Original Research Article

Women Who Deliver Twins Are More Likely to Smoke and Have High Frequencies of Specific SNPs: Results From a Sample of African–American Women Who Delivered Preterm, Low Birth Weight Babies

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Objectives: We examine if there are genetic and environmental differences between mothers of singleton and multiple pregnancies in a sample of African–American mothers.

Methods: We focus on genomic areas suggested to increase or decrease the odds of multiple pregnancies. We computed the odds ratio (OR) and the 95% confidence interval (CI) for each SNP unadjusted or adjusted with smoking. SNPs' allelic differences between mothers of multiple pregnancies and singletons were also tested using Fisher's exact test. We considered additive terms for the SNPs' genotypes, smoking, and a multiplicative interaction term of two selected SNPs' genotypes.

Results: We found significant interactions between smoking and SNPs of the *CYP19A*, *MDM4*, *MTHFR* and *TP53* genes which correlated with higher odds of twinning. We also found a significant interaction between SNPs at the *TP53* (rs8079544) and *MTHFR* gene (rs4846049), where the interaction between the homozygotes (TT for rs8079544, GG for rs4846049) correlated with lowered odds of multiple pregnancy.

Conclusions: We provide a mechanistic explanation and preliminary evidence for previous reports that mothers of twins are more likely to have smoked, despite seemingly conflicting evidence for the fertility-reducing effects of nicotine. Nicotine, as an aromatase inhibitor, inhibits estrogen synthesis and may allow for greater production of gonadotropins. While smoking may have deleterious effects on fertility across many genotypes, in women of specific genotypes it may raise their odds of producing twins. *TP53* involvement suggests the necessity of future work examining relationships between women who bear multiples and cancer risk. Am. J. Hum. Biol. 27:605–612, 2015.

There is wide agreement that human population differences in twin pregnancy frequencies result from differences in the rates of dizygotic twin (DZ) pregnancies, as the frequency of monozygotic (MZ) twins is stable throughout the world (Hoekstra et al., 2008). Women who produce twin dizygotic pregnancies have been hypothesized to have certain traits in common, such as having higher parity (Satija et al., 2008), increased maternal age (Satija et al., 2008; Smits and Monden, 2011), a higher frequency of smoking (Hoekstra et al., 2010; Parazzini et al., 1996; Smits and Monden, 2011), and possibly consumption of vitamins, particularly folic acid (Czeizel and Vargha, 2004; Hoekstra et al., 2008; Kallen, 2004; Reilly et al., 2014), although some studies have failed to find an association with folic acid (Li et al., 2003; Mathews et al., 1999; Shaw et al., 2003; Vollset et al., 2005). Some have attempted to determine if mothers of twins have genomic areas associated with polyovulation or with increased probability of intrauterine survival of multiple pregnancy (Busjahn et al., 2000). Possible genomic areas explored are the follicle stimulating hormone (*FSH*) gene, which has yielded conflicting results (Al-Hendy et al., 2000; Liao et al., 2001; Painter et al., 2010), the PPARG gene (Busjahn et al., 2000), the Fragile X syndrome region (Healey et al., 1997), the *GDF9* gene (Palmer et al., 2006), the *TP53* and the *MDM4* genes (Tagliani-Ribeiro et al., 2012).

In general, African populations have the highest (40–50 per thousand), Asian and Native American have the lowest (3–4 per thousand) and European and Middle-Eastern populations have an intermediate frequency of twinning (8 per thousand) (Bulmer, 1970; Hoekstra et al., 2008). The assumption has been that environmental and genetic factors which predispose women to higher rates of dizygotic twinning are found at higher frequency in African populations and presumably in African-derived populations (Madrigal, 1994; Smits and Monden, 2011; Vinet et al., 2012).

