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Oral Contraceptives Modify the Effect of GATA3 Polymorphisms on the Risk of Asthma at Age 18 Years via DNA Methylation

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ORAL CONTRACEPTIVES MODIFY THE EFFECT OF *GATA3* POLYMORPHISMS ON THE RISK OF ASTHMA AT AGE 18 YEARS VIA DNA METHYLATION

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Abstract

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Prevalence of asthma is higher in boys than girls before puberty, while a shift between the genders is found after the puberty. However, the actual mechanism behind this gender shift is not yet clear. We hypothesize that, in girls, oral contraceptive (OCP) use and age at menarche modify the effect of GATA3 polymorphisms on the risk of asthma at age 18 via DNA methylation. Blood samples were collected at age 18 years from 245 female participants randomly selected for methylation analysis from the Isle of Wight birth cohort, UK. In the first stage, OCP use and age at menarche in interaction with rs1269486 were significantly associated with differential methylation of cg17124583, which subsequently modified the association of rs422628 with the risk for asthma at age 18 years. A two-stage model consisting of genetic variants in the *GATA3* gene, OCP use, age at menarche, and DNA methylation may explain how female sex hormones can be linked to explain the gender differences in asthma before and after puberty.

List of Abbreviations

BMI: Body mass index

CI: Confidence Interval

CpG: Cytosine-phosphate-guanine dinucleotide

DNA-M: DNA methylation

FDR: False discovery rate

ICC: Intraclass correlation coefficient

ISAAC: International Study of Asthma and Allergies in Childhood

LD: Linkage disequilibrium

MethQTL: Methylation quantitative trait locus

ModGV: Modifiable genetic variant;

OCP: Oral Contaceptive Pill

RR: Risk Ratio

SNP: Single nucleotide polymorphisms

Th: T helper

5'UTR: Five prime untranslated region

3'UTR: Three prime untranslated region

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Introduction

Asthma is a complex, multifactorial disease that is influenced by the interplay between genetic and environmental factors. Studies have shown that the prevalence of asthma is higher in boys than girls before puberty, while a shift between the genders is found after puberty (Postma, 2007; Soto-Ramirez et al., 2013; Tollefsen et al., 2007; Wada, Okuyama, Ohkawara, Takayanagi, & Ohno, 2010). The actual mechanism behind this gender switch is not yet clear. In addition, some studies suggest that asthma and lung function vary during different phases of the menstrual cycle, suggesting a role of sex hormones in asthma (Balzano, Fuschillo, Melillo, & Bonini, 2001; Siroux, Curt, Oryszczyn, Maccario, & Kauffmann, 2004; Tam et al., 2011). The biological effects of female sex hormones estrogen and progesterone include a decrease in the contractility and thus an increase in the relaxation of airway smooth muscles (Haggerty, Ness, Kelsey, & Waterer, 2003). Progesterone is considered to stimulate the IL-4 production and promotes the development of human Th2 cells (Piccinni et al., 1995). Immunological effects of estrogen include increased production of TNF-α by the lungs, increased production of IL-4 by the bone marrow and thus migration of eosinophils during allergic inflammation (Ana Paula Ligeiro de Oliveira, Adriana Lino dos Santos Franco, & Bernardo Boris Vargaftig, 2007), decreased expression of T-regulatory cells (Arruvito, Sanz, Banham, & Fainboim, 2007), and increased expression of IL-5 and IL-13 (Cai, Zhou, & Webb, 2012), differentiation of naive $CD4^+$ cells in to type 2 T helper (Th2) cells (Cai et al., 2012), and Th2 responses by augmenting the production of dendritic cells (Uemura, Liu, Narita, Suzuki, & Matsushita, 2008). Hence, estrogen may be linked to potential immunological effects and variations in airway responses. In addition, it has

been reported that both exogenous and endogenous sex hormones influence the occurrence of asthma in young women (Salam, Wenten, & Gilliland, 2006).

Oral contraceptive pills (OCPs) are exogenous sex hormone preparations used by adolescent girls for irregular menstruation, hirsutism, polycystic ovarian disease, dysmenorrhoea, and primarily for birth control. There are limited and conflicting reports on the association between OCPs and asthma. One study has shown that OCPs diminished the hormonal fluctuations and decreased the exacerbation of symptoms in women with asthma (Tan, McFarlane, & Lipworth, 1997). In an another study, OCPs were considered to increase the total lung capacities in non-asthmatic women during the follicular but not during the luteal phases of the menstrual cycle (Seaton, 1972). More wheezing among non-asthmatics and less wheezing among asthmatics it has been reported for women who use OCPs (Salam et al., 2006). Additionally, an increased production of suppressor T cells is reported in asthmatic women taking OCPs (Velez-Ortega et al., 2013). Finally, one study in premenopausal women reported no association between the use of OCPs and asthma (Lange, Parner, Prescott, Ulrik, & Vestbo, 2001).

