Second, it is clearly established that meiosis I and meiosis II nondisjunctions are biologically different phenomena, with different recombination and maternal age risk factors, and thus it is of interest to analyze their other risk factors separately. Finally, because our study design did not allow recombination to be calculated for controls, interactions between other factors and recombination can be considered only in this case-only analysis.

Consistent with previous studies (4, 5), this study model showed a maternal age effect ($P = 0.06$), with older mothers more likely to be meiosis II cases. Once this maternal age effect is accounted for, we did not observe any statistically significant effect of oral contraceptive use on the relative likelihood of meiosis I versus meiosis II. We did, however, observe a pattern of oral contraceptive exposure in both meiosis I and meiosis II categories (Web Table 2), with a gradual increase in the proportion of oral contraceptive users with age (with frequencies of risk-positive cases of 0.15, 0.33, and 0.5 for young, middle, and old users, respectively, for meiosis I and of 0.29, 0.35, and 0.67, respectively, for young, middle, and old users for meiosis II) (Table 2). This is in contrast with controls, for whom oral contraceptive use was absolutely constant with age. This observation suggests that oral contraceptive use probably imparts an age-dependent risk of nondisjunction. An interesting interaction between smokeless chewing tobacco use and maternal age was evident. Smokeless chewing tobacco use had a borderline significant association with meiosis II (vs. meiosis I) ($P = 0.08$), and this association was strongest in the young age group ($P = 0.006$ for age × smokeless chewing tobacco interaction). There is a gradual decrease in the proportion of smokeless chewing tobacco user meiosis II women with advancing age (with frequencies of risk-positive cases of 0.93, 0.64, and 0.5, respectively, for young, middle, and old users) (Table 2). Controls showed a constant frequency of about 0.04 in all age groups. This observation fits well with a model in which age and smokeless chewing tobacco use are independent risk factors for meiosis I and meiosis II nondisjunction.

Model 3: recombination in the meiosis I group

In models 3 and 4, we asked how smokeless chewing tobacco and oral contraceptive use relate to the previously established recombination risk factors for Down syndrome. For meiosis I nondisjunction, absence of recombination and telomeric single chiasma have been established as risk factors (4–6) and, for meiosis II nondisjunction, pericentromeric recombination has been established as a risk factor (4, 5). Model 3 used a binary outcome of presence or absence of observed recombination in meiosis I mothers only and tested whether this outcome was predicted by age, smokeless chewing tobacco use, or oral contraceptive use. We observe a greater likelihood of recombination among oral contraceptive users ($P = 0.01$). This suggests a model in which oral contraceptive use and lack of recombination are independent risk factors for meiosis I nondisjunction, if one assumes

Table 2. Proportions of Smokeless Chewing Tobacco and Oral Contraceptive Users in Control and Meiotic Outcome Groups as a Function of Maternal Age Categories, Kolkata and Adjoining Area, India, 2003–2009

	Meiotic I Nondisjunction		Meiotic II Nondisjunction		Controls	
	Sample Size, no.	Frequency of Risk-positive Cases	Sample Size, no.	Frequency of Risk-positive Cases	Sample Size, no.	Frequency of Risk-positive Cases
Smokeless chewing tobacco user						
Young (≤28 years)	62	0.67	14	0.93	59	0.37
Middle (29–34 years)	45	0.42	14	0.64	72	0.4
Old (≥35 years)	36	0.63	12	0.5	64	0.4
Oral contraceptive user						
Young (≤28 years)	62	0.15	14	0.29	59	0.17
Middle (29–34 years)	45	0.33	14	0.35	72	0.17
Old (≥35 years)	36	0.5	12	0.67	64	0.17

that oral contraceptive use and recombination are uncorrelated in the control population. We were not able to test that assumption in our data because we do not have recombination data for controls, but we are not aware of any literature suggesting such an association. We did not observe any interaction between oral contraceptive use and maternal age in this model (Web Table 3).

Smokeless chewing tobacco use showed an extremely strong effect in this model. Overall, smokeless chewing tobacco use was associated with lack of recombination ($P = 0.007$) among meiosis I mothers, but this effect was limited to younger and middle-aged mothers ($P = 0.009$ for interaction) (Table 3). About 60% of younger and 36% of middle-aged women in the meiosis I group exhibited achiasmate meioses at oogenesis.