The purpose of this study is to explain within-population variation in pregnancies in a subsample of African–American women who had low birth weight babies, and to examine possible genetic and environmental differences between mothers of singletons and multiples from this group. African–American women can have poor pregnancy outcomes due to low prenatal care access, socioeconomic class stressors, and weathering from discrimination (Rosenthal and Lobel, 2011; Wakeel et al., 2013; Woods et al., 2010). They are also expected to have high frequencies of multiple pregnancies because they may carry higher frequencies of African-derived genes than more European-derived counterparts in the USA. The combination of these two factors puts this particular set of women and their newborns in a highly vulnerable position, as they are at risk for multiples, for low birth weight, and for preterm birth.

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TABLE 1. Bibliographic source of SNPs and function of gene

SNP ID (rs#)	Literature reported related SNPs	Gene	Chromosome location	SNP position	SNP type/ Function class
rs284847	Minarik et al., 2006; Oikonen et al., 2011	*CYP19A1*	10	104595828	Intron-variant
rs7588807	Lin et al., 2011; Palmieri et al., 2008	*INHA*	2	2.2E+08	Intron-variant
rs17037563	Canzian et al., 2010; Eriksson et al., 2009	*LHCGR*	2	48926788	Intron-variant
rs10900595	Bryois et al., 2014; Chen et al., 2011; Moen et al., 2013; Westra et al., 2013	*MDM4*	1	204511602	Intron-variant
rs4846049	Chen et al., 2011; Velez et al., 2009; Wu et al., 2012	*MTHFR*	1	11850365	Utr-variant-3-prime
rs10739779	Ganesh et al., 2013; Kiefer et al., 2013	*TGFBR1*	9	101882199	Intron-variant
rs8079544	Anunciaçao et al., 2010; Anuncia-ao and Oliveira, 2010; Arcand et al., 2008; Feng et al., 2011; Garritano et al., 2009; Schildkraut et al., 2009	*TP53*	17	7580052	Intron-variant, upstream-variant-2KB

We take a two-pronged approach by determining if the above-mentioned phenotypic variables, and specific single nucleotide polymorphisms (SNPs), are associated with multiple pregnancies. We also examine interactions between the two to see if certain variables lessen or increase the impact of a particular SNP on the odds for multiple pregnancies.

MATERIALS AND METHODS

Data set

Access to the data was obtained via the DbGAP website (http://www.ncbi.nlm.nih.gov/gap), which distributes the results of studies that investigate the interaction of genotype and phenotype. The data are part of a larger study called the GENEVA study of Preterm Delivery, which enlisted 2200 African American mothers with term or preterm labor at three different research sites. All of the 2200 participants who took part in this study at the University of Iowa, as well as other collaborative medical centers located in Pittsburgh, North Carolina, the state of New York, and Missouri, were genotyped. A subset of the participants (∼800) was recruited by the Neonatal Research Network as part of a study on cytokines and infection in low birth weight infants. Our analysis focuses on 227 mothers of infants with extremely low birth weight (401–1,000 g and <72 h of age). These 227 mothers were chosen because the original study also asked them if they had had one or more multiple pregnancies. According to principal investigators of the Preterm Delivery study, these 227 mothers were asked this question as a sample of convenience (Jeffrey Murray, personal communication). For more information on the data and on the IRB approval for the data collection, see (Schelonka et al., 2011). Although the variables recorded at the three sites are very similar, they are not always the same. Therefore, the sample size fluctuates depending on how many variables were recorded at each site. The original study did not record the mother's use of folate supplements, nor her height or BMI. The outcome of the pregnancy is recorded as singleton or multiple, but the type of multiple pregnancy, and whether the multiple births are dizygotic or monozygotic is not specified. Thus, we can only test for an association between multiple pregnancy and the following variables: (1) smoking during pregnancy, (2) parity, and (3) maternal age. Our aim is to determine if some of the factors thought to predispose mothers to multiple term pregnancies (as reported by previous researchers) are also present among women who deliver prematurely.