Some studies found early menarche to be associated with the risk of adult asthma (Al-Sahab, Hamadeh, Ardern, & Tamim, 2011; Macsali et al., 2011; Salam et al., 2006), while other studies reported no association (Burgess et al., 2007; Jartti et al., 2009). Overall, there is a lack of understanding of the association between age at menarche, sex hormones, and asthma.

The transcription factor *GATA3*, located on chromosome 10, is a master regulator of Th2 cell differentiation (Zheng & Flavell, 1997) and plays an important role in the production of cytokines (Lee et al., 2000; Zhu et al., 2004). A study by Wada *et al* in a

mouse model of asthma demonstrated that there is an increased production of antigen induced Th2 cytokines from the bronchial lymph node cells of female mice and that this was associated with enhanced *GATA3* expression, compared to male mice (Wada et al., 2010), suggesting a possible role of *GATA3* in asthma specific to females.

The term epigenetics refers to the study of changes in phenotype or expression of genes that are not due to changes in the sequence of DNA (Egger, Liang, Aparicio, & Jones, 2004). Epigenetics is considered to play an important role in regulation and differentiation of T cells and asthma (Kumar, Hitchins, & Foster, 2009; Runyon et al., 2012). In particular, DNA methylation (DNA-M) may regulate genes associated with asthma and allergy (Kim et al., 2010). Single nucleotide polymorphisms called methylation quantitative trait loci (methQTLs) may influence the DNA-M at specific CpG sites, which may be conditional of environmental exposures (Bell et al., 2011; Hellman & Chess, 2010; Shoemaker, Deng, Wang, & Zhang, 2010). To reflect both the genetic and the environmental influences, we call these loci conditional methQTLs.

The role of sex hormones and the gender-specific role of *GATA3* in asthma motivated us to study a possible interaction between oral contraceptives and *GATA3* and further its association with asthma. It is also important to understand whether the change in DNA-M is a cause or a consequence of the disease. To address this issue we use a two stage model proposed by Karmaus et al., which incorporates both methQTLs and genetic variants (Karmaus, Ziyab, Everson, & Holloway, 2013). In stage one; we identify the conditional methQTLs (influenced by the use of OCPs) that may result in a change of the DNA-M of specific CpG sites of the *GATA3* gene. These differentially methylated CpG sites by OCP may modify the penetrance/activity of SNPs, which are then called modifiable genetic

variants (modGV) (Malousi & Kouidou, 2012; Oberdoerffer, 2012). Therefore in stage two, we evaluate the association between the differentially methylated CpG sites on the modGVs and asthma at age 18 years.

Age at menarche is related to changes in endogenous sex hormones and reflects body changes. In girls, the earlier the onset of puberty, the longer the exposure to sex hormones. Hence, we reran our stage one model using age at menarche as an alternate indicator of a possible endocrine effect. We believe that agreement of both exposures will provide credence to our findings.

Methods

Study design and population

A whole population birth cohort was established in Isle of Wight, UK, in 1989 to prospectively study the natural histories of asthma and allergic conditions. The local research ethics committee approved the study and written informed consent was obtained from 1456 children, who were followed up at ages 1, 2, 4, 10 and 18 years. The birth cohort has been described in detail elsewhere (Arshad & Hide, 1992; Kurukulaaratchy et al., 2003). Questionnaires were completed by the parents for each child at every follow-up. Blood samples were collected at age 18 years from 245 randomly selected female offspring, and were stored for subsequent DNA analysis. In addition, in a small sample of 34 girls at 10 years of age, blood samples were collected to analyze DNA-M.

Exposures

Information on OCP was collected at age 18 years. The question was: 'Are you on the contraceptive pill?' Regarding age at menarche, we used the National Institute of Child and Human Development (NICHD) questionnaire from the Study of Early Child Care

and Youth Development, which is based on the Pubertal Development Scale (PDS) method ("National Institute of Child and Human Development; NICHD Study of Early Child Care and Youth Development"). Among other questions on pubertal signs, we asked: 'How old were you when you started to menstruate?'

Outcome

Asthma information was collected using the International Study of Asthma and Allergies in Childhood (ISAAAC) questionnaire (Asher et al., 1995). The question for asthma was based on physician diagnosed asthma and the questions were as follows: "history of physician diagnosed asthma?", "wheezing or whistling in the chest in the last 12 months?" and "asthma treatment in the last 12 months?" Asthma at age 18 years was defined by diagnosis of asthma plus current symptoms and/or asthma medication.

Genotyping

Genomic DNA was isolated from blood samples by using QIAamp DNA Blood Kits (Qiagen, Valencia, Calif) or the ABI PRISM 6100 Nucleic Acid PrepStation (Applied Biosystems, Foster City, Calif). Polymorphisms in the *GATA3* gene were examined using the SNPper and Applied Biosystems databases. Genotyping was conducted by fluorogenic 5' nuclease chemistry PCR using Assays on Demands kits cycled on a 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA), or biotin-streptavidin based pyro sequencing performed on PSQ- 6 instrumentation (Biotage AB, Uppsala, Sweden). SNPs (n=17) that tagged the *GATA3* gene were identified using a tagger implemented in Haploview 4.2 using Caucasian Hapmap data, including 10 kb upstream and downstream of the *GATA3* gene (Barrett, Fry, Maller, & Daly, 2005). Estimates of linkage disequilibrium (LD) between SNPs were calculated using D^1 and r^2.