Model 4: chiasma position within meiosis I and meiosis II groups

Model 4 used the location of single recombinants as the outcome variable in meiosis I and meiosis II mothers separately. In meiosis I mothers, neither age nor smokeless chewing tobacco use nor oral contraceptive use was a statistically significant predictor of the location of recombination (Tables 4 and 5). In meiosis II mothers, older age was highly significantly associated with pericentromeric recombination ($P < 0.001$), as has been established in previous studies (4, 5), but neither smokeless chewing tobacco use nor oral contraceptive use was a statistically significant predictor (Web Table 4).

Model 5: interaction between maternal age and combined exposure to smokeless chewing tobacco and oral contraceptives

Finally, we observed an increase in the proportions of women who were exposed to both smokeless chewing tobacco and oral contraceptives with increasing age in each meiotic outcome group (with frequencies of risk-positive cases of 0.11, 0.18, and 0.33, respectively, for young, middle, and old users for meiosis I and of 0.21, 0.29, and 0.5, respectively, for young, middle, and old users for meiosis II). We fit a model with combined smokeless chewing tobacco and oral contraceptive exposures as the outcome variable and maternal age group as the predictor, which showed a statistically significant increase in this combined exposure with

Table 3. Proportion of Observed Recombination Among Different Maternal Age Groups in Interaction With Smokeless Chewing Tobacco and Oral Contraceptive Use, Kolkata and Adjoining Area, India, 2003–2009

Type of Nondisjunction and Maternal Age Group	Sample Size, no.	Observed No. of Recombination Events								
		0			1			≥2		
		Total	Smokeless Chewing Tobacco	Oral Contraceptive	Total	Smokeless Chewing Tobacco	Oral Contraceptive	Total	Smokeless Chewing Tobacco	Oral Contraceptive
Meiotic I nondisjunction										
Young (≤28 years)	62	0.81	0.6	0.08	0.16	0.05	0.03	0.03	0.01	0.03
Middle (29–34 years)	45	0.69	0.36	0.18	0.24	0.04	0.1	0.07	0.02	0.04
Old (≥35 years)	36	0.42	0.22	0.19	0.31	0.22	0.14	0.28	0.19	0.17
Meiotic II nondisjunction										
Young (≤28 years)	14	NA			0.64	0.64	0.14	0.36	0.29	0.14
Middle (29–34 years)	14	NA			0.71	0.43	0.21	0.29	0.21	0.14
Old (≥35 years)	12	NA			0.83	0.42	0.5	0.17	0.08	0.17

Abbreviation: NA, not applicable.

Table 4. Distribution of Single Detectable Recombination Frequency on Nondisjoined Chromosome 21 in the Oocyte of Smokeless Chewing Tobacco User/Nonuser Women Having a Down Syndrome Child, Stratified by Stage of Nondisjunction and Age, Kolkata and Adjoining Area, India, 2003–2009

Marker Intervals (Centromere to Telomere)	Meiotic I Nondisjunction Group[a]						Meiotic II Nondisjunction Group[a]					
	Young		Middle		Old		Young		Middle		Old	
	User	Nonuser	User	Nonuser	User	Nonuser	User	Nonuser	User	Nonuser	User	Nonuser
1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.05	0.2
3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.03	0.0	0.3	0.2
4	0.0	0.14	0.0	0.0	0.4	0.0	0.0	0.0	0.03	0.0	0.3	0.2
5	0.33	0.0	0.0	0.11	0.04	0.0	0.0	0.0	0.36	0.25	0.25	0.2
6	0.11	0.0	0.0	0.11	0.07	0.11	0.0	0.0	0.25	0.13	0.1	0.2
7	0.11	0.0	0.16	0.03	0.06	0.11	0.08	0.0	0.15	0.26	0.0	0.0
8	0.0	0.0	0.17	0.25	0.32	0.11	0.08	0.0	0.14	0.26	0.0	0.0
9	0.0	0.0	0.17	0.14	0.19	0.45	0.07	0.0	0.04	0.05	0.0	0.0
10	0.0	0.0	0.0	0.09	0.03	0.11	0.29	0.0	0.0	0.05	0.0	0.0
11	0.0	0.0	0.0	0.09	0.04	0.11	0.15	0.0	0.0	0.0	0.0	0.0
12	0.33	0.28	0.5	0.09	0.17	0.0	0.11	0.0	0.0	0.0	0.0	0.0
13	0.34	0.43	0.0	0.09	0.04	0.0	0.11	0.0	0.0	0.0	0.0	0.0
14	0.0	0.14	0.0	0.0	0.0	0.0	0.11	0.0	0.0	0.0	0.0	0.0

[a] Young (≤28 years); middle (29–34 years); old (≥35 years).

age, particularly in the older mothers ($P = 0.002$, combining both meiosis I and meiosis II).

