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Genetic Influences on Preterm Birth

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GENETIC INFLUENCES ON PRETERM BIRTH

by Jevon Anastasia Plunkett

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ABSTRACT OF THE DISSERTATION

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by

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Professor Louis Muglia, Chairperson

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Preterm birth (PTB) is a leading cause of mortality and disease burden globally; however, determinants of human parturition remain largely uncharacterized, making prediction and prevention of difficult. Genetic studies are one way in which we can attempt to better understand this disorder.

We first sought to develop a model for the genetic influences on PTB to facilitate gene discovery. Study of standard measures of familial aggregation, the sibling risk ratio and the sibling-sibling odds ratio, and segregation analyses of gestational age, a

quantitative proxy for preterm birth, lend support to a genetic component contributing to birth timing, since preterm deliveries cluster in families and models in which environmental factors alone contribute to gestational age are strongly rejected. Analyses of gestational age attributed to the infant support a model in which mother's genome and/or maternally-inherited genes acting in the fetus are largely responsible for birth timing.

We also aimed to discover specific genes associated with PTB by screening genes selected based on an evolutionary-motivated filter, rather than known parturition physiology. Because humans are born developmentally less mature than other mammals, birth timing mechanisms may differ between humans and model organisms that have been typically studied; as a result, we screened 150 genes, selected because of their rapid evolution along the human lineage. A screen of over 8000 SNPs in 165 Finnish preterm and 163 control mothers identified an enrichment of variants in *FSHR* associated with PTB and prompted further study of the gene. Additionally, *PLA2G4C*, identified as the gene with the most statistically significant evidence for rapid evolution that was also included in a list of preterm birth candidate genes, was examined further. Three SNPs in *PLA2G4C* and one SNP in *FSHR* were statistically significant across populations after multiple testing corrections. Additional work to identify variants in these genes with functional effects was also initiated, including comparisons of prostaglandin metabolite levels among genotype classes for significantly associated SNPs in *PLA2G4C* and sequencing of *FSHR* to identify functional coding variants. Together, these experiments better characterize the nature of genetic influences on PTB and support the role of *PLA2G4C* and *FSHR* in PTB.

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Dedication

To my sister, Jamie Plunkett, my first, and very best, friend.

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Chapter 1: Introduction[*]

Clinical aspects of preterm birth (PTB)

Human parturition typically occurs between 37-42 weeks of gestation, with 40 weeks being the most common time of delivery (Figure 1.1). Deliveries before 37 weeks are designated as "preterm" births by the World Health Organization [1] and represent an important public health concern. Approximately one-third of infant deaths are attributable to prematurity [2]. Preterm infants also have an increased risk of serious health problems, such as respiratory illness, blindness and cerebral palsy [3]. Moreover, the severity and incidence of these problems worsen with decreasing gestational age [4].

A variety of subtypes of PTB can be described. For example, preterm births may be spontaneous or medically indicated. For 20-30% preterm births, women are delivered early to minimize complications from maternal conditions, like preeclampsia, or fetal distress [4]. However, most preterm births result from spontaneous preterm labor or preterm premature rupture of membranes (PPROM) [4]. Preterm labor or PPROM may arise in response to various stimuli, such as damage to the placental unit, intrauterine infection or changes in cervical length, but the mechanisms by which these processes are initiated are unknown. Additionally, early births of multiple gestations, which are generally delivered earlier than singletons, may occur by different mechanisms than singleton PTB. Hence, considerable heterogeneity in etiology may exist among various subgroups of PTB and warrants careful consideration of phenotype in studies of PTB.

PTB is common, with rates consistently rising in recent decades. In 2006, 12.8% of births in the United States occurred before 37 weeks, a 21% increase since 1990 [5].

[*] This chapter is adapted from: Plunkett J & Muglia LJ. (2008) Genetic Contributions to PTB: Implications from Epidemiological and Genetic Association Studies. Ann Med., 40(3):167-95.

While the recent rise in multiple births, which are generally delivered earlier than singletons, contributes to this increase, the rate among singleton gestations has risen 13% since 1990 and is now 11.1%. Among singleton PTB, late-preterm births (34-36 weeks) account for all of the increase, with essentially no change in the rate of births <34 weeks during this period. Importantly, cesarean-delivery rates have also increased since 1990, such that medically-indicated PTB may represent a greater portion of the increase in overall PTB rate. Consequently, distinguishing spontaneous from medically-indicated PTB may be important in identifying risk factors for this condition.

Of note, rates differ among racial groups. Blacks experience approximately twice the rate of PTB than that of Whites. The highest PTB rates are observed when both parents are Black and remain higher when one parent is Black, whether that parent is the mother or father [6; 7]. According to a study by Goldenberg and colleagues [8], these racial disparities are not explained entirely by measured medical and environmental risk factors, such as smoking, hypertension, education level or socioeconomic status, suggesting that other differences among races, such as genetic ancestry, contribute to the disorder. As a result, careful consideration of important differences in social, cultural and biological factors among races also are important in studies of PTB risk.

Motivation for studying genetics

While a number of risk factors have been identified, accurate prediction and prevention of PTB are difficult [4]. For example, biomarkers, such as serum protein concentrations of IL-6, IL-8, TNFα or relaxin, while strong predictors of PTB, may not be useful in large low-risk populations [9]. One problem may be that such markers vary

over time or among individuals, making it difficult to determine levels at which risk for PTB is increased.

In contrast, genetic factors are stable over time and therefore may be better predictors of risk. As a result, genetic studies may identify markers which more accurately predict PTB than currently known risk factors. Genetic studies may also identify novel proteins and/or pathways involved in the disorder. This new information will augment our general understanding of parturition and provide new targets for drug therapies, potentially improving both prevention and treatment of PTB.

Evidence for genetic influences on birth timing

Birth timing across pregnancies in the same woman

A wealth of evidence suggests that genetics are important in birth timing. For example, both preterm and postterm births tend to recur in mothers [10-15]. Moreover, the most likely age for a recurrent PTB is same week as the first PTB [12; 16; 17], suggesting that factors that are stable over time, such as genetics, affect birth timing.

Birth timing trends among family members

Familial trends for birth timing also suggest that genetics influence this trait. Women who are born preterm are more likely to have a preterm delivery themselves [18], indicating that mothers and their daughters share risk. Sisters of women who have had a preterm delivery also have an increased risk for preterm delivery [15]. Due to the nature of family studies, environmental factors shared between mothers and daughters or between sisters cannot be untangled from genetic influences. As a result, it is difficult to

determine the relative importance of genetic versus environmental factors from these studies alone.

Partitioning variance in birth timing into genetic versus environmental components

In contrast to family studies, twin studies measure the relative importance of genes in overall trait variance within a population. By comparing concordance rates between monozygotic and dizygotic twins, which share 100% and approximately 50% of their genes, respectively, one can model the genetic and environmental factors that influence a trait. Such studies indicate that genes account for about 30% of variation in preterm delivery [19; 20] and child's gestational age as continuous trait [19; 21], when the mother is considered the proband of a delivery.

A similar method was used to estimate the influence of maternal and fetal genetic factors by Lunde and colleagues [22]. Comparing concordance rates among full and half siblings for gestation age, the authors estimated that 11% of variation for this trait is due to fetal genetic factors and 14% of variation is due to maternal genetic factors [22]. Such comparisons use the degree of genetic relatedness (on average 50% for full siblings and 25% for half siblings) and trait concordance to estimate the relative importance of genetic versus environmental factors. Because siblings that are not monozygotic twins display some variability in their percent genetic identity and may differ in important dominance or interactive genetic effects, these estimates are more difficult to make using non-twin siblings. Despite the limitations in estimating the heritability, each study suggests that genetics play an important role in PTB.

Another approach to separating genetic and environmental factors is the coefficient of kinship. This measure depicts the degree of genetic relatedness within a

population. Ward and colleagues [23] used this measure to examine genetic influences on PTB in a Utah population. The Utah population from which the families were drawn was established by 10,000 people who moved to the state to establish the Mormon religion [23]. Because Mormons are discouraged from using alcohol or tobacco and have low rates of substance abuse and sexually-transmitted diseases, this population may represent individuals with relatively few environmental risk factors for PTB [23]. As a result, detecting genetic effects may be easier in this cohort of Utah preterm families. In this study, Ward and colleagues found that families with preterm deliveries had a significantly lower coefficient of kinship than controls [23], indicating that these families are more closely genetically-related than control families. This evidence suggests that the increased rate of PTB in these families can be explained by genetic factors. It is important to note that the authors of this study did not report the relative abundance of any environmental risk factors for PTB in the two populations. It is possible that one or more important environmental risk factors differ between these groups, in addition to genetic relatedness. Hence, the results of Ward et al. [23] support the significance of genetics in PTB, but do not address their relative importance compared to known environmental risk factors.

Mendelian disorders

Certain Mendelian disorders are associated with PTB, further supporting genetic effects on birth timing. Ehlers-Danlos Syndrome (EDS) represents a diverse group of Mendelian disorders affecting connective tissue, primarily inherited in an autosomal dominant manner [24]. Women with vascular EDS have an increased risk of delivering preterm, primarily due to PPROM [24]. Since this disorder is inherited in an autosomal

dominant pattern, there is a 50% chance the fetus has inherited the disorder, making it less clear whether the mother's or infant's genome contributes to the increase in PPROM risk.