An r^2 value of 0.85 was the threshold for tagging, and 7 SNPs were selected (1 SNP from each of the 5 haplotype blocks and 2 SNPs that did not have strong linkage disequilibrium with other SNPs, Figure 1).

DNA Methylation

DNA was extracted from the blood/saliva samples using a standard salting out procedure (Miller, Dykes, & Polesky, 1988). DNA samples were interrogated using GoldenGate Genotyping Assays (Illumina, Inc, SanDiego, CA, USA) on the BeadXpress Veracode platform (Illumina, Inc, SanDiego, CA, USA) per Illumina's protocol. Data were analyzed using the genotyping module of the GenomeStudio Software package (Illumina, Inc, SanDiego, CA). The quality threshold for allele determination was set at a GenCall score 0.25 (scores #0.25 were ''no calls'') with 98.3% retained for further analysis. One microgram DNA was bisulfite-treated for cytosine to thymine conversion using the EZ 96-DNA methylation kit (Zymo Research, CA, USA), following the manufacturer's standard protocol. Arrays were processed using a standard protocol as described elsewhere (Bibikova, 2009). The Bead Chips were scanned using a Bead Station, and the methylation level (beta (β) value) was calculated for each queried CpG locus using Methylation Module of BeadStudio software.

Covariates

Maternal history of asthma and maternal smoking during pregnancy was assessed by a questionnaire administered after birth. Information about the child's active smoking status and their body mass indexes (BMI) were collected from the 18-year questionnaire and anthropometric measurements conducted at age 18 years. ''Family social status cluster'' is a composite variable derived from a combination of family income, parental

occupation (socioeconomic status) and number of children in a child's bedroom. The British Registrar General's social classification grouping places professional occupations in group 1 and unskilled manual occupations in group 5, with two subgroups within group 3 (3.1= non-manual and 3.2 = manual). Family income at age 10 years was recorded under six categories: class 1=less than £12 000; 2 = £12 000 to £17 999; 3 = £18 000 to £29 999, 4 = £30 000 to £41 999, 5 = £42 000 and greater. The number of children who shared one bedroom ranged from 1 to 3. A cluster analysis of these three variables yielded six groups, one clearly ''highest'' and one clearly ''lowest'' status, and four ''middle'' clusters representing a diversity of middle class living conditions (Ogbuanu, Karmaus, Arshad, Kurukulaaratchy, & Ewart, 2009).

In addition, we adjusted the stage-one models for cell mixture using the method proposed by Houseman et al (Houseman et al., 2012). This method identified CpG loci from our data which were within differentially methylated regions known to distinguish between white blood cell types, then utilized our Beta-values to predict the proportions of CD8+ T-cells, CD4+ T-cells, natural killer cells, B-cells, monocytes and granulocytes for each sample.

Statistical analysis

The preprocessing of the DNA-M data was done using the IMA (Wang D, 2012) package implemented in the R statistical computing package ("R: A language and environment for statistical computing. R Foundation for Statistical Computing.," 2012). To identify tag-SNPs, linkage disequilibrium (LD) between SNPs was calculated using D^1 and r^2 (Hill & Robertson, 2007) and they were tested for Hardy-Weinberg equilibrium using Haploview 3.2 software (Barrett et al., 2005). To assess whether the subset

population (n=245) represents the total cohort of girls at age 18 years chi square tests were used. DNA-M levels were quantified using β values that present the proportion of methylated (M) over the sum of methylated and unmethylated (U) sites (β=M/[C+M+U]), with c being a constant to prevent dividing by zero (Kuan PF, 2010). As the β value method has severe heteroscedasticity, it is recommended to use M-values for differential methylation analysis (Du, 2010). A logit transformation was employed for all β-values to normalize their distribution, which are then called M-values.

In our study, 16 CpG sites that spanned the *GATA3* gene were analyzed, out of which one CpG site was removed due to the presence of a probe SNP. Another CpG site was removed because of the low methylation level (<0.05%). In addition, we tested in 34 female participants, to determine whether DNA-M from blood was stable between 10 and 18 years. Stability was estimated via the intraclass correlation coefficient (ICC).

The aim of the first stage of the two-stage model is to detect CpG sites that were affected by an interaction of SNPs and OCP. We ran linear regression models, in which each of the 14 CpG sites were modeled against seven SNPs, each interacting with OCPs. Since we performed 98 tests (14 × 7) we adjusted for multiple testing using the FDR approach (p= 0.05) (Benjamini & Hochberg, 1995).

Focusing on the CpG site(s) detected to OCPs, we then reran the analyses of stage 1 using age at menarche as exposure. This allowed us to check whether we can find similar associations with CpG sites with another marker of endocrine changes.