DISCUSSION

The results of our case-control study (model 1) suggest a role for smokeless chewing tobacco and oral contraceptive habits as risk factors for Down syndrome birth, with smokeless chewing tobacco use primarily a risk factor in younger women and oral contraceptive use primarily a risk factor in older women. These patterns of interaction with maternal age suggest that the adverse effect of smokeless chewing tobacco use is apparently maternal age independent, whereas oral contraceptive use may affect the normal segregation of

Table 5. Distribution of Single Detectable Recombination Frequency on Nondisjoined Chromosome 21 in the Oocyte of Oral Contraceptive User/Nonuser Women Having a Down Syndrome Child, Stratified by Stage of Nondisjunction and Age, Kolkata and Adjoining Area, India, 2003–2009

Marker Intervals (Centromere to Telomere)	Meiotic I Nondisjunction Group[a]						Meiotic II Nondisjunction Group[a]					
	Young		Middle		Old		Young		Middle		Old	
	User	Nonuser	User	Nonuser	User	Nonuser	User	Nonuser	User	Nonuser	User	Nonuser
1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.04	0.25
3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.28	0.25
4	0.5	0.0	0.0	0.0	0.07	0.0	0.0	0.0	0.0	0.0	0.3	0.25
5	0.5	0.0	0.0	0.17	0.07	0.0	0.0	0.0	0.34	0.33	0.21	0.25
6	0.0	0.0	0.0	0.17	0.11	0.0	0.0	0.0	0.42	0.1	0.17	0.0
7	0.0	0.0	0.06	0.04	0.1	0.07	0.0	0.11	0.08	0.26	0.0	0.0
8	0.0	0.0	0.07	0.38	0.1	0.4	0.0	0.11	0.08	0.23	0.0	0.0
9	0.0	0.0	0.27	0.04	0.5	0.07	0.0	0.08	0.08	0.04	0.0	0.0
10	0.0	0.0	0.0	0.13	0.05	0.07	0.5	0.23	0.0	0.04	0.0	0.0
11	0.0	0.0	0.07	0.07	0.0	0.1	0.0	0.19	0.0	0.0	0.0	0.0
12	0.0	0.35	0.37	0.0	0.0	0.22	0.0	0.14	0.0	0.0	0.0	0.0
13	0.0	0.48	0.16	0.0	0.0	0.7	0.5	0.0	0.0	0.0	0.0	0.0
14	0.0	0.17	0.0	0.0	0.0	0.0	0.0	0.14	0.0	0.0	0.0	0.0

[a] Young (≤28 years); middle (29–34 years); old (≥35 years).

chromosomes in an age-dependent fashion. Considering maternal age as predictor, we observed an increasing frequency of oral contraceptive user with advancing maternal age (Table 2) that further confirmed that oral contraceptive use may impart an age-dependent risk for Down syndrome birth. However, our oral contraceptive results are contradictory to what was reported by Martínez-Frías et al. (11). That study suggested a manyfold risk increment of long-term oral contraceptive use among the women of <35 years age, which corresponds to our younger and middle age definitions. However, our present analysis exhibits a strong effect in the ≥ 35-year age group. Although difficult to explain, this discrepancy may arise because of differences in the oral contraceptive exposure pattern in these 2 populations. Most of our cases who are oral contraceptive positive used a short-term, irregular dose of pills as they described at the time of interview. The irregular dose of oral contraceptive might exacerbate the adverse effect of natural aging-related hormonal imbalance in the ovary and lead to an increased anomaly in follicles.