Possible role of fetal genome in birth timing

Several lines of evidence further suggest that fetal genetic effects may influence birth timing. First, fetal genes that are paternally imprinted mainly control placental and fetal membrane growth [25]. Because the placenta and fetal membranes likely play a role in PTB, fetal genes controlling these tissues may also contribute. Additionally, several studies suggest that paternity affects risk for the disorder. For example, several studies indicate that partner changes between pregnancies reduced risk of PTB [26; 27]; however, changes in paternity may reflect association with long interpregnancy intervals rather than paternity effects *per se*. Paternal race also has been associated with PTB risk [6; 7], suggesting that fetal race may influence birth timing. However, father's family history of PTB has been shown to have only a weak association with risk. While an early study of a Norwegian birth registry demonstrated a correlation between fathers' and children's gestational ages [28], a more recent and extensive study of this registry suggested fathers contributed little to no risk to preterm delivery risk [29]. Similarly, recent studies [21; 30] suggested that paternal genetics contributed little to gestational age, but could not refute the possible role of maternally-inherited genes expressed in the fetus. Hence, while paternally-inherited genes may contribute little to PTB or other disorders, maternally-inherited genes expressed in the fetus may still be important. Together, these data suggests that the fetal genome may contribute to birth timing, motivating further study defining the infant as the proband.

Complexity of genetic effects on birth timing

There is increasing evidence that PTB can be conceptualized as a common, complex disorder. In contrast to Mendelian disorders in which alterations of a single gene can lead to disease, complex diseases are influenced by a variety of factors, none necessary and sufficient to cause the disorder itself. As a result, there is not a direct relationship between genotypes and phenotypes [31]. These disorders likely depend on a number of interacting factors, including genetic, epigenetic and environmental risk factors[31]. Modeling procedures used by twin studies suggest that additive genetic factors and environmental risk factors that are not shared among siblings both influence PTB [19; 20; 32]. Additionally, interactions between genes [33; 34] have been associated with PTB risk. Several studies suggest that gene-environment interactions, such as interactions between inflammatory gene risk alleles and bacterial infections [35-37], also influence the disorder. Together, these studies imply that the etiology of PTB likely involves genetic as well as environmental factors in complex interactions.

In addition to the complexity of genetic effects described above, several issues complicate how investigators think about the disorder. As mentioned above, it is not clear whether the mother or infant from a preterm delivery should be considered the proband. As a result, it is not clear which individual's DNA should be interrogated. Additionally, preterm delivery as a trait can be thought of in two ways. First, PTB can be thought of as discrete, resulting from genetic factors that lead to either term or early delivery. Alternatively, gestational age can be thought of as a quantitative trait, with preterm ages as extremely low values; hence, genetic effects may be quantitative trait loci (QTLs) that

influence the value of gestational age in both term and preterm deliveries. As disease models shape how one approaches identifying genes, it is important to consider the uncertainty about how to conceptualize PTB when evaluating various approaches taken to study this trait.

Identifying specific genes associated with PTB

Functional candidate gene studies have identified few genes consistently associated with PTB

Candidate genes in a variety of pathways believed to be important in parturition have been tested with mixed results (summary in Table 1.1). Few positive association findings for PTB have been consistent. To illustrate, for *TNF*, the most extensively studied gene, 11 studies report major effects of the gene[34; 36; 38-46]; yet, 13 others report no major effects of the gene [35; 47-56], including a meta-analysis of 7 studies [51]. Similarly, *IL1RN* was associated in 4 studies [57-60], but not in another 6 [39; 46; 53; 56; 61]. One possible explanation may be that some polymorphisms are significant only in the context of a particular environmental factor, such as infection. For example, *IL6* has been associated in 2 studies without considering environmental influences [62; 63], associated in another 2 studies only in the context of infection [35; 37], associated in another study only in interaction with other genes [41], and not associated in an additional 10 studies [34; 39; 42; 46; 53; 54; 56; 64; 65].

A number of problems in previous studies' design may limit their ability to detect true genetic effects. Most of these studies have been underpowered, because of small sample sizes and/or incomplete sampling of genetic variation in a gene of interest.

Phenotypic heterogeneity also may confound many studies' results, as most consist of mothers and/or children collected using a variety of phenotype definitions that may have different etiologies. In addition, genetic etiologies may differ across ethnic groups, since pregnancies in which either the mother or father is Black are at increased risk for preterm delivery, regardless of which parent is Black; however, few studies have included analyses separately by race or attempted to correct for possible population substructure, further questioning the validity of many genes associated to date. Moreover, no genetic model has been identified for PTB to suggest what nature of genetic effects is expected, limiting investigators' ability to appropriately design such studies. This proposal considers such issues to better identify PTB genes.

Alternative approaches

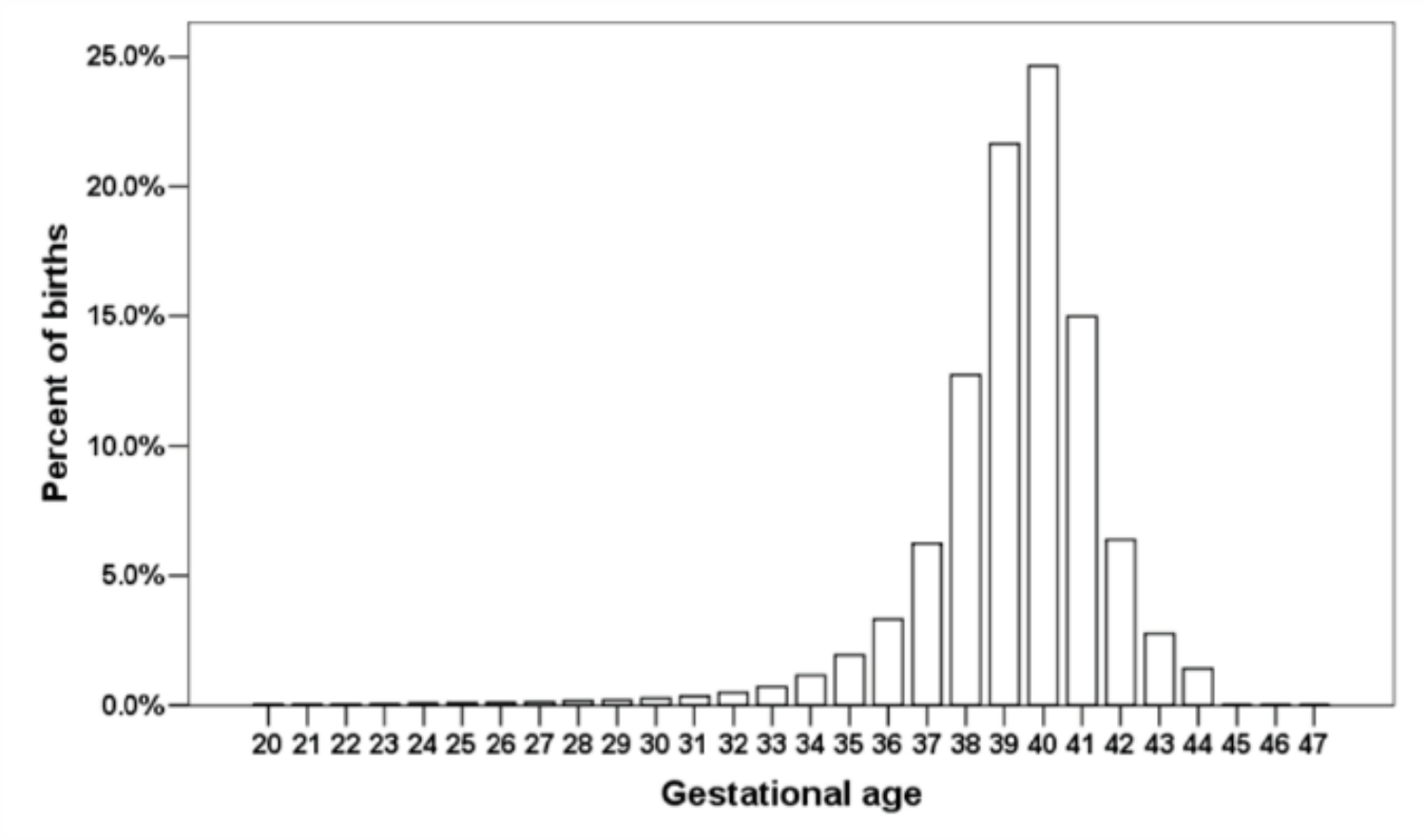
A variety of alternative genetic approaches may be undertaken to identify specific genes involved in PTB. For example, unbiased genome-wide screens, such as the screen conducted on a Danish cohort as part of the National Institutes of Health Gene Environment Association Studies (GENEVA) program, may identify novel genes and pathways. Additionally, non-additive genetic effects, such as copy number or structural variation, may be important avenues for future research. Such approaches may enable investigators to identify novel genes and pathways involved in birth timing with important clinical applications.