In the second stage with asthma as a categorical variable, we used log-linear models (GENMOD procedure in SAS 9.3) to estimate statistical interactions between the

methylation level of selected CpG sites (from stage 1) and the *GATA3* SNPs on the risk for asthma at age 18 years.

In these models, confounders include maternal history of asthma, maternal smoking during pregnancy, BMI at age 10 and 18, smoking at age 18, and prior socioeconomic status. All confounders were simultaneously entered as covariates into the log-linear model. Those that changed the association of interest by 10% or more were retained as confounders in the final model. All tested hypotheses were penalized for multiple testing using the FDR. We tested the CpG sites associated with OCP with another exposure, which similarly indicates endocrine changes, namely age at menarche. The statistical analyses were performed using the SAS statistical package (version 9.3; SAS Institute, Cary, NC, USA).

Results

There were no significant differences in the prevalence of asthma, BMI at age 10 years, BMI, and smoking at 18 years, maternal history of asthma, socioeconomic status, early menarche and OCP use between female offspring of the whole cohort (n=750) and those who were randomly selected for the DNA-M analysis (n=245;Table 1). Among the female participants with available methylation data, 12% had maternal history of asthma, 19% had mothers that smoked during pregnancy, 14.3% (35/245) had asthma at age 18 years, 48% used OCPs at age 18, and the median age at menarche was found to be 13 years. Use of oral contraceptives and age at menarche in our sample are significantly associated (Wilcoxon test: p=0.001). 62% of the participants with age at menarche $\leq$ 11 years, 45.8% of those between 12 to 14 years, and 36% of those with $\geq$14 years used OCPs (data not shown).

Of the 13 SNPs that were genotyped, 7 SNPs (rs1269486, rs3802604, rs3824662, rs422628, rs434645, rs12412241, and rs406103) were selected for further analysis since these were uncorrelated (Figure 1). Of the 7 SNPs that were analyzed, only rs1269486 spanned the promoter region, followed by four SNPs (rs3802604, rs3824662, rs422628, and rs406103) in the intron region, and the two other SNPs (rs434645, and rs12412241) in the downstream of *GATA3* gene (Table 2). Interestingly, the mean methylation (ß-value, %) of six of the 14 CpG sites of the *GATA3* gene was low (<.10; Table 3), four were higher methylated (>0.90), and another four ranged between >0.10 and <0.55.

In stage one, after controlling for cell type composition in peripheral blood using the Houseman formula (Houseman et al., 2012), the interaction term 'OCP use × rs1269486' was found to be associated with differential methylation of cg17124583 (p-value=0.002; FDR *p*-value = 0.04; table 4), indicating that rs1269486 represents a conditional methQTL. OCP users with the 'AA' (the difference in a logit scale is -0.86; *p*-value=0.03) and 'AG' (the difference in a logit scale is -0.57; *p*-value=0.002) genotypes for rs1269486 had decreased methylation compared to those with the 'GG' genotype. The association was adjusted for potential confounders including the socioeconomic status, maternal smoking during pregnancy, smoking at age 18, and BMI at age 18. However, none changed the interaction effect by more than 10 percent.

To replicate our significant OCP model with an alternate indicator for endocrine effects, we investigated whether we can see a similar effect with age at menarche (Table 4). Cg17124583 was also differentially methylated by the interaction of same SNP rs1269486 and age at menarche. Methylation levels at cg17124583 are found to be increased with every one year increase in age at menarche, in those with the 'AA' and

'AG' genotypes for rs1269486. However, the interaction remained statistically significant only in those with the 'AA' genotype (the difference in a logit scale is 0.42; *p*-value =0.003). Finally, both OCP use and age at menarche, in interaction with rs1269486 showed a differential methylation of cg17124583.

Interestingly, in a small sample of 34 paired DNA-M measurements, the differentially methylated CpG site cg17124583 show some variability from age 10 to age 18 (test for stability: ICC=0.39, *p*=0.01) with mean methylation levels of 0.06 and 0.05, respectively. This CpG site shows both stability and variability, but the variability is not explained, either by OCP use or by age at menarche (data not shown). Also, there were no main effects for the OCP use and the SNP rs1269486 of the *GATA3* gene on the risk for asthma at age 18 years.

In the second stage, we analyzed whether the OCP fingerprint on the differentially methylated CpG site cg17124583 modifies the association between SNPs (modGVs) and asthma at age 18. We tested the interaction between seven SNPs and the methylation levels of cg17124583 (differentially methylated in stage 1), and its association with asthma at age 18. We found statistically significant interactions between SNPs rs434645, and rs422628 and cg17124583 that modify the risk of asthma at age 18 (Table 5). For rs434645, we combined the 'AA and AG' genotypes since the direction of effect on methylation is same for both, and checked its interaction with cg1712583 and its association with asthma at age 18, using 'GG' as the reference. The interaction was found to be significant (*p*= 0.01; Table 5), however, it did not survive multiple testing with FDR. For the SNP rs422628, we used the co-dominant model and compared participants who had the 'AG' and 'GG' genotypes, with those who have 'AA' genotype. The