In elucidating the possible effect of risk factors on the relative likelihood of the meiotic outcome groups (meiosis I or meiosis II), we did not find any effect of oral contraceptive use once maternal age was controlled for (Web Table 2). We did find smokeless chewing tobacco use to be associated with meiosis II (vs. meiosis I), particularly in younger women (Table 2). This is consistent with our case-control results and again suggests a model in which smokeless chewing tobacco use is a risk factor (especially for meiosis II), independent of maternal age. When we examined the relation between smokeless chewing tobacco and oral contraceptive use and meiotic recombination, we did not find an association between either of the risk factors and the location of recombination (Web Table 4). We did, however, find associations between both the risk factors and the amount of recombination (Web Table 3). Oral contraceptive use was positively correlated with recombination (with no age interaction) (Table 3), suggesting that lack of recombination and oral contraceptive use may be independent risk factors. By contrast, smokeless chewing tobacco use was associated with absence of recombination, with a much larger effect in younger women and a gradual decrease with age (Table 3). One possible explanation for this type of effect might be if smokeless chewing tobacco use actually causes lack of recombination and thus is a risk for younger mothers, and the older mothers have some other age-related risks. This would be consistent with our risk prediction model, which suggests that smokeless chewing tobacco is a maternal age independent risk factor. However, other explanations could also fit the data. The genotoxicity of smokeless chewing tobacco is either maternal or grandmaternal in origin (as grandmothers were also smokeless chewing tobacco positive), and it probably overrides the surveillance system (27) that ensures achiasmate chromosome biorientation and disjunction at the meiotic anaphase (as chiasma formation and recombination occur in the embryonic ovary, but exposure to smokeless chewing tobacco usually occurs much later in the lifetime). Support for this prediction is available in reports of research on the Chinese hamster (28, 29). Importantly, the observation that smokeless chewing tobacco use is not a predictor of chiasma position for meiosis I and meiosis II errors suggests that, regardless of oocyte age and the amount and location of recombination, it probably affects some molecular components common to meiosis I and meiosis II stages that may be the spindle apparatus. Though again, other mechanisms may also be possible at the molecular level.

Finally, we found a very interesting result in model 5 that suggests that exposure to both smokeless chewing tobacco and oral contraceptives is a risk, which fits with our age-dependent risk model. A similar trend was observed by Yang et al. (10) for exposures to both cigarette smoking and oral contraceptives. This observation is very intriguing as smokeless chewing tobacco appears to have an age-independent effect alone. It might be possible that, in the presence of smokeless chewing tobacco, the deleterious effect of oral contraceptives gets exacerbated, particularly in older women for whom other age-related challenges may also be present.

Although caution should be taken not to overinterpret specific details of our results until they are replicated in other populations, we have clearly shown highly statistically significant interactions between both smokeless chewing tobacco and oral contraceptive use and maternal age. Overall, our findings demonstrate that the risk environment in the oocyte for nondisjunction of chromosome 21 is extremely complicated and arises from complex, multidimensional interactions among genetic and environmental predisposing factors.

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REFERENCES

1. Sherman SL, Allen EG, Bean L, et al. Epidemiology of Down syndrome. *Ment Retard Dev Disab Res Rev*. 2007;13(3):221–227.
2. Allen EG, Freeman SB, Druschel C, et al. Maternal age and risk for trisomy 21 assessed by the origin of chromosome nondisjunction: a report from the Atlanta and National Down Syndrome Projects. *Hum Genet*. 2009;125(1):41–52.
3. Ghosh S, Bhaumik P, Ghosh P, et al. Chromosome 21 nondisjunction and Down syndrome birth in an Indian cohort: analysis of incidence and aetiology from family linkage data. *Genet Res (Camb)*. 2010;92(3):189–197.
4. Oliver TR, Feingold E, Yu K, et al. New insights into human nondisjunction of chromosome 21 in oocytes [electronic article]. *PLoS Genet*. 2008;4(3):e1000033. (doi:10.1371/journal.pgen.1000033).