Gene selection and SNPs

We focus on genomic areas previously suggested to increase or decrease the odds of having a multiple pregnancy. Some of these genes were mentioned in the introduction. Other genes which could affect the frequency of multiple pregnancies, whether by affecting ovulation, by being involved in the metabolic pathway, or by influencing levels of circulating steroid hormones, were also considered. SNPs related to these genes were searched in the literature (see references in Table 1). Beyond the literature we reviewed above, we found few SNPs in genes that had been previously researched in order to establish an association with multiple pregnancies. However, these SNPs have been typed and researched in other association studies. Of all of these, only a set of seven SNPs related to seven genes (*MTHFR*, *MDM4*, *CYP19A1*, *TP53*, *LHCGR*, *TGFBR1*, and *DDX17*) had been typed in the sample we studied in this paper (Bribiescas and Muehlenbein, 2010; Jasienska, 2010; Tagliani-Ribeiro et al., 2012). These SNPs were retrieved using the PLINK program to extract the SNP list from the dbGap website.

Statistical analysis

The seven investigated SNPs and their genotype values were extracted and recoded as dummy variables using the PLINK program (version 1.07), where the lower risk genotype serves as the reference. For the SNP data, Hardy-Weinberg equilibrium was also tested using PLINK for the total sample. We divided the participants by pregnancy type (single or multiple), we conducted descriptive statistical analysis (e.g., Mann-Whitney *U*-test and Fisher's exact test) to determine associations between pregnancy type (multiple or single) and maternal age, gestation age, parity, and smoking.

We computed the OR and the 95% CI for each SNP unadjusted or adjusted with smoking using logistic regression models. In this manner, the OR shows the

TABLE 2. Descriptive statistics

		All mothers	Mothers of multiple pregnancies	Mothers of singleton pregnancies	M-W U-test for group difference
Maternal age	Obs	214	29	185	
	Mean	26.96	26.93	26.97	Z = 0.008
	Std. Dev.	5.92	4.88	6.09	$P = 0.9938$
	Range	(15, 44)	(18,38)	(15,44)	
Gestation age (week)	Obs	224	29	198	Z = 2.67
	Mean	30.21	27.72	30.58	$P = 0.0075$
	Std. Dev.	4.82	2.55	4.97	
	Range	(23,40)	(24,40)	(23,40)	
Parity	Obs	140	16	124	Z = 2.25
	Mean	2.2	2.94	2.1	$P = 0.0244$
	Std. Dev.	1.38	1.53	1.34	
	Range	(0,8)	(1,5)	(0,8)	
Smoked during pregnancy	Obs	205	26	179	Fisher's exact test
					$P = 0.0011$
	# Smoked (%)	47 (22.92)	13 (50.0)	34 (18.99)	OR = 4.226

probability of producing multiple pregnancies associated with a specific SNP genotype with or without smoking as the control. SNPs' allelic differences between mothers of multiple pregnancies and singletons were also tested using Fisher's exact test. All the tests were with Bonferroni correction for multiple testing.

To test the interaction between a SNP and smoking, or a pair of SNPs representing their respective two genes, we considered additive terms for the SNPs' genotypes, smoking, and a multiplicative interaction term of two selected SNPs' genotypes (Crawford et al., 2007; Hartwig, 2013). Statistical analyses were performed using the STATA package (version11, College Station, Texas, USA).

RESULTS

The number of observations for each variable differs because some study participants had missing observations. There were a total of 227 pregnancies, 29 of which were multiple, 198 of which were singleton. The mean gestation age was significantly shorter for multiple pregnancies than for singleton pregnancies ($z = 2.67$, $P = 0.0075$). Women with multiple pregnancies had a significantly higher parity than women with singleton pregnancies ($z = 2.25$, $P = 0.0244$). More women with multiple pregnancies smoked than women with singleton pregnancies (OR = 4.226, $P = 0.002$, by Fisher's exact test). The two groups were not different in maternal age (Table 2).

We found evidence of unequal distribution of alleles by singleton vs. multiple pregnancies in the genes *CYP19A* and *MTHFR* genes (Table 3). Since we had shown that the two groups differed for smoking, we wished to explore the effect of smoking by SNP. Table 3 shows the ORs of having a multiple pregnancy with a specific SNP, computed unadjusted and adjusted for smoking.