interaction term ‘cg17124583 x rs422628’ was found to be statistically significant in those with the heterozygous genotype after adjusting for multiple comparisons (p= 0.0045; FDR adjusted p=0.03; Table 5). The DNA-M level range for cg17124583 was 0.01 to 0.46. Since the number of participants at methylation levels of 0.02 or less and 0.14 or more were less, we grouped these methylation levels into ≤0.02 (n=4) and ≥0.14 (n=9). Descriptively, 157 participants had methylation levels of 0.05 and less, 71participants of 0.06 to 0.09, and 17 participants of 0.1 to 0.46. For the ‘AG’ genotype, compared to ‘AA’, we found that at methylation levels of 0.02, 0.04, 0.06, 0.08, 0.10, and 0.12, the RRs of asthma were 0.31, 0.61, 1.21, 2.42, 4.83, and 9.64 respectively (Figure 2). The risk of asthma linked with the rs422628 ‘AG’ genotype compared to ‘AA’ increases as methylation of cg17124583 increases.

Discussion

Of the 14 CpGs and the seven SNPs that were analyzed, we identified a conditional methQTL (rs1269486) interacting with OCPs and with age at menarche leading to a differential DNA-M of cg17124583. The same differentially methylated CpG site cg17124583 in interaction with another SNP rs422628 was found to modify the association of asthma at age 18 years. This association remained statistically significant after adjusting for multiple comparisons using the FDR. This is the first study to identify SNPs on the *GATA3* gene, which in interaction with oral contraceptive use, and with age at menarche, are associated with differential methylation of *GATA3* CpG sites. Although the CpG site cg17124583 is located 13,768 base pairs away from rs422628, we can see that the risk of asthma is modulated by this CpG site. It is possible that the SNP is

in linkage disequilibrium with a genetic variant adjacent to the cg17124583. Such an explanation was recently shown in another study (Grundberg et al., 2013).

The probability of a selection bias is minimal in our study as the study participants were randomly selected for the DNA-M analysis and no significant differences were found between the study population and the whole cohort. As the information on the use of OCPs is self-reported by the participants, there is a possibility of a recall bias. However, previous studies have shown high agreement between questionnaire data and medical records for any OCP use, current use, and time since first use (Bean, Leeper, Wallace, Sherman, & Jagger, 1979; Norell, Boethius, & Persson, 1998). We repeated the analyses with a different exposure marker, namely age at menarche, which showed similar result.

Age at menarche is an important event in a women's life, thus, recall bias also is considered to be minimal (Must et al., 2002). In addition, since women were neither aware of their SNPs nor the methylation status, a recall bias would result in a non-differential misclassification and likely underestimate the true association.

The DNA-M profiles in our study are obtained by using the Infinium Human Methylation 450 array, which is demonstrated to have high validity and high reproducibility (Bibikova et al., 2011). As DNA-M is established with tissue specific patterns, it is important to consider whether the DNA-M obtained from peripheral blood represents the methylation in other tissues, and is currently under debate (Heijmans & Mill, 2012; Ma et al., 2014; Talens & Putter, 2010; Terry, Delgado-Cruzata, Vin-Raviv, Wu, & Santella, 2011). In addition, peripheral blood leukocyte represents a mixture of cells (Houseman et al., 2012). Using CpG site information, we estimated the relative contribution of cell type

composition in peripheral blood using the Houseman approach (Houseman et al., 2012). The estimated cell type composition had only a minor influence on the DNA-M of *GATA3* CpG sites.

In the regression models, we observed that, although the main effects of OCP, age at menarche, and SNPs were not significantly associated with DNA-M of cg17124583, their interactions were found to be significantly associated even after penalizing for multiple testing. Similarly, no main effects were seen for the association of OCP, cg17124583, and the SNPs on the risk for asthma at age 18. However, the interaction of the SNP and DNA-M were found to be statistically significant.

Asthma being considered a 'Th2-disease', we focused on the GATA3 gene because it is known to be the master regulator of Th2 cell differentiation (Zheng & Flavell, 1997) and has been linked to endocrine responses (Parikh, Palazzo, Rose, Daskalakis, & Weigel, 2005). Estrogen is an immune modulator and is known to stimulate the production of Th2 cytokines which include IL-4, IL-5, and IL-13 (Ana Paula Ligeiro de Oliveira et al., 2007; Cai et al., 2012). Our findings show that OCP use modifies the DNA-M of *GATA3* gene. We speculate that OCPs, which also contain estrogen and progesterone (Miyaura & Iwata, 2002; Salem, 2004), may influence Th2 cytokine production via the differential methylation of *GATA3* gene. Also similar findings are seen with age at menarche altering the DNA-M of *GATA3* gene. Statistically, although early age at menarche is related to use of OCP, the two variables are not in complete agreement and seem to measure different features. Age at menarche is known to be related to endogenous sex hormones (Apter, Reinila, & Vihko, 1989) and OCP to exogenous sex hormones. We believe that the agreement of our stage one findings between OCP use and

age at menarche provides credence to our results. Our two stage model suggests a potential pathway from exposures such as OCP and age at menarche altering the DNA-M of *GATA3* to subsequently changes in the risk for asthma at age 18 years.