Objectives of dissertation

The etiology of PTB is complex and likely involves both genetic and environmental risk factors. A variety of evidence supports genetic influences on PTB, yet

few specific genes have been associated with PTB. Developing a model for the genetic influences on PTB may facilitate gene discovery. As little work had been done to systematically identify a genetic model for PTB, we used sibling risk estimates and segregation analyses to identify one. Another method to facilitate discovery of specific genes associated with PTB is using *a priori* methods. Using information from comparative genomic studies, we conducted a screen of genes minimally biased by our current understanding of parturition to identify novel PTB genes. In order to validate our findings, we replicated genes identified in this screen in additional populations. Of note, genes encoding the follicle-stimulating hormone receptor, *FSHR*, and a phospholipase, *PLA2G4C*, showed evidence of association across populations and was investigated further. Together, these experiments better characterize the nature of genetic influences on PTB and provide evidence for novel genes involved in this disorder.

Figure 1.1: Birth timing in Missouri (1978-1997).



Human parturition typically occurs between 37-42 weeks of gestation, with 40 weeks being the most common time of delivery.

Table 1.1: Summary of candidate gene association studies' findings as of May 2010.

Gene Symbol	Gene Name	Number of Studies	Studies reporting positive findings
ABCA1	ATP-binding cassette, sub-family A, member 1	1	0
ACE	angiotensin I converting enzyme 1	7	1
ADD1	adducin 1 (alpha)	2	0
ADH1B	alcohol dehydrogenase 1B	4	0
ADH1C	alcohol dehydrogenase 1c	5	0
ADRB2	adrenergic, beta-2, receptor, surface	8	3
AGT	angiotensinogen	3	2
AGTR1	angiotensin II receptor, type 1	2	0
ALOX5AP	arachidonate 5-lipoxygenase-activating protein	1	0
ANXA5	annexin A5	1	0
APOA1	apolipoprotein A-I	1	0
APOA4	apolipoprotein A-4	1	0
APOA5	apolipoprotein A-5	1	0
APOB	apolipoprotein B	1	0
APOC2	apolipoprotein C2	1	0
APOC3	apolipoprotein C3	1	0
APOE	apolipoprotein E	1	0
BHMT	betaine-homocysteine methyltransferase	1	0
CBS	cystathionine-beta-synthase	5	1
CCL2	chemokine (C-C motif) ligand 2	4	0
CCL3	chemokine (C-C motif) ligand 3	4	0
CCL8	chemokine (C-C motif) ligand 8	4	0
CD14	monocyte differentiation antigen CD14	5	1
CETP	cholesteryl ester transfer protein, plasma	1	0
COL1A1	collagen, type I, alpha 1	3	0
COL1A2	collagen, type I, alpha 2	3	0
COL3A1	collagen, type 3, alpha 1	3	0
COL5A1	collagen, type 5, alpha 1	4	0
COL5A2	collagen, type 5, alpha 2	4	0
CRH	corticotropin releasing hormone	4	0
CRHBP	corticotropin releasing hormone binding protein	5	1
CRHR1	corticotropin releasing hormone receptor 1	4	0
CRHR2	corticotropin releasing hormone receptor 2	4	0
CRP	C-reactive protein, pentraxin-related	4	0
CSF3	colony stimulating factor 3	1	0
CTGF	connective tissue growth factor	1	0
CTLA4	cytotoxic T-lymphocyte-associated protein 4	4	1
CYP19A1	cytochrome P450, family 19, subfamily A, polypeptide 1	4	0
CYP1A1	cytochrome P450, family 1, subfamily A,	7	2

	polypeptide 1		
CYP2C19	cytochrome P450, family 2, subfamily C, polypeptide 19	1	0
CYP2D6	cytochrome P450, family 2, subfamily D, polypeptide 6	4	0
CYP2E1	cytochrome P450, family 2, subfamily E, polypeptide 1	2	0
CYP3A4	cytochrome P450, family 3, subfamily A, polypeptide 4	1	0
DHCR24	24-dehydrocholesterol reductase	1	0
DHCR7	7-dehydrocholesterol reductase	1	0
DHFR	dihydrofolate reductase	4	0
DRD2	dopamine receptor D2	1	0
EDN2	endothelin 2	4	0
PROCR	protein C receptor, endothelial (EPCR)	1	0
EPHX1	epoxide hydrolase 1, microsomal (xenobiotic)	5	0
EPHX2	epoxide hydrolase 2, microsomal (xenobiotic)	4	0
F13A1	coagulation factor XIII, A1 polypeptide	1	0
F2	coagulation factor II (thrombin)	9	0
F5	coagulation factor V	12	4
F7	coagulation factor VII	7	1
FAS	Fas	4	0
FASLG	Fas ligand	4	0
FGB	fibrinogen beta chain	2	0
FLT1	fms-related tyrosine kinase 1	1	0
GNB3	guanine nucleotide binding protein, beta polypeptide 3	1	0
GSTM1	glutathione S-transferase mu 1	4	2
GSTP1	glutathione S-transferase pi 1	5	0
GSTT1	glutathione S-transferase theta 1	5	2
GSTT2	glutathione S-transferase theta 2	4	0
HMGCR	3-hydroxy-3-methylglutaryl-Coenzyme A reductase	1	1
HSD11B1	hydroxysteroid (11-beta) dehydrogenase 1	4	0
HSD17B7	hydroxysteroid (17-beta) dehydrogenase 7	4	0
HSPA14	heat shock 70kDa protein 14	4	0
HSPA1A	heat shock 70kDa protein 1A	4	0
HSPA1B	heat shock 70kDa protein 1B	4	0
HSPA1L	heat shock 70kDa protein 1-like	4	1
HSPA4	heat shock 70kDa protein 4	4	0
HSPA6	heat shock 70kDa protein 6	4	0
ICAM1	intercellular adhesion molecule 1	2	1
ICAM3	intercellular adhesion molecule 3	1	0
IFNG	interferon, gamma	6	2
IFNGR1	interferon, gamma receptor 1	1	0

IGF1	insulin-like growth factor 1	4	0
IGFBP3	insulin-like growth factor binding protein 3	4	0
IL10	interleukin 10	9	2
IL10RA	interleukin 10 receptor, alpha	5	1
IL10RB	interleukin 10 receptor, beta	4	0
IL11	interleukin 11	1	0
IL12A	interleukin 12A	1	0
IL13	interleukin 13	5	1
IL15	interleukin 15	4	1
IL18	interleukin 18	5	0
IL1A	interleukin 1, alpha	8	1
IL1B	interleukin 1, beta	9	1
IL1R1	interleukin 1 receptor, type I	5	0
IL1R2	interleukin 1 receptor, type 2	5	1
IL1RAP	interleukin 1 receptor accessory protein	4	0
IL1RN	interleukin 1 receptor antagonist	10	4
IL2	interleukin 2	6	1
IL2RA	interleukin 2 receptor, alpha	4	1
IL2RB	interleukin 2 receptor, beta	4	1
IL4	interleukin 4	8	3
IL4R	interleukin 4 receptor	4	0
IL5	interleukin 5	4	1
IL6	interleukin 6	15	5
IL6R	interleukin 6 receptor	7	3
IL8	interleukin 8	7	0
IL8RA	interleukin 8 receptor alpha	4	0
ITGA2	integrin, alpha 2	1	0
ITGB3	integrin, beta 3	1	0
KL	Klotho	4	1
LCAT	lecithin-cholesterol acyltransferase	1	0
LDLR	LDL receptor	1	0
LIPC	lipase, hepatic	1	0
LNPEP	leucyl/cystinyl aminopeptidase	1	0
LOXL1	lysyl oxidase-like 1	1	0
LPL	lipoprotein lipase	1	0
LST1	leukocyte specific transcript 1	4	0
LTA	lymphotoxin alpha	5	1
MASP2	mannan-binding lectin serine peptidase 2	1	0
MBL2	mannose-binding lectin 2, soluble	10	6
MMP1	matrix metallopeptidase 1	5	1
MMP2	matrix metallopeptidase 2	4	1
MMP3	matrix metallopeptidase 3	5	0
MMP8	matrix metallopeptidase 8	4	0
MMP9	matrix metallopeptidase 9	5	2
MTHFD1	methylenetetrahydrofolate dehydrogenase	4	0

MTHFR	5,10-methylenetetrahydrofolate reductase	11	0
MTR	5-methyltetrahydrofolate-homocysteine methyltransferase	1	0
MTRR	5-methyltetrahydrofolate-homocysteine methyltransferase reductase	2	1
NAT1	N-acetyltransferase 1	5	0
NAT2	N-acetyltransferase 2	6	0
NFKB1	nuclear factor of kappa light polypeptide gene enhancer in B-cells 1	4	0
NFKB2	nuclear factor of kappa light polypeptide gene enhancer in B-cells 2	4	0
NFKBIA	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	4	0
NFKBIB	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, beta	4	0
NFKBIE	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, epsilon	4	0
NOD2	nucleotide-binding oligomerization domain containing 2	2	1
NOD2/CARD15	nucleotide-binding oligomerization domain containing 2	4	0
NOS2A	nitric oxide synthase 2, inducible	2	2
NOS3	nucleotide-binding oligomerization domain containing 3	7	1
NPPA	natriuretic peptide precursor A	1	0
NQO1	NAD(P)H dehydrogenase, quinone 1	1	0
NR3C1	glucocorticoid receptor	4	0
OPRM1	opioid receptor, mu 1	1	1
OXT	oxytocin	1	0
OXTR	oxytocin receptor	1	1
PAFAH1B1	platelet-activating factor acetylhydrolase, isoform Ib, subunit 1	4	0
PAFAH1B2	platelet-activating factor acetylhydrolase, isoform Ib, subunit 2	4	0
PDE4D	phosphodiesterase 4D, cAMP-specific	1	0
PGEA1	chibby homolog 1	4	0
PGR	progesterone receptor	9	1
PGRMC1	progesterone receptor membrane component 1	4	0
PGRMC2	progesterone receptor membrane component 2	4	0
PLA2G4A	phospholipase A2, group IVA	4	0
PLAT	plasminogen activator, tissue	6	2
POMC	proopiomelanocortin	4	0
PON1	paraoxonase 1	7	3
PON2	paraoxonase 2	6	2
PPARG	peroxisome proliferator-activated receptor	1	1