For several genes, we found that the significant effect was for smoking, rather than for the SNP. This is the case for *CYP19A*: rs284847 (where smoking increases the odds of twinning 5.52 times); for *INHA*: rs7588807 (where smoking increases the odds of twinning 5.22 times, but where it significantly reduces the odds of twinning for the GT genotype); for *LHCGR*: rs17037563 (where smoking increases the odds of twinning 5.23 times); for *MDM4*: rs10900595 (where smoking increases the odds of twinning 6.77 times); for *TGFBR1*:rs10739779 (where smoking increases the odds of twinning 6.76 times and 5.05 times for the CA genotype), and for *TP53*: rs8079544 (where smoking increases the odds of twinning 9.24 times, but where the TT genotype with smoking together increase the odds of twinning 62.23 times specifically). The odds for twinning for women with the AC genotype for the rs17037563 SNP for the *LHCGR* gene were 5.6, with a Bonferroni corrected P value of 0.002 (95% CI: 1.96–16.82), but this disappeared after we adjusted for smoking. We subsequently tested for a gene X smoking interaction but none of the *LHCGR* SNPs had a significant interaction with smoking (Table 4). The gene X smoking significant interactions were for SNPs of the *CYP19A* gene, the *MDM4*, the *MTHFR* and the *TP53* genes, after Bonferroni correction.

Tests of gene-gene interactions were also conducted after adjusting for smoking. After Bonferroni-correction for multiple comparisons, there was one significant gene-gene interaction among these SNPs: between a SNP at the *TP53* gene (rs8079544) and the *MTHFR* gene (rs4846049), in which the interaction was between the homozygotes (TT for rs8079544, GG for rs4846049) for both systems, and whose effect was protective against multiple pregnancies (OR = 0.316, $P = 0.001$, CI: 95% = 0.168–0.595).

DISCUSSION

This is the first paper to investigate if a sample of African-American women has higher frequencies of genetic markers associated with multiple pregnancies. This is surprising, as populations of African ancestry are known to have a higher frequency of multiple pregnancies. Although most human genetic diversity is shared (Barbujani et al., 2013; Madrigal and Barbujani, 2007), and although African-American populations have received much gene flow from their European ancestral populations, they may still have higher frequencies of any genes which predispose African women to frequent multiple pregnancies. It is crucial to study this group of women, since African-American women are already under increased risk of poor birth outcomes, particularly preterm delivery associated with social and maternal stress-related factors. While it is not surprising that the multiple pregnancies in our sample ended at a significantly lower

TABLE 3. ORs, P values and 95% CIs for having a multiple pregnancy associated with the seven SNPs with or without smoking adjusted