5. Ghosh S, Feingold E, Dey SK. Etiology of Down syndrome: evidence for consistent association among altered meiotic recombination, nondisjunction, and maternal age across populations. *Am J Med Genet A*. 2009;149A(7):1415–1420.
6. Hultén MA, Patel S, Jonasson J, et al. On the origin of the maternal age effect in trisomy 21 Down syndrome: the oocyte mosaicism selection model. *Reproduction*. 2010;139(1):1–9.
7. Chen CL, Gilbert TJ, Daling JR. Maternal smoking and Down syndrome: the confounding effect of maternal age. *Am J Epidemiol*. 1999;149(5):442–446.
8. Kaufman MH. Ethanol-induced chromosomal abnormalities at conception. *Nature*. 1983;302(5905):258–260.
9. Padmanabhan VT, Sugunan AP, Brahmaputhran CK, et al. Heritable anomalies among the inhabitants of regions of normal and high background radiation in Kerala: results of a cohort study, 1988–1994. *Int J Health Serv.* 2004;34(3):483–515.
10. Yang Q, Sherman SL, Hassold TJ, et al. Risk factors for trisomy 21: maternal cigarette smoking and oral contraceptive use in a population-based case-control study. *Genet Med*. 1999;1(3):80–88.
11. Martínez-Frías ML, Bermejo E, Rodríguez-Pinilla E, et al. Periconceptional exposure to contraceptive pills and risk for Down syndrome. *J Perinatol*. 2001;21(5):288–292.
12. Boué A, Boué J. Chromosomal and anatomic studies of pregnancies after discontinuation of steroid contraceptives [in French]. *J Gynecol Obstet Biol Reprod (Paris)*. 1973;2(2): 141–154.
13. Cooper AR, Moley KH. Maternal tobacco use and its preimplantation effects on fertility: more reasons to stop smoking. *Semin Reprod Med*. 2008;26(2):204–212.
14. Trofor A, Man MA, Miron R. Smoking during pregnancy—a challenge to practitioners. *Pneumologia*. 2009;58(4):247–249, 251.
15. Jurisicova A, Taniuchi A, Li H, et al. Maternal exposure to polycyclic aromatic hydrocarbons diminishes murine ovarian reserve via induction of Harakiri. *J Clin Invest*. 2007;117(12): 3971–3978.
16. Hassa H, Gurer F, Tanir HM, et al. Effect of cigarette smoke and alpha-tocopherol (vitamin E) on fertilization, cleavage, and embryo development rates in mice: an experimental in vitro fertilization mice model study. *Eur J Obstet Gynecol Reprod Biol*. 2007;135(2):177–182.
17. Kline J, Levin B, Shrout P, et al. Maternal smoking and trisomy among spontaneously aborted conceptions. *Am J Hum Genet*. 1983;35(3):421–431.
18. Hook EB, Cross PK. Maternal cigarette smoking, Down syndrome in live births, and infant race. *Am J Hum Genet*. 1988; 42(3):482–489.
19. Kline J, Levin B, Stein Z, et al. Cigarette smoking and trisomy 21 at amniocentesis. *Genet Epidemiol*. 1993;10(1):35–42.
20. Lejeune J, Prieur M. Oral contraceptives and trisomy 21. A retrospective study of 730 cases [in French]. *Ann Genet*. 1979;22(2):61–66.
21. Källén K. Down's syndrome and maternal smoking in early pregnancy. *Genet Epidemiol*. 1997;14(1):77–84.
22. Torfs CP, Christianson RE. Effect of maternal smoking and coffee consumption on the risk of having a recognized Down syndrome pregnancy. *Am J Epidemiol*. 2000;152(12): 1185–1191.
23. Janerich DT, Piper JM, Glebatis DM. Oral contraceptives and birth defects. *Am J Epidemiol*. 1980;112(1):73–79.
24. Ericson A, Källén B, Lindsten J. Lack of correlation between contraceptive pills and Down's syndrome. *Acta Obstet Gynecol Scand*. 1983;62(5):511–514.
25. Feingold E, Brown AS, Sherman SL. Multipoint estimation of genetic maps for human trisomies with one parent or other partial data. *Am J Hum Genet*. 2000;66(3):958–968.
26. Piegorsch WW, Weinberg CR, Taylor JA. Non-hierarchical logistic models and case-only designs for assessing susceptibility in population-based case-control studies. *Stat Med*. 1994; 13(2):153–162.
27. Cheslock PS, Kemp BJ, Boumil RM, et al. The roles of *MAD1, MAD2* and *MAD3* in meiotic progression and the segregation of nonexchange chromosomes. *Nat Genet*. 2005; 37(7):756–760.
28. Yildiz D. Nicotine, its metabolism and an overview of its biological effects. *Toxicon*. 2004;43(6):619–632.
29. Yildiz D. Comparison of pure nicotine and smokeless tobacco extract induced formation of 8-OH-dG. *Toxicol Mech Methods*. 2004;14(4):253–256.