	gamma		
PRKCA	protein kinase C, alpha	1	1
PROC	protein C	1	0
PTCRA	pre T-cell antigen receptor alpha	4	0
PTGER2	prostaglandin E receptor 2	5	1
PTGER3	prostaglandin E receptor 3	4	2
PTGES	prostaglandin E synthase	5	0
PTGFR	prostaglandin F receptor	5	0
PTGS1	prostaglandin G/H synthase (cyclooxygenase)	4	0
PTGS2	prostaglandin-endoperoxide synthase 2	4	0
PTPN22	protein tyrosine phosphatase, non-receptor type 22	4	0
REN	renin	1	0
RFC1	replication factor C (activator 1) 1	1	0
RLN1	Relaxin 1	1	0
RLN2	Relaxin 2	1	0
RLN3	Relaxin 3	1	0
SCGB1A1	secretoglobin, family 1A, member 1 (uteroglobin)	4	0
SCNN1A	sodium channel, nonvoltage-gated 1 alpha	1	0
SELE	selectin E	2	0
SERPINB2	serpin peptidase inhibitor, clade B (ovalbumin), member 2	1	0
SERPINE1	serpin peptidase inhibitor, clade E, member 1	6	1
SERPINH1	serpin peptidase inhibitor, clade H, member 1 (collagen binding protein 1)	6	2
SFTPC	Surfactant protein C	2	0
SFTPD	Surfactant protein D	1	0
SHMT1	serine hydroxymethyltransferase 1	1	1
SLC23A1	solute carrier family 23 (nucleobase transporters), member 1	5	0
SLC23A2	solute carrier family 23, member 2	1	1
SLC6A4	solute carrier family 6, member 4	4	0
TCN2	transcobalamin II	4	0
TFPI	tissue factor pathway inhibitor	1	0
TGFA	transforming growth factor, alpha	1	0
TGFB	transforming growth factor, beta	1	0
TGFB1	transforming growth factor, beta 1	6	0
THBD	thrombomodulin	1	1
TIMP3	TIMP metallopeptidase inhibitor 3	4	0
TIMP4	TIMP metallopeptidase inhibitor 4	4	0
TLR10	toll-like receptor 10	1	1
TLR2	toll-like receptor 2	6	1
TLR3	toll-like receptor 3	4	0
TLR4	toll-like receptor 4	9	2

TLR7	toll-like receptor 7	4	0
TLR8	toll-like receptor 8	4	0
TLR9	toll-like receptor 9	4	0
TNF	tumor necrosis factor	24	11
TNFR1	tumor necrosis factor receptor 1	7	4
TNFR2	tumor necrosis factor receptor 2	3	2
TNFRSF1A	tumor necrosis factor receptor superfamily, member 1A	4	0
TNFRSF1B	tumor necrosis factor receptor superfamily, member 1B	4	0
TNFRSF6	tumor necrosis factor receptor superfamily, member 6b, decoy	2	1
TRAF2	TNF receptor-associated factor 2	4	0
TREM1	triggering receptor expressed on myeloid cells 1	4	1
TSHR	thyroid stimulating hormone receptor	4	0
UGT1A1	UDP glucuronosyltransferase 1 family, polypeptide A1	4	0
VEGF	vascular endothelial growth factor A	6	1

Chapter 2: Increased risk to siblings of preterm infants suggests genetic factors may influence PTB[†]

Abstract

Adverse pregnancy outcomes, such as PTB, PPROM, placental abruption, and preeclampsia, are common and contribute to spontaneous and medically-indicated preterm deliveries, with acute and long-term complications for both the mother and infant. Etiologies underlying such adverse outcomes are not well understood. As maternal and fetal genetic factors may influence these outcomes, we estimated the magnitude of familial aggregation as one index of possible heritable contributions. Using the Missouri Department of Health's maternally-linked birth certificate database, we performed a retrospective population-based cohort study of births (1989-1997), designating an individual born from an affected pregnancy as the proband for each outcome studied. We estimated the increased risk to siblings compared to the population risk, using the sibling risk ratio, λs, and sibling-sibling odds ratio (sib-sib OR), for the adverse pregnancy outcomes of PTB, PPROM, placental abruption, and preeclampsia. Risk to siblings of an affected individual was elevated above the population prevalence of a given disorder, as indicated by λ_S (λ_S (95% CI): 4.3 (4.0-4.6), 8.2 (6.5-9.9), 4.0 (2.6-5.3), and 4.5 (4.4-4.8), for PTB, PPROM, placental abruption, and preeclampsia, respectively). Risk to siblings of an affected individual was similarly elevated above that of siblings of unaffected individuals, as indicated by the sib-sib OR (sib-sib OR adjusted for known risk factors

[†] This chapter is adapted from: Plunkett J, et al. (2008) Population-based estimate of sibling risk for adverse pregnancy outcomes. BMC Genetics, 9:44.

(95% CI): 4.2 (3.9-4.5), 9.6 (7.6-12.2), 3.8 (2.6-5.5), 8.1 (7.5-8.8) for PTB, PPROM, placental abruption, and preeclampsia, respectively). These results suggest that the adverse pregnancy outcomes of PTB, PPROM, placental abruption, and preeclampsia aggregate in families, which may be explained in part by genetics.

Introduction

In the United States, 12.7% of births occur preterm (<37 weeks) [66], approximately one-fourth of which occur due to PPROM [67]. Preeclampsia, high blood pressure and fluid retention in pregnancy, and placental abruption, early detachment of the placenta from the uterus, affect approximately 7% [68] and 1% [69] of all pregnancies, respectively. While many pregnancies share more than one of these complications, together they affect a significant portion of pregnancies and represent the most common reasons for early delivery. Moreover, adverse pregnancy outcomes are important causes of perinatal morbidity and mortality. For example, placental abruption, while uncommon, accounts for 12% of all perinatal deaths [25]. The incidence of PTB [66] and placental abruption [25] have increased over recent decades, further motivating additional study to understand susceptibility factors which contribute to these outcomes.

Prediction and prevention of these adverse outcomes is difficult. Etiologies underlying PTB, PPROM, placental abruption and preeclampsia are not well understood. Genetic studies are one way in which we can attempt to better understand these disorders. Such studies may identify genetic markers that can predict one's risk for a particular pregnancy outcome. Genetic studies may also identify novel proteins and/or pathways involved in the disorder.

Both maternal and fetal genetic factors may influence adverse pregnancy outcomes. Evidence suggests that maternal genetic factors contribute to PTB [70; 71], PPROM [71-73], placental abruption [74; 75] and preeclampsia [76-79]. In contrast, fetal effects on these outcomes have not been well studied. Several lines of evidence suggest that fetal genetic effects may influence adverse pregnancy outcomes. First, fetal genes that are paternally imprinted mainly control placental and fetal membrane growth [25]. Because the placenta and fetal membranes likely play a role in adverse pregnancy outcomes, fetal genes controlling these tissues may also contribute. Additionally, heritability studies, which estimate the relative portion of population variation in a trait due to genetics, suggest that PTB [22] and preeclampsia [80] are influenced in part by fetal genetic factors. Lastly, several studies suggest that paternity affects risk for PTB and preeclampsia. For example, several studies indicate that partner changes between pregnancies reduce risk of PTB [26; 27] and preeclampsia [80-83]. Changes in paternity may reflect association with long interpregnancy intervals rather than paternity effects *per se*; however, for preeclampsia [78; 84], fathers' family history affects risk for the disorder in their partners' pregnancies. For PTB, father's family history has been shown to have only a weak association with risk. While an early study of a Norway birth registry demonstrated a correlation between father and children's gestational ages [28], a more recent and extensive study of this registry suggested fathers contributed little to no risk to preterm delivery risk [29]. Also, paternal race has been associated with PTB risk [6; 7]. Together, this data suggests that paternal genes expressed in the fetus may contribute to these disorders, motivating study of maternal-fetal influences, assessed by defining the infant as the proband, in addition to influences that are maternal-specific.

While multiple lines of evidence suggest the importance of genetic contributors in adverse pregnancy outcomes, observing clustering of such outcomes in families is necessary to assert genetic influences on a disorder. A disorder that does not aggregate in families is unlikely to be influenced by inherited factors. Hence, detecting an increased risk for a disorder among siblings or other family members of an individual born from a pregnancy affected by the same adverse outcome would further support genetic influences on these conditions. However, familial aggregation is not sufficient to claim genetic influences on a disorder. Since family members share both genes and environment, any similarities seen in families may be explained by genetic or shared environmental factors (such as *in utero* environment) or by their interaction.