	Singleton *n* (%)	Multiple pregnancies *n* (%)	Smoking unadjusted			Smoking adjusted		
			OR	*P*-value	95% CI	OR	*P*-value	95% CI
rs284847 (*CYP19A*)	*n* = 158	*n* = 17						
CC	156 (98.7)	15 (88.2)	1			1		
CT	2 (1.3)	2 (11.8)	10.4	0.024	(1.37, 79.21)	8.79	0.055	(0.95, 80.99)
Smoking						5.52	0.003[a]	(1.78, 17.19)
C	312 (99.4)	30 (93.8)		0.044[1]				
T	2 (0.6)	2 (6.2)						
rs7588807(*INHA*)	*n* = 158	*n* = 17						
GG	40 (25.3)	9 (52.9)	1			1		
GT	90 (57.0)	4 (23.5)	0.19	0.009	(0.06, 0.68)	0.15	0.007[a]	(0.04, 0.60)
TT	28 (17.7)	4 (23.5)	0.63	0.484	(0.18, 2.27)	0.53	0.402	(0.12, 2.32)
Smoking						5.22	0.005[a]	(1.64, 6.56)
G	170 (53.8)	22 (64.7)		0.277				
T	146 (46.2)	12 (35.3)						
rs17037563 (*LHCGR*)	*n* = 158	*n* = 17						
AA	105 (66.5)	5 (29.4)	1			1		
AC	45 (28.5)	12 (70.6)	5.6	0.002[a]	(1.86, 16.82)	3.63	0.031	(1.12, 11.73)
CC	8 (5.1)	0 (0.0)	N/R			N/R		
Smoking						5.23	0.005[a]	(1.67, 16.41)
A	255 (80.7)	22 (64.7)		0.043				
C	61 (19.3)	12 (35.3)						
rs10900595 (*MDM4*)	*n* = 156	*n* = 17						
CC	55 (35.3)	2 (11.8)	1			1		
AC	72 (46.2)	14 (82.3)	5.35	0.031	(1.17, 24.51)	5.11	0.045	(1.04, 25.25)
AA	29 (18.5)	1 (5.96)	0.95	0.966	(0.08, 10.90)	0.86	0.966	(0.07, 9.60)
Smoking						6.77	0.001[a]	(2.11, 21.67)
C	182 (58.3)	18 (52.9)		0.543				
A	130 (41.6)	16 (47.1)						
rs4846049 (*MTHFR*)	*n* = 157	*n* = 17						
TT	36 (22.9)	2 (11.7)	1			1		
GT	82 (52.2)	4 (23.5)	0.89	0.884	(0.15, 5.01)	0.64	0.645	(0.10, 4.17)
GG	39 (24.8)	11 (64.7)	5.08	0.043	(1.05, 24.48)	3.25	0.158	(0.63, 16.72)
Smoking						4.45	0.011	(1.40, 14.10)
T	154 (49.0)	8 (23.5)		0.006[a]				
G	160 (51.0)	26 (76.5)						
rs10739779(*TGFBR1*)	*n* = 158	*n* = 17						
CC	121 (76.6)	9 (52.9)	1			1		
CA	31 (19.6)	8 (47.1)	3.47	0.018	(1.24, 9.73)	5.05	0.007[a]	(1.55, 16.44)
AA	6 (3.8)	0 (0.0)	N/R			N/R		
Smoking						6.76	0.001[a]	(2.11, 22.23)
C	273 (86.4)	26 (76.5)		0.127				
A	43 (13.6)	8 (23.5)						
rs8079544(*TP53*)	*n* = 158	*n* = 17						
CC	133 (84.1)	13 (76.5)	1			1		
CT	24 (15.2)	2 (11.8)	0.85	0.84	(0.18, 4.02)	1.66	0.562	(0.30, 9.12)
TT	1 (0.6)	2 (11.7)	20.46	0.016	(1.73, 241.20)	62.23	0.002[a]	(1.73, 867.06)
Smoking						9.24	0.001[a]	(2.58, 33.05)
C	290 (91.8)	28 (82.4)		0.11				
T	26 (8.2)	6 (17.6)						

[a]Significant after Bonferroni-correction for multiple comparisons (corrected *P*-value is 0.01/7 at 1% level and 0.05/7 at 5% level). [1]Fisher's exact test by 2X2 contingency table.

number of gestational weeks, such a significant difference emphasizes how compromised these premature and multiple-pregnancy babies are, since they were delivered at an average of three weeks earlier than were the singleton babies.

Although we have demonstrated that there are significant differences in SNP frequencies in mothers of twins and mothers of singletons in this sample, perhaps the most important difference that emerges between our two groups of mothers is whether or not these mothers smoked. Previous studies had indicated that smoking affected the probability of twinning (Hoekstra et al., 2008; Kallen, 1998; Parazzini et al., 1996), but no previous paper had suggested a mechanism to account for this statistical probability. James (1981) guessed that twinning and smoking were related because mothers of twins had earlier menarche, they smoked more and engaged in more frequent coital behavior (James, 1997). No study before ours had been able to demonstrate that there is a gene by environment (smoking) interaction, meaning smoking may differentially affect either the probability of multiple ovulation, or the ability to sustain a multiple pregnancy, depending on one's genotype. For *CYP19A*, *INHA*, *LHCGR*, *MDM4*, *TGFBR1* and *TP53*, the effect odds of twinning adjusted for smoking ranged from 5.52 to 9.24, all of which were significant after Bonferroni adjustment.