A limitation of our study is that the RRs at methylation levels larger than 9% are high, which is due to the limited number of individuals (n=17) with methylation levels larger than 9%. Another limitation is the lack of availability of the information on the type of OCP (estrogen/progesterone only pills or a combined pill), which can further help to elucidate the role of either estrogen/progesterone or both in genetic polymorphisms and on asthma.

Conclusion

The present study findings suggest a potential pathway in which OCP exposure and age at menarche may alter the DNA-M of *GATA3* CpG site, which subsequently may influence the risk of asthma at age 18 years. These findings may provide one among multiple explanations for the gender differences in asthma after puberty. However, a replication of our findings is warranted. This study represents the first report of an association of the interaction of SNP and DNA-M of *GATA3* on the risk for asthma at age 18 years, that is modified by the use of OCP and age at menarche. Also, this study provides the motivation for other researchers to search for interactions between genetic variants, sex hormones, and DNA-M, in particular to explain the gender shift in asthma after puberty.

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Appendices

Table 1

Subjects' Characteristics with Available Methylation Data Compared to the Female Participants of the Total Cohort

Factors	Total female participants n (%) n=750	Female participants with DNA-M data n (%) n=245	P value
Maternal smoking during pregnancy			
Yes	188 (25.3)	47 (19.3)	
No	555 (74.7)	197 (80.7)	0.055
Missing	7	1	
Maternal history of asthma			
Yes	80 (10.8)	30 (12.3)	
No	662 (89.2)	213 (87.7)	0.50
Missing	8	2	
Asthma at age 18 years			
Yes	128 (19.4)	35 (14.3)	
No	531 (80.6)	210 (85.7)	0.07
Missing	91	0	
Oral contraceptive use at age 18 years			
Yes	293 (46.7)	117 (48	
No	335 (53.3)	126 (52)	0.75
Missing	122	2	
Smoking at age 18 years			
Yes	192 (29.7)	63 (25.8)	
No	455 (70.3)	181(74.2)	0.29
Missing	103	1	
Socioeconomic status			
High	50 (7.4)	22 (9.1)	
Medium	520 (77.3)	182 (74.6)	0.69
Low	103 (15.3)	37 (15.2)	
Missing	77	1	
	n (Median; 5, 95%)		
Body mass index at age 18 years (kg/m^2)	499 (22.2;18.2, 32)	240 (22.9;19.05, 32.93)	0.56
Missing	251	5	
Age at menarche	631(13.0;11.0,15.0)	233 (13.0; 10.0, 15.0)	0.32
Missing	119	12	

Table 2

Single Nucleotide Polymorphisms (SNPs) for GATA3 and their Genotypes

SNP	Position	Location	Genotype	Frequency (%)
rs1269486	8096199	Promoter	AA	13 (5.7)
			AG	87 (37.8)
			GG	130 (56.5)
			Total	230 (100.0)
rs3802604	8102272	Intron	AA	88 (38.4)
			AG	103 (45.0)
			GG	38 (16.6)
			Total	229 (100.0)
rs3824662	8104208	Intron	AA	8 (3.4)
			AC	69 (29.6)
			CC	156 (67.0)
			Total	233 (100.0)
rs422628	8111409	Intron	AA	128 (55.0)
			AG	93 (39.9)
			GG	12 (5.1)
			Total	233 (100.0)
rs406103	8111621	Intron	AA	13 (5.6)
		(boundary)	AG	78 (33.5)
			GG	142 (60.9)
			Total	233 (100.0)
rs434645	8121451	Downstream	AA	4 (1.7)
			AG	67 (29.3)
			GG	158 (69.0)
			Total	229 (100.0)
rs12412241	8127139	Downstream	AA	19 (8.1)
			AG	92 (39.7)
			GG	121 (52.1)
			Total	232 (100.0)

Table 3

Distribution of Methylation on CpG Sites of GATA3 Gene

CpG site	Location	Coordinate	Mean Methylation	5%	95%
cg18599069	5'UTR	8096991	0.06	0.04	0.07
cg10008757	5'UTR	8097183	0.07	0.05	0.09
cg14327531	5'UTR	8097331	0.06	0.04	0.08
cg17124583	Body	8097641	0.05	0.02	0.10
cg19883813[a]	Body	8098005	0.04	0.02	0.08
cg11430077	Body	8099018	0.11	0.05	0.20
cg01255894	Body	8099218	0.06	0.03	0.09
cg10089865	Body	8100286	0.93	0.91	0.95
cg22770911	Body	8101307	0.52	0.44	0.60
cg04492228	Body	8101513	0.19	0.13	0.26
cg17489908	Body	8101566	0.25	0.17	0.34
cg03669298	Body	8102210	0.06	0.04	0.09
cg00463367	Body	8103673	0.20	0.11	0.31
cg04213746	Body	8106003	0.95	0.93	0.96
cg27409129	Body	8111731	0.93	0.92	0.94
cg07989490[b]	3'UTR	8117026	0.95	0.94	0.97

[a] CpG site is deleted because of a low methylation level (<5%)

[b] CpG site is deleted because of a probe SNP

Table4

Assessment of Interaction of Single Nucleotide Polymorphisms with Oral Contraceptive Use, and with Age at Menarche on the Methylation of the CpG Site cg17124583 using Linear Regression[&]

Parameter		**Estimate (Standard error)**		**P value**	
		Not adjusted for cell type	**Adjusted for cell type**	**Not adjusted for cell type**	**Adjusted for cell type**[#]
OCP use		0.12 (0.11)	0.16 (0.11)	0.28	0.16
rs1269486	AA	0.64 (0.24)	0.68 (0.25)	0.009	0.006
	AG	0.41 (0.12)	042 (0.13)	0.001	0.001
	GG	Reference			
OCP use × rs1269486	AA	-0.72 (0.39)	-0.86 (0.40)	0.06	0.03
	AG	-0.55 (0.18)	-0.57 (0.18)	0.002	0.002
	GG	Reference			
Age at menarche		-0.09 (0.04)	-0.09 (0.04)	0.05	0.06
rs1269486	AA	-5.28 (1.77)	-4.87 (1.78)	0.003	0.006
	AG	-1.12 (0.86)	-1.06 (0.86)	0.19	0.21
	GG	Reference			
Age at menarche × rs1269486	AA	0.45 (0.14)	0.42 (0.14)	0.001	0.003
	AG	0.09 (0.06)	0.09 (0.06)	0.14	0.16
	GG	Reference			

[&] Adjusted for socioeconomic status, smoking at age 18, and BMI at age 18

[#] Additionally adjusted for cell mixture of the peripheral blood using the Houseman formula (Houseman et al., 2012)

Table5

Log-Linear Model of Interaction between Genetic Variants with DNA Methylation of the GATA3 Gene on the occurrence of Asthma at Age 18 Years[#]

Parameter		**Estimate**[Ψ]	**95% CI**	***P* value**
cg17124583		-22.19		
rs422628	AG	-1.87	-3.26 -0.48	0.008
	GG	-0.68	-3.83 2.47	0.67
	AA	Reference		
cg17124583 × rs422628	AG	34.49	10.7 58.27	0.004
	GG	-1.86	-60.69 56.96	0.95
	AA	Reference		
rs434645	AA and AG	-1.11	-2.47 0.25	0.11
	GG	Reference		
cg17124583 × rs434645	AA and AG	31.13	-6.68 55.59	0.01
	GG	Reference		

[#] Adjusted for socioeconomic status, maternal smoking during pregnancy, smoking at age 18, and BMI at age 18

[Ψ] The estimate needs to be exponentiated to calculate the risk ratio. In addition, to estimate the risk ratio due to the interaction, we need to take the two main effects and the interaction effect into account. The information is provided in Figure 2.

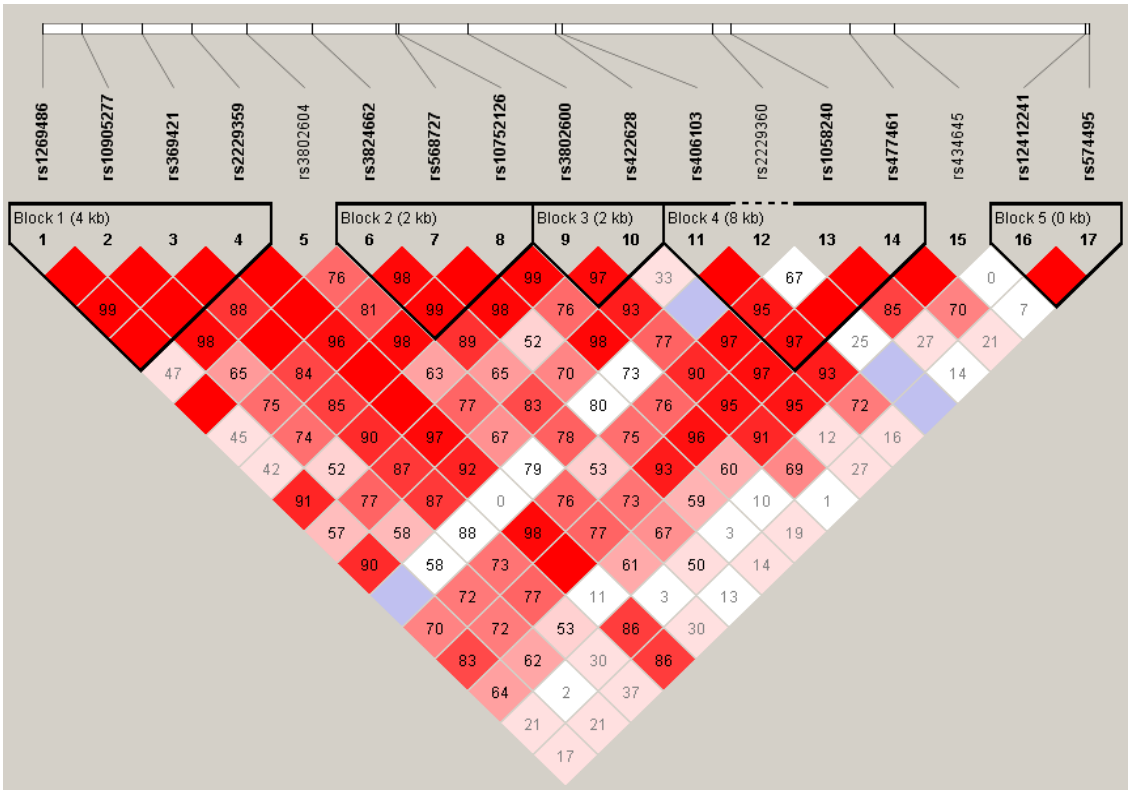

Figure 1. Linkage Disequilibrium of *GATA3* Single Nucleotide Polymorphisms

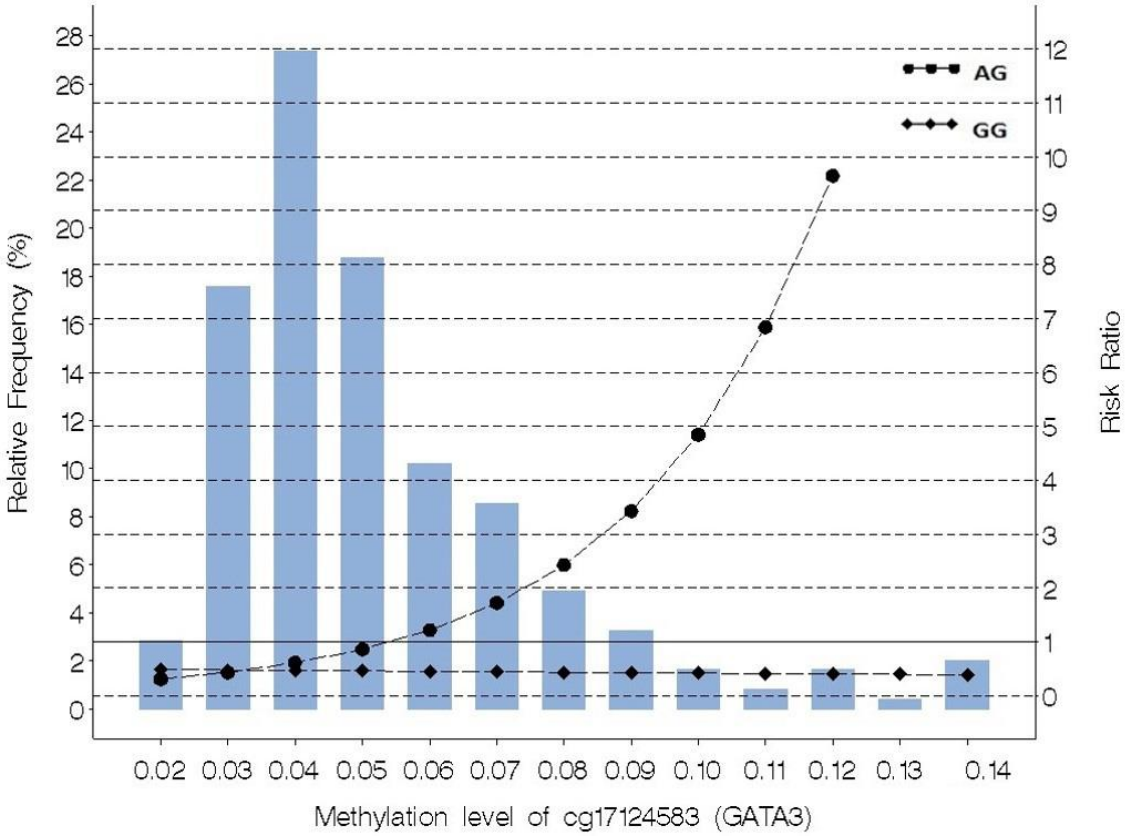

Figure 2. Risk Ratio of Asthma at Age 18 Years Versus Methylation at Different Genotypes of GATA3rs422628

The blue bars present the relative frequency of the DNA methylation levels. For instance, 4% methylation is found in 28% of the participants. The reference genotype is 'AA', which is a solid horizontal line that indicates a risk ratio value of 1.

IRB waiver form

Beverly Jacobik (bjacobik)
on behalf of
Institutional Review Board
Tue 11/19/2013 13:22
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Dear Dr. Karmaus and Kranthi Guthikonda,

The IRB Administrator has reviewed your determination request for the study, "Effect of genetic polymorphisms of GATA3 and Oral contraceptives on Asthma." Based on the information contained on that form it is determined that you are using coded private information that was not collected for your current research project and the data contains no identifiers that would allow you to readily ascertain the identity of individuals in your study. Therefore, you are not conducting Human Subjects research and 45 CFR 46 does not apply.

This research does not require IRB review nor approval.

Best regards,

Beverly Jacobik, IRB Administrator
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