Two standard measures of familial aggregation are increase in risk to siblings of affected individuals, compared to the population risk for the disorder, the sibling risk ratio, λs [85], and compared to siblings of unaffected individuals, the sibling-sibling odds ratio (sib-sib OR) [86]. These measures have been estimated for a variety of disorders, ranging from single locus Mendelian disorders, such as cystic fibrosis [87], to complex disorders, including hypertension [88], type 2 diabetes [89], and myopia [90; 91]. These familial aggregation measures have been incompletely documented in pregnancy outcomes. When considering mother as the affected individual, investigators have reported increased risk among first-degree relatives of women affected with PTB [15; 18], placental abruption [75] and preeclampsia [78; 92]; however, few of these studies have scaled the increase in risk among relatives by the population prevalence for a given pregnancy outcome (placental abruption [75] , preeclampsia [92]), as done to calculate λs. Maternal recurrence risk, similar in calculation to the sib-sib OR, has previously been

reported for these disorders [73; 93-98]. Yet, only one study of PTB and PPROM [73] scaled maternal recurrence relative to population prevalence of the disorder and did not consider this measure as an indication of familial aggregation. λs and sib-sib OR, defining the infant of an affected pregnancy as proband, have not been reported for these disorders. Estimating λs, in which the increased risk for a disorder is scaled by population prevalence, is particularly important, as population prevalence can vary by race. While there may be a significant increase in risk among siblings or a significant maternal recurrence risk, such a risk may reflect high population prevalence, rather than familial effects, *per se*. As a result, calculating λs may lead to different conclusions that those made by previous reports of maternal recurrence risk. Since individual demographic factors, such as socioeconomic status or body mass index, may also contribute to risk, we calculate sib-sib OR adjusted for important medical and environmental risk factors to assess to what extent genetic effects may account for familial aggregation.

In order to test whether genetic effects may influence these outcomes, our analyses define the infant of an affected pregnancy as the proband. We estimate λs and sib-sib OR to determine whether each outcome clusters in families.

Results

Preterm birth. The population risks for PTB at <35 gestational weeks were estimated as 3.6%, 2.8%, and 7.8%, in all races, Whites and Blacks, respectively. Among second-born siblings in the sibling subcohort whose older sibling was affected, rates of PTB for all races, Whites and Blacks, respectively, were used to estimate the sibling risk (see Table 2.1). λ_S and its 95% CI were 4.3 (4.0-4.6), 4.4 (4.0-4.7), and 2.8 (2.6-3.1) for

all races, Whites, and Blacks, respectively, indicating a significant increase in risk to siblings of PTB patients compared to the population.

Individuals whose older sibling was affected by PTB were also at significantly higher risk compared to individuals whose older sibling was unaffected (see Table 2.1). This increase in risk persisted after adjusting for known risk factors. Adjusted OR with 95% CI were 4.2 (3.9-4.5), 5.1 (4.6-5.7), and 3.3 (2.9-3.7) for all races, Whites and Blacks, respectively.

PPROM. The population risks for PPROM were estimated as 0.8%, 0.6% and 1.9%, in all races, Whites and Blacks, respectively. Among second siblings in the matched sibling sub-cohort whose older sibling was affected, rates of PPROM were used to estimate sibling risk (see Table 2.2). λ_S and its 95% confidence interval were 8.19 (6.50-9.88), 6.75 (4.59-8.91), and 6.40 (4.66-8.14) for all races, Whites, and Blacks, respectively, indicating a significant increase in risk to siblings of PPROM patients compared to the population.

Individuals whose older sibling was affected by PPROM were also at significantly higher risk compared to individuals whose older sibling was unaffected (see Table 2.2). This increase in risk persisted after adjusting for known risk factors. Adjusted OR with 95% CI were 9.6 (7.6-12.2), 8.5 (6.0-12.1), and 8.9 (6.4-12.5) for all races, Whites and Blacks, respectively.

Placental Abruption. Population rates of placental abruption were estimated as 0.8%, 0.7%, 1.0%, in all races, Whites and Blacks respectively. Among second siblings in the matched sibling sub-cohort whose older sibling was affected, rates of placental abruption were used to estimate risk to siblings (see Table 2.3). λ_S and its 95%

confidence interval were 3.95 (2.63-5.27) and 4.93 (3.18-6.68), for all races and Whites, respectively, indicating a significant increase in risk to siblings of placental abruption patients compared to the population.

We found that individuals whose older sibling was affected by placental abruption were also at significantly higher risk compared to individuals whose older sibling was unaffected (see Table 2.3). This increase in risk persisted after adjusting for known risk factors. Adjusted OR with 95% CI: 3.8 (2.6-5.5) and 5.0 (3.4-7.4) for all races and Whites, respectively.

Blacks did not show a significant increase in risk to siblings of placental abruption births either compared to the population (λ_S= 1.64 (0.04-3.24)) or compared to siblings of births unaffected by this disorder (unadjusted OR: 1.4 (0.5-3.7), adjusted OR: 1.2 (0.4-3.9)).

Preeclampsia. Population rates of preeclampsia were estimated as 3.2%, 3.1%, and 4.1%, in all races, Whites and Blacks, respectively. Among second siblings in the matched sibling sub-cohort whose older sibling was affected, rates of preeclampsia were used to calculate sibling risk (see Table 2.4). λ_S and its 95% confidence interval were 4.51 (4.24-4.78), 4.52 (4.21-4.83), and 4.11 (3.59-4.63) for all races, Whites, and Blacks, respectively.

We found that individuals whose older sibling was affected by preeclampsia were also at significantly higher risk compared to individuals whose older sibling was unaffected (see Table 2.4). This increase in risk persisted after adjusting for known risk factors. Adjusted OR with 95% CI were 8.1 (7.5-8.8), 9.0 (8.2-9.8), and 5.8 (4.9-7.0) for all races, Whites and Blacks, respectively.

Discussion

We hypothesized that siblings of individuals who were products of pregnancies affected by one of several adverse outcomes, PTB, PPROM, placental abruption and pre-eclampsia, would be at increased risk for the same outcome. λs and sib-sib OR values significantly greater than one indicate that risk to siblings of adverse pregnancy outcome births is elevated compared to the population rate and to the rate in siblings of unaffected individuals, respectively. None of the 95% CI for λs or sib-sib OR values overlap with one, with the exception of placental abruption in Blacks. The lack of evidence for familial aggregation of placental abruption in Blacks may be explained by the rarity of the event and the relatively small racial subgroup (see Table 2.3). These data suggest that genetic and/or environmental risk factors shared among siblings affect these disorders.

Estimates of sib-sib OR are consistent with previous studies of maternal recurrence risk in the Missouri birth certificate database [93; 94], and of maternal recurrence risk scaled to the population prevalence for PTB [73]. Our estimate of λs is noticeably smaller than the maternal recurrence risk, scaled by population prevalence of PPROM estimated in [73] (OR (95% CI): 20.6 (4.7, 90.2)). This difference likely reflects the larger and population-based cohort used in our study, in contrast to [73] in which relatively small groups of PPROM (n=114) and normal term (n=208) deliveries were selected from a hospital population.

The utility of these measures lies primarily in establishing familial aggregation of a disorder, a prerequisite to claiming genetic influences on any trait. Yet, λs values may also be used to make tentative assessments of future genetic studies. The magnitude of λs values may reflect the mode of genetic etiology, influencing future studies' design. For

example, for complex disorders, to which multiple genetic and environmental factors likely contribute, reported λs values range from 1.3-75, with peaks at 3-4 and 10-15 [88]; in contrast, monogenic Mendelian disorders show λs values an order of magnitude higher or more (e.g. cystic fibrosis λs ~500 [87]). Thus, moderate values for λs, such as those reported for the adverse pregnancy outcomes studied (see Tables 2.1-2.4), are consistent with complex genetic and environmental etiologies. Among complex disorders, λs has been used to estimate the ability of a study to detect specific genes [99]. However, large values of λs do not necessarily predict linkage [88; 100] or association [101] studies' success. Additionally, measures that reflect the strength of a genetic effect detected either by linkage, λs calculated with respect to a specific locus, or by association, genotype relative risk, γ, which measures the ratio of disease risks between individuals with and those without the susceptibility genotypes, have only an indirect correlation with λs [101]. Moderate λs values may correspond with high γ values (e.g. rheumatoid arthritis [101]) and vice versa. While limitations in interpreting λs values exist, disorders with similar λs values to the adverse pregnancy outcomes reported here have had specific genes mapped (e.g. hypertension, obesity [88]), suggesting that identification of specific genes influencing these conditions may be possible.

While the increased risk to siblings may be explained in part by shared genetics, some evidence suggests that multiple interacting environment factors can account for familial clustering [102]. Hence, the clustering of multiple non-genetic risk factors in families may account for these results. In order to distinguish genetic from other familial risk factors, we calculated sib-sib OR unadjusted and adjusted for important known environmental risk factors. Overall, the elevated risk to siblings persists after adjustment

for such factors. While there may be important non-genetic factors affecting each outcome for which we have not accounted, we believe these results suggest that genetic influences may contribute to each of the adverse pregnancy outcomes studied.

Interestingly, λs and sib-sib OR estimates in Blacks are generally smaller than those for Whites. For PROM and preeclampsia, the 95% CI for λs and sib-sib OR estimates for the two racial groups overlap; however, these CI do not overlap for PTB or placental abruption. Hence, it is difficult to determine to what extent family clustering of these outcomes may differ among races. Differences in the magnitude of λs and sib-sib OR estimates between Blacks and Whites may be explained in part by the higher population prevalence for Blacks compared to Whites for each outcome studied (non-overlapping 95% CI, see Tables 2.1-2.4), which may reflect higher overall rates of genetic and/or environmental risk factors in this population.