The interaction of smoking with the TT genotype of the rs8079544 SNP of the *TP53* gene was profoundly significant, with a twinning OR of 62.23. The *TP53* gene, which codes for the p53 protein, makes important, broad contributions to health and reproduction. Often called the "guardian of the genome," the p53 protein regulates the

TABLE 4. ORs, P values and 95% CIs for having smoking-gene interaction associated with significant SNPs

	Odds	*P* value	[95% Interval Conf.]	
rs284847 (*CYP19A*) CT	0.95	0.791	0.65	1.39
Smoking	1.15	0.006[a]	1.04	1.28
CT X Smoking	2.36	0.002[a]	1.37	4.06
rs7588807(*INHA*) GT	0.87	0.024	0.77	0.98
TT	0.95	0.556	0.81	1.12
Smoking	1.24	0.02	1.03	1.48
GT X Smoking	0.95	0.686	0.76	1.2
TT X Smoking	0.87	0.369	0.63	1.19
rs17037563(*LHCGR*) AC	0.98	0.758	0.89	1.09
CC	0.94	0.682	0.71	1.25
Smoking	1.30	0.001[a]	1.14	1.48
AC X Smoking	0.74	0.010	0.59	0.93
CC X Smoking	1.07	0.746	0.69	1.67
rs10900595 (*MDM4*)				
AC	1.04	0.464	0.93	1.17
AA	1.02	0.753	0.88	1.19
Smoking	1.06	0.527	0.89	1.26
AC X Smoking	1.38	0.007[a]	1.09	1.73
AA X Smoking	0.9	0.462	0.67	1.20
rs4846049(*MTHFR*) GT	1.03	0.606	0.91	1.17
GG	1.15	0.063	0.99	1.33
Smoking	1.4	0.008	1.09	1.79
GT X Smoking	0.74	0.051	0.55	1.00
GG X Smoking	0.87	0.360	0.65	1.17
rs10739779(*TGFBR1*)				
CA	1.13	0.049	1.00	1.27
AA	0.98	0.890	0.71	1.35
Smoking	1.17	0.011	1.04	1.32
CA X Smoking	1.16	0.237	0.91	1.47
AA X Smoking	0.86	0.635	0.45	1.63
rs8079544 (*TP53*)				
CT	0.96	0.525	0.84	1.09
TT	1.87	0.001[a]	1.37	2.54
Smoking	1.17	0.003[a]	1.06	1.30
CT X Smoking	1.66	0.004[a]	1.18	2.34
TT X Smoking	N/R			

[a]Significant after Bonferroni-correction for multiple comparisons (corrected *P*-value is 0.01/7 at 1% level and 0.05/7 at 5% level).

cell cycle, arresting it in the event of DNA damage, regulating apoptosis, and thus preventing genome mutation. *TP53* is activated under many stress conditions and operates largely as a tumor suppressor. *TP53* also plays a direct role in maternal reproduction, affecting implantation of the embryo (Levine et al., 2011). P53 protein-deficient mice have small litter sizes and inefficient embryo implantation; this can be resolved by injection of leukemia inhibitory factor (LIF) which is regulated by *TP53* (Levine et al., 2011). LIF is a cytokine critical to uterine conditions for implantation as well as embryo development as it regulates cell differentiation. LIF is also found in lower concentrations in pregnancies with multiples (Perni et al., 2005). There are several other mechanisms by which *TP53* and reproduction interact; most notably, in that estradiol induces *TP53* inactivation (Molinari et al., 2000). In breast cancer cell lines, increasing levels of estrogen have been shown to increase p53 protein levels, which increases p53's responsiveness to DNA damage compared to estrogen-deprived lines (Fernandez-Cuesta et al., 2011). P53-deficient mouse models have greater problems achieving ovulation (Alexander et al., 2007), suggesting another possible mechanistic avenue for exploration. Finally, some variants of *TP53* are found to increase risk of miscarriage and recurrent pregnancy loss (Coulam et al., 2006; Firouzabadi et al., 2009; Fraga et al., 2014).

The reproductive process must strike a fine balance between rapid growth and control over that growth—between activation and suppression. *TP53* is not only a tumor suppressor but a major regulator of that balance in reproduction (Toledo and Wahl, 2006). Women in our study who had a specific SNP for *TP53* and who smoked were more likely to produce twins. One possible explanation could be that a mutagenic environment (as with smoking), along with a particular variant of *TP53*, disrupts the normal balance that leads to gestation of a singleton, making twinning more likely. *TP53* has been implicated in processes of ovulation, implantation, and gestation (Birgander et al., 1996; Coulam et al., 2006; Murdoch, 2003). In particular, we suggest further study of TP53 variants and ovarian processes that lead to single versus polyovulation, particularly in the context of a mutagenic environment that may increase the risk of mutations that lead to rapid, uncontrolled growth. We hypothesize that some *TP53* variants may regulate atresia differently or less strongly (Hatzirodos et al., 2014), making ovulation of more than one follicle more possible. This research, then, interfaces nicely with existing work on relationships between *TP53*, mutagens in the environment, and risk for cancer, as the balancing act required to suppress tumors yet regulate proliferation are similar (Saleemuddin et al., 2008; Schildkraut et al., 1997; Toledo and Wahl, 2006).

After we controlled for smoking, we found a significant association between the *TP53* and the *MTHFR* genes which was protective against twinning. While the *TP53* gene had been found to be associated with twinning at the "twin town" of Candido Godoi in Brazil (Tagliani-Ribeiro et al., 2012) the *MTHFR* gene was not investigated in the Brazilian population. The *TP53* SNP available to us in the sample analyzed in this paper was not the same analyzed by the authors of the Brazilian study. However, the SNP we analyzed has been researched, sequenced and validated in work related to female reproduction, health and cancerous growth (Anunciaçao et al., 2010; Arcand et al., 2008; Feng et al., 2011; Garritano et al., 2009; Schildkraut et al., 2009). Relationships between *TP53* and *MTHFR* genes have been studied in both breast cancer and recurrent pregnancy loss. A rather common mutation in the gene (C677T) interferes with the remethylation of homocysteine to methionine. The *MTHFR* enzyme is also important to the folate and methionine pathways, which together are linked to an increased risk of neural tube defects (Zhang et al., 2013). Several variants of the *MTHFR* gene also interact with low folate status to produce even greater odds for this condition (Christensen et al., 1999). Neural tube defects are found in higher frequencies among twins and triplets. Some folate supplementation studies have found that supplementation can lead to greater number of twin births among supplemented participants than among non-supplemented controls (Lumley et al., 2001; Werler et al., 1997), although this result has not been replicated in every study (Li et al., 2003). In our study we found a significant difference in the distribution of *MTHFR* alleles between case and control groups. Because folate status was not collected in this dataset, the nutrient-gene interaction could not be assessed. Future work should involve a well-controlled study in which the participants are genotyped for this common mutation, and their folate status closely followed.