The Missouri database provides many of this study's strengths. The large number of first recorded siblings in the population cohort (n=267,472) and matched sibpairs in the sibling cohort (n=163,826) provides a large sample size from which to estimate λs and sib-sib OR. Additionally, because this database represents a population cohort of births, rather than births selected based on any particular pregnancy outcome, biases due to ascertainment and overreporting, which can inflate λs values [103], should be minimal.

However, using a birth certificate database like this one also presents several limitations. First, complications of labor and delivery and maternal and infant medical conditions recorded in such databases may be underreported [104]; as a result, population and/or sibling risk for a particular disorder may be underestimated, potentially biasing our results. For example, the relative rarity of placental abruption in the population makes

concordant sibships, particularly in Blacks, rare, thereby reducing sample sizes for risk estimates for this disorder. Additionally, gestational age estimates contained in birth certificate databases are based primarily on the date of the last menstrual period, which may be recalled inaccurately or misclassified due to postconceptional bleeding [104], potentially influencing estimates of PTB and PPROM prevalence in this dataset. We also acknowledge that each of the categories of PTB that we analyzed may in themselves be rather heterogeneous. For example, initiation of spontaneous labor may result in PTB in each of the categories, though for some etiologies, particularly preeclampsia, iatrogenic delivery could contribute significantly. Our utilization of a more rigorous definition of PTB at less than 35 weeks should minimize the contribution of iatrogenic delivery. A final important limitation to this database is the limited amount of information on race. Maternal race is self-reported and possibly subject to population stratification and/or admixture. Additionally, information on paternal race is incomplete, further affecting the accuracy of infants' reported race.

The Missouri database also does not document relationships between mothers; as a result, similar calculations cannot be made to estimate familial clustering when the mother of an affected pregnancy is considered the proband. Moreover, the database contains little information on fathers, making it impossible to distinguish full from half siblings in most sibships. Because we cannot distinguish siblings that share both maternal and paternal factors from those that share maternal factors only, we cannot assess to what extent the increased risk can be attributed to factors unique to the fetus, rather than those shared with its mother. Due to these limitations, we cannot examine the relative importance of maternal versus fetal genetic effects, studied by Wilcox et al. [29] and

Cnattingius et al. [80], for PTB and preeclampsia, respectively. Cnattingius et al. [80] reports 20% of variation in preeclampsia risk is due to fetal genetic effects and the combined effect of fetal genetic factors and couple effects are as important as maternal genetic effects. In contrast, Wilcox and colleagues [29] report only a weak association between father's family history and risk for PTB (RR (95% CI): 1.12 (1.01-1.25)), which became nonsignificant at earlier gestational ages (RR (95% CI): 1.06 (0.77-1.44). From this trend, the authors conclude that fetal genes may contribute to normal labor, but, not preterm delivery [29]; however, Wilcox and colleagues [29] have relatively few early preterm offspring of early preterm mothers (n=91) and fathers (n=39) from which risk was estimated, and do not stratify based upon race/ethnicity. Similarly, a recent study [21] suggested that paternal genetics contributed little to gestational age, but could not refute the possible role of maternally-inherited genes expressed in the fetus. Hence, while paternally-inherited genes may contribute little to PTB or other disorders, maternally-inherited genes expressed in the fetus may still be important. Because of our study's limitations, we may be detecting effects due to shared uterine environment, shaped in part by maternal genes, rather than maternally-inherited genes in siblings. Hence, fetal genetic effects may make contributions of lesser magnitude than maternal genetic factors, with fetal genetic factors having a more prominent role in certain etiologies of PTB.

We have observed familial aggregation of PTB, PPROM, placental abruption and preeclampsia. Overall, siblings are at increased risk for each outcome, even after adjusting for important known environmental risk factors. While the influence of shared unmeasured environmental risk factors on sibling risk cannot, and should not, be discounted, we hypothesize that maternal and/or fetal genetic influences account for some

of the increased risk to siblings observed. Moreover, though it is difficult to determine to what extent fetal and maternal effects overlap in these analyses, we postulate that fetal genetic factors may contribute to these disorders and suggest that they are studied further.

Methods

Study design. A protocol was approved by the Missouri Department of Health and Senior Services and by Washington University School of Medicine to analyze the state's maternally linked birth-death certificate database. We analyzed this database to assess the recurrence risk for a discrete group of adverse pregnancy outcomes, including PTB, PPROM, placental abruption, and pre-eclampsia, in maternally-linked siblings. Births to the same mother were linked by a unique identifier called a sibship number, described elsewhere [105]. Full siblings and half-siblings resulting from pregnancies in the same mother were not distinguished. All protected health information with personal identifiers was removed before distributing the data for analysis.

This analysis was restricted to births that occurred between 1989 and 1997, since births that occurred before 1989 lacked complete medical and social histories. Fetal deaths occurring at <20 weeks gestation, multiple gestation pregnancies and individuals with no maternally-linked siblings recorded in the database were excluded from this analysis. After excluding such cases, the remaining cohort consisted of 473,881 births, of which 383,812 (81.2%) were White and 81,889 (17.3%) were Black. 267,472 births (220,728 (82.5%) White and 42,899 (16.0%) Black) were the first maternally-linked sibling in the database and used to estimate the population prevalence for each outcome.

A second cohort of matched siblings was constructed from this dataset to analyze sibling risk for each outcome. The two oldest siblings born to the same mother during the

study period were included. The dataset was not restricted to parity 0 and parity 1 women, in order to be as unbiased as possible in estimating risk for siblings and providing the best index of population prevalence. Additional siblings born to the same mother were excluded to simplify the statistical model. This cohort comprised of 327,652 matched siblings, of which 265,947 (81.2%) were White and 55,555 (17.0%) were Black. Second-born siblings whose older sibling was affected by a particular outcome were used to estimate sibling risk for λs and sib-sib OR.

Definitions. PTB is defined by the World Health Organization as delivery <37 weeks [1]. To avoid inclusion of borderline gestational ages which may introduce misclassification bias, we defined PTB as delivery <35 weeks in this study. Information from the last menstrual period and clinical data were used to calculate the best estimate of gestational age. PPROM was defined as births delivered <35 weeks complicated by premature rupture of membranes. For PPROM, births complicated by pre-eclampsia, insulin-dependent and other diabetes, or eclampsia were excluded from analysis due to the potential for these births being delivered for medical reasons. First-born sibling and second-born sibling refer to the two oldest siblings recorded in database.

Statistical analysis

$$\lambda s = \frac{\mathrm{P(affected \mid affected\ \ sibling)}}{\mathrm{Population\ prevalence}}$$

λ_S was calculated as the frequency of an outcome in the individuals whose older sibling was affected with the disorder in the sibling cohort divided by the frequency of the outcome in first siblings in the larger cohort. 95% confidence intervals (CI) for sibling risk, population risk and sibling risk ratio were calculated by standard procedures for a binomial variable.

$$\text{Sib} - \text{sib OR} = \frac{\text{P(affected} \mid \text{affected sibling)}}{\text{P(affected} \mid \text{unaffected sibling)}}$$

Sib-sib OR was calculated as the odds of a child being affected with a particular adverse outcome, given that their older sibling was affected, divided by the odds of a child being affected with a particular adverse outcome, given that their older sibling was unaffected. Sib-sib OR were adjusted for known medical and environmental risk factors for the outcome to most conservatively estimate residual familial effects on risk. For preterm-birth and PPROM, OR were adjusted for: mother's age <20 years old, mother <12 years of education, recipient of state-funded assistance (an index of low socioeconomic status), no prenatal care, mother's body mass index (BMI) <20 kg/m^2, and cigarette smoking during pregnancy. In addition to these risk factors, preeclampsia ORs were corrected for: mother's age >35years old, insulin-dependent diabetes mellitus, chronic hypertension. ORs for placental abruption were corrected for hydraminos/oligohydraminos in addition to the risk factors listed above.

Frequencies for λ_S and logistic regression analyses for the sib-sib OR were performed using Stata 9 [106]. Each calculation was made for PTB, PPROM, placental abruption, and preeclampsia in all races (including individuals whose race was neither Black nor White), as well as stratified by Black or White race. λ_S and sib-sib ORs calculated by race compare siblings of affected individuals designated as Black or White to the siblings of unaffected individuals of the same race or the population prevalence for that race.

Table 2.1: Estimated sibling risk ratio (λs) and sibling-sibling odds ratio (sib-sib OR) for PTB <35 weeks.

	All Races	White	Black
Population: Preterm births	9759	6232	3354
Population: Total births	268103	220728	42899
Population Risk	0.036 (0.035-0.037)	0.028 (0.027-0.029)	0.078 (0.075-0.081)
Siblings: sibpairs with both siblings delivered preterm	1020	514	489
Siblings: sibpairs with first sibling delivered preterm	6522	4181	2210
Sibling Risk	0.156 (0.147-0.165)	0.123 (0.113-0.133)	0.221 (0.204-0.238)
λs (95% CI)	4.3 (4.0-4.6)	4.4 (4.0-4.7)	2.8 (2.6-3.1)
Sib-sib unadjusted OR (95% CI)	5.6 (5.2-6.0)	5.7 (5.2-6.3)	3.6 (3.2-4.0)
Sib-sib adjusted OR (95% CI)#	4.2 (3.9-4.5)	5.1 (4.6-5.7)	3.3 (2.9-3.7)

#Adjusted for mother <20 years old, mother <12 education, Medicaid (index of low SES), no prenatal care, mother BMI <20 kg/m^2, cigarette smoking

Table 2.2: λs and sib-sib OR (with 95% CI) for PPROM.

	All Races	White	Black
Population: PPROM	2105	1311	763
Population: N	254740	211308	39190
Population risk	0.008 (0.008-0.008)	0.006 (0.006-0.006)	0.019 (0.018-0.020)
Siblings: sibpairs with both siblings delivered after PPROM	88	37	49
Siblings: sibpairs with first sibling delivered after PPROM	1300	883	393
Sibling risk	0.068 (0.054-0.082)	0.042 (0.029-0.055)	0.125 (0.092-0.158)
λs	8.2 (6.5-9.9)	6.8 (4.6-8.9)	6.4 (4.7-8.1)
Sib-sib unadjusted OR (95% CI)	10.8 (8.6-13.5)	8.8 (6.3-12.4)	8.8 (6.4-12.1)
Sib-sib adjusted OR (95% CI)#	9.6 (7.6-12.2)	8.5 (6.0-12.1)	8.9 (6.4-12.5)

#Adjusted for mother <20 years old, mother <12 education, Medicaid (index of low SES), no prenatal care, mother BMI <20 kg/m^2, cigarette smoking

Table 2.3: λs and sib-sib OR (with 95% CI) for placental abruption.

	All races	White	Black
Population: placental abruption	2050	1579	428
Population: N	268002	220641	42888
Population risk	0.008 (0.008-0.008)	0.007 (0.007-0.007)	0.010 (0.009-0.011)
Siblings: sibpairs with both siblings from pregnancy affected with placental abruption	34	30	4
Siblings: sibpairs with first sibling delivered from pregnancy affected with placental abruption	1124	851	245
Sibling risk	0.030 (0.020-0.040)	0.035 (0.023-0.047)	0.016 (0-0.032)
λs	4.0 (2.6-5.3)	4.9 (3.2-6.7)	1.6 (0.0-3.2)
Sib-sib unadjusted OR (95% CI)	4.1 (2.9-5.8)	5.4 (3.8-7.9)	1.4 (0.5-3.7)
Sib-sib adjusted OR (95% CI)#	3.8 (2.6-5.5)	5.0 (3.4-7.4)	1.2 (0.4-3.9)

#Adjusted for mother <20 or >35years old, mother <12 education, Medicaid (index of low SES), no prenatal care, mother BMI <20 kg/m^2, cigarette smoking, insulin-dependent diabetes mellitus, chronic hypertension, hydraminos/oligohydraminos

Table 2.4: λs and sib-sib OR (with 95% CI) for preeclampsia.

	All races	White	Black
Population: preeclampsia	8600	6749	1736
Population: N	267840	220505	42861
Population risk	0.032 (0.031-0.033)	0.031 (0.031-0.031)	0.041 (0.039-0.043)
Siblings: sibpairs with both siblings from pregnancy affected with preeclampsia	1070	821	233
Siblings: sibpairs with first sibling from pregnancy affected with preeclampsia	7384	5869	1400
Sibling risk	0.145 (0.137-0.153)	0.140 (0.131-0.149)	0.166 (0.146-0.186)
λs	4.5 (4.2-4.8)	4.5 (4.2-4.8)	4.1 (3.6-4.6)
Sib-sib unadjusted OR (95% CI)	9.2 (8.5-9.9)	10.0 (9.1-10.9)	6.7 (5.7-7.9)
Sib-sib adjusted OR (95% CI)#	8.1 (7.5-8.8)	9.0 (8.2-9.8)	5.8 (4.9-7.0)

#Adjusted for mother <20 or >35years old, mother <12 education, Medicaid (index of low SES), no prenatal care, mother BMI <20 kg/m^2, cigarette smoking, insulin-dependent diabetes mellitus, chronic hypertension

Chapter 3: Patterns of inheritance suggest familial PTB is complex genetic disorder[‡]

Abstract

While multiple lines of evidence suggest the importance of genetic contributors to risk of PTB, the nature of the genetic component has not been identified. We perform segregation analyses to identify the best fitting genetic model for gestational age, a quantitative proxy for PTB. Because either mother or infant can be considered the proband from a preterm delivery and there is evidence to suggest that genetic factors in either one or both may influence the trait, we performed segregation analysis for gestational age either attributed to the infant (infant's gestational age), or the mother (by averaging the gestational ages at which her children were delivered), using 96 multiplex preterm families.

These data lend further support to a genetic component contributing to birth timing since sporadic (i.e. no familial resemblance) and nontransmission (i.e. environmental factors alone contribute to gestational age) models are strongly rejected. Analyses of gestational age attributed to the infant support a model in which mother's genome and/or maternally-inherited genes acting in the fetus are largely responsible for birth timing, with a smaller contribution from the paternally-inherited alleles in the fetal genome. Our findings suggest that genetic influences on birth timing are important and likely complex.

[‡] This chapter is adapted from: Plunkett J, et al. (2009) Mother's genome or maternally-inherited genes acting in the fetus influence gestational age in familial PTB. Hum Hered 68:209-219.

Introduction

While multiple lines of evidence suggest genetic contributors are important in PTB, a specific mode of inheritance has not been identified. No prominent simple Mendelian pattern of inheritance has been observed in multiplex pedigrees identified to date. Modeling procedures used by twin studies suggest that additive genetic factors and environmental risk factors that are not shared among siblings both influence PTB [19-21]. Moderate values of sibling risk ratio (λ_S), a measure of risk to siblings of affected individuals compared to the population risk for a disorder, estimated for PTB (λ_S (95% CI): 4.3 (4.0-4.6)) [107] are also consistent with complex genetic and environmental etiologies [88]. Moreover, association studies have reported gene-gene [33; 34] and gene-environment [35; 108; 109] interactions with PTB. Together, these studies imply that the etiology of PTB likely involves genetic as well as environmental factors in complex interactions. However, there has not been a systematic study of possible genetic models for PTB to date.

In this study, we performed segregation analyses to identify the best fitting genetic model for gestational age, a quantitative proxy for PTB. Because either mother or infant can be considered the proband from a preterm delivery, and there is evidence to suggest that genetic factors in either one or both may influence the trait, we performed segregation analysis for gestational age as a quantitative trait either attributed to the infant, infant's gestational age, or to the mother, by averaging the gestational ages at which her children were delivered, using 96 multiplex preterm families. We also tested parent of origin models for infant's gestational age to examine whether mother's genotype is the sole determinant of variation in this trait. Additionally, as pregnancies in

which either the mother [10; 13] or father [6; 7] is Black are at increased risk for preterm delivery, we performed segregation analysis for each phenotype in the total sample, as well as stratified by Black and White race, to test for heterogeneity between these two groups.

Results

Modeling of gestational age attributed to the infant. We first analyzed gestational age of the infant. The number of subjects and descriptive statistics for this phenotype are shown in Table 3.1. Table 3.2 presents the likelihood ratio tests (LRTs) and Akaike's Information Criterion (AIC) values for segregation analysis of infant's gestational age for 17 models of inheritance. The parameter estimates for segregation analysis of infant's gestational age are listed in Table 3.1. The hypotheses of no familial resemblance (model 2), no major gene effect (model 3), and no multifactorial effect (model 4) are rejected, suggesting the presence of both a major gene and a multifactorial effect. Additionally, the equal τ's hypotheses (models 10, 12 and 14) are rejected for the mixed, recessive mixed and dominant mixed models, respectively. In the free τ's models, the estimated τ_{Aa} differed from 0.5, expected under the Mendelian model, and fit the data better than their respective general models (models 1, 7 and 8) for all groups. Together, this evidence supports a genetic component for PTB transmitted from parents to offspring and suggests that the transmitted effect is complex. Similar models were tested for general parent of origin effects, as well as maternal-specific and paternal-specific effects under Mendelian transmission (models 15-17). The parent of origin model (model 15) best fit the data as judged by AIC values and was chosen as the most parsimonious model (Table 3.3). This model suggests that, when attributing gestational age to the infant of a

delivery, genetic factors influence this trait and the parent from whom such factors are inherited influences the overall trait value.

Modeling of gestational age attributed to the mother. We also analyzed gestational age attributed to the mother, by averaging the gestational ages at which her children were delivered (see Table 3.1). Table 3.2 presents the likelihood ratio tests and AIC values for segregation analysis on mother's average gestational age of children for 14 models of inheritance. The parameter estimates for the combined dataset are listed in Table 3.4. The hypotheses of no familial resemblance (model 2), no major gene effect (model 3), and no multifactorial effect (model 4) are rejected, suggesting the presence of both a major gene and a multifactorial effect. The equal τ's (models 10, 12 and 14) are rejected. None of the free τ's models (models 9, 11 and 13) converged initially. In order to estimate these models, τ_{AA} and τ_{aa} were fixed to 1 and 0, respectively. Only τ_{Aa} was estimated and, in each case, differed from 0.5, expected under the Mendelian model. Additionally, the free τ's models fit the data better than their respective general models (models 1, 7 and 8), perhaps suggesting that the major effect observed is more complex than the single biallelic locus modeled here. Together, this evidence supports a genetic component for PTB transmitted from parents to offspring and suggests that the transmitted effect is complex. The mixed free τ's model (model 9) best fit the data as judged by the AIC values and was chosen as the most parsimonious model (Table 3.5). This model suggests that a multifactorial genetic model most likely best accounts for variation in gestational age, when the trait is attributed to the mother of a delivery.

Heterogeneity between Blacks and Whites. Since pregnancies in which either the mother [10; 13] or father [6; 7] is Black are at increased risk for preterm delivery, we

tested for evidence of genetic heterogeneity between these two groups. Segregation analyses of infant's gestational age and mother's average gestational age over all her children also were performed in Black and White subgroups. Table 3.1 documents the number of subjects and descriptive statistics for both phenotypes by race. The segregation analyses of infant's gestational age supported similar conclusions in the combined sample and Black and White subgroups. However, several results differed in the segregation analysis of mother's average gestational age of children when the sample was stratified by Black and White race, compared to the combined sample. In the race-stratified samples, final estimates of the free τ's models had higher -2ln likelihoods than did similar models with fewer parameters, indicating that the maximum likelihood was not reached. As a result, the equal τ's hypotheses (models 10, 12 and 14) and the free τ's hypotheses (models 9, 11 and 13) were not rejected for the mixed, recessive mixed and dominant mixed models, respectively, in Black and White subgroup analysis. The best fitting model according to AIC values was the mixed equal τ's model and was selected as the most parsimonious model in both race subgroups (data not shown).

To test formally for heterogeneity between Blacks and Whites, we used the heterogeneity χ^2 test [110; 111] in which the -2lnL value under a given model for the combined data is subtracted from the summed -2lnL values from stratified analyses. For infant's gestational age, evidence for genetic heterogeneity among races was observed when comparing values under the multifactorial model ($\chi^2 = \Sigma$ (698.07 Black + 2010.02 White) -2690.72 combined =17.37, 3 df, p=0.0006). Of note, parameter estimates for Blacks and Whites differed, particularly estimates of p, H and μ_{AA} which were generally higher in Blacks than Whites. For the multifactorial model, H was estimated as 0.46

(95% CI: 0.27, 0.65) for Blacks and 0.23 (95% CI: 0.13, 0.33) for Whites. While these point estimates are quite different, the 95% CI overlap, indicating that this difference may not be statistically significant. For mother's average gestational age of children, evidence for genetic heterogeneity among races was observed when comparing values under the multifactorial model ($\chi^2 = \Sigma$ (54.92 Black +213.58 White)-216.37 combined =52.13, 3 df, $p=2.81x10^{-11}$). Of note, parameter estimates for Blacks and Whites differed, particularly estimates of H, which are generally higher in Blacks than Whites. For the mixed free τ's model, H was estimated as 0.70 (95% CI: 0-1) for Blacks and 0.23 (95% CI: 0-0.57) for Whites. As the 95% confidence intervals overlap, this difference is not significant in the sample size analyzed here.

Discussion

PTB likely has a complex etiology involving both genetic and environmental risk factors, based on evidence from previous studies. This study is the first to explicitly test different modes of inheritance for birth timing, by assessing gestational age as a phenotype of either mother or infant. These data lend further support to a genetic component contributing to birth timing since sporadic (i.e. no familial resemblance) and nontransmission models, in which gestational age is attributed to environmental factors alone, are strongly rejected (Tables 3.2 and 3.4). Our findings suggest that genetic influences on birth timing are important and likely complex.

For infant's gestational age, the parent of origin model (model 15) best fit the data according to the AIC values and was chosen as the most parsimonious model (Table 3.2). Based on the mean estimates for AA, Aa, aA and aa under this model (Table 3.3), it appears that this parent of origin effect is largely maternal. Heterozygotes who inherit the

A allele from their mother (Aa) have a mean closer to, but not equal to, AA. Under a strict maternal model, AA and Aa would be equivalent, since mother's A allele would be expected to be the sole determinant of phenotype. In contrast, heterozygotes who inherited the A allele from their father (aA) have a mean that is approximately equivalent to that of aa, suggesting that father's A allele has little effect on phenotype. A model in which the maternally-inherited allele was the sole determinant of the phenotype (model 16) did not fit the data better than the parent of origin model in which the entire genotype was considered. Importantly, father's genes also affect phenotype in this model, aligning with previous work showing paternity [26; 27] as well as paternal race [6; 7] influence PTB risk. Hence, both maternal and paternal alleles seem to contribute to infant's gestational age, with maternally-inherited alleles having a stronger effect on phenotype than those inherited from the father. This finding may support previous studies that have observed stronger effects of mother's race [6; 7] and family history on risk for PTB [29] than those of the father.

These data suggest that maternally-inherited genes acting in the fetus and/or maternal genes acting in the mother are largely responsible for birth timing; however, these two possibilities are not easily distinguished. Maternal genetic effects can create the same pattern of phenotypic variation as genomic imprinting [112]. These two classes of effects can be distinguished by comparing the offspring of heterozygous mothers [112]; however, such comparisons are not possible in our dataset in which individuals were assigned probabilities for each possible genotypic state, rather than having known genotypes measured empirically. Previous studies in cattle [113; 114] have observed maternal genetic effects on gestational age, but did not consider parent of origin effects.

Consequently, further study is needed to determine whether maternal effects or imprinting account for our observations. In either case, considering the mother of a preterm delivery as proband may be most useful in identifying genetic contributions to PTB.

Segregation analysis of mother's average gestational age of children also supported a complex genetic model. The mixed free τ's model (model 9) best fit the data according to the AIC values and was chosen as the most parsimonious model (Table 3.4). This model, in combination with results from LRTs, suggests that genetic influences on birth timing are important and likely complex. Importantly, fewer individuals are informative when mother is considered the proband in a preterm delivery (Figure 3.1), and the smaller sample size affects the power of the analysis. As a result, the parameter and likelihood estimates made when considering mother as proband are more affected by sampling variance; however, conclusions made by comparing across models should be less affected by sample size.

Overall, mother-based and infant-based analyses both support the importance of genetic factors, perhaps primarily acting in the mother or maternally-inherited alleles acting in the fetus, in birth timing. The genetic component influencing PTB likely involves many genes in interaction with environmental and other genetic factors. These results are consistent with previous studies suggesting that genes and environments [19-21], as well as gene-gene [33; 34] and gene-environment [35; 108; 109] interactions influence PTB. Estimates from the multifactorial model (model 3, Tables 3.3 and 3.5) indicate 30-40% of variation in gestational age, attributed to either mother or infant, can be explained by genetics, consistent with estimates from previous twin studies [19; 21].

As twins may not be representative of the population as a whole, our heritability estimates corroborate the general importance of genetics in birth timing. Heritability estimates were generally higher in mother-based analyses (0.44 (95% CI: 0.11, 0.77)) than in infant-based analysis (0.33 (95% CI: 0.24, 0.42)), but not significantly different (Tables 3.3, 3.5). While many genes may be contributing to the observed genetic influence on birth timing, the moderate heritability observed suggests that the cumulative effect of these genes accounts for an important amount of variation in gestational age.

Although alternative methods for segregation analysis exist, we considered the Pedigree Analysis Package (PAP) to be the best method for our primary goal of identifying the best-fitting genetic model for birth timing. Using PAP, we were able compare models directly and identify the most parsimonious model. In contrast, Markov Chain Monte Carlo (MCMC) methods, such as those used by the Loki [115] and Morgan [116] packages, generate a series of posterior probabilities for various models, but no one model is identified as superior. Moreover, MCMC methods model several Mendelian loci simultaneously but do not include a polygenic component, which we wanted to include in the models we examined. Furthermore, one cannot correct for ascertainment within MCMC analysis, in contrast to PAP. One of the disadvantages of using PAP exclusively was that we were not able to estimate the approximate number of loci contributing to birth timing, as could be done with MCMC methods. Additionally, MCMC methods may be better at handling large, complex pedigrees than PAP [117]; however, since our families were relatively small and simple (e.g. containing no inbreeding), we did not consider this a limitation.

We have likely enriched for genetic and/or common environmental effects by using 96 multiplex families, all of whom were recruited based on having two or more first degree relatives delivered preterm. These multiplex families provided a large sample size from which genetic effects could be examined. However, the ascertainment scheme also introduces bias, as the families were not collected randomly and may overestimate the importance of certain genetic models. To minimize errors due to such bias, all analyses were corrected for ascertainment by conditioning on the initial proband, either the mother or the offspring depending which phenotype was considered, that led to the ascertainment of the family, assuming single ascertainment. However, if our assumption of single ascertainment is incorrect, conditioning on probands in this manner may create bias in estimating model parameters [118], leading to inaccurate conclusions about the best-fitting model for familial PTB. Because we believe our ascertainment scheme is consistent with single ascertainment, these results appear to be most appropriate for modeling genetic effects in familial PTB. Yet, it is possible that our conclusions drawn from familial cases of PTB may not generalize to all instances of this disorder. Familial cases of PTB may have a different genetic contribution than isolated cases, perhaps having different etiologies than isolated cases.

This study is also limited by the phenotypes studied. Information on gestational age was