Smoking during pregnancy has been linked to increases in time to conception and risk of miscarriage and infertility (Detmar et al., 2006; Prelog, 2011). Several mechanisms have also been described by which other substances in cigarettes reduce fetal size (Infante-Rivard et al., 2006). The contrast between seemingly fertility-enhancing and fertility-diminishing relationships with smoking made us wonder if underlying gene variants might make one more susceptible to the different effects of smoking on reproduction. Therefore we tested for interactions between smoking and SNPs in an effort to clarify the pathway by which smoking increases the odds of multiple pregnancies and found significant interactions with SNPs from *MDM4* and *CYP19A*. *CYP19A* encodes for the enzyme aromatase, which is involved in estrogen synthesis, specifically the aromatization of testosterone to estrogen. Interestingly, nicotine has been found to block aromatase in the human trophoblast (Barbieri et al., 1986) as well as to inhibit progesterone synthesis in human granulosa cells (Gocze et al., 1999), making it something of an aromatase inhibitor. Aromatase inhibitors are not uncommon in assisted reproductive technologies because they can lower estrogen concentrations, thereby lowering estrogen's negative feedback mechanism to the anterior pituitary's gonadotropin secretion and thus potentially elevating luteinizing hormone (LH) and FSH secretion (Yapura et al., 2011). They also tend to support ovulation without suppressing endometrial function, a common side effect of other ovulation induction methods (Al-Fozan et al., 2004; Atay et al., 2006; Badawy et al., 2009; Healey et al., 2003; Nahid and Sirous, 2012). Therefore, the inhibiting action of nicotine may allow for greater production of gonadotropins, which has been proposed to support polyovulation in cattle (Echternkamp et al., 2012). The *CYP19A* variant we found in our sample with an interactive effect with smoking may be more sensitive to this inhibition, and thus more likely to lead to a strong enough LH surge to promote polyovulation, supporting a relationship to multiple pregnancy. Because our sample was taken from a hospital population, it sampled the genetic variation present at the hospital at the time of the study. Unfortunately none of our participants carries the TT genotype in *CYP19A* (rs284847) so we are unable to determine if such genotype interacts with smoking to affect the odds of twinning. Our results speak about the strength of the relationship between the *CYP19A* gene and smoking in a natural population, although the analysis of allelic differences and smoking interactions for *CYP19A* (rs284847) was focused on genotypes of CC, CT, but not TT ($n = 157$).

Our findings were observed in a small sample with a very specific ethnic background. Given that some of the genetic variants we analyzed here (i.e. *TP53* and *MDM4*) have different gene frequencies across human populations, others might not observe the associations we documented in this paper (Asai et al., 2014; Vijai et al., 2010) However, our study and those of others confirm that there is ample genetic variance in humans on which natural selection may act either to reduce the frequency of multiple births (because the mortality and morbidity associated with these pregnancies is higher than that associated with singleton births) or to increase the frequency (if the achieved fertility of mothers of multiple births is greater than that of mothers of singletons). Indeed, previous researchers have established that under certain conditions, mother of twins achieve a higher lifetime reproductive success than do mothers of singletons (Madrigal, 1995; Sear et al., 2001).

A limitation of our study is that we do not know if the mothers smoked during the periconceptional period of their multiple pregnancy as well as during the rest of the pregnancy. However, it makes sense to assume that if a woman smoked during her pregnancy, she would have done so during the periconceptional period, when she was unaware of her pregnancy. Another limitation of the study is that BMI, a known risk factor for multiple pregnancies was not available for analysis.

Given that most human genetic diversity is shared across populations (Barbujani and Colonna, 2010; Colonna et al., 2011; Madrigal and Barbujani, 2007; Schlebusch et al., 2012), we expect to find at least some of the genetic markers reported by us or by others (Tagliani-Ribeiro et al., 2012) in other human populations, including those reported as "twin towns" such as Candido Godoi in Brazil (De Oliveira et al., 2013; Tagliani-Ribeiro et al., 2012), Eftimie Murgu in Romania (Schmidt et al., 1983), Mohammad Pur Umri in India (Cyranoski, 2009) and the Southwestern area of Nigeria (Akinboro et al., 2008). It would be particularly interesting to determine if these polymorphisms are highly frequent in these "twin-towns" due to natural selection or genetic drift. Twinning is a complex phenotype, which is amply illustrated by the numerous studies attempting to understand it. The results presented in this study show the potential for genetic, environmental, and interactive bases for human twinning rate differences.

CONCLUSIONS

Previous studies had noted that mothers of twins are more likely to smoke (Hoekstra et al., 2010; Parazzini et al., 1996; Smits and Monden, 2011). In this paper we provide evidence that in a sample of African-American mothers of low birth weight babies, those with specific SNPs do have greater odds of having twin pregnancies. Smoking does not affect all pregnant women in the same manner because it interacts with the women's genotype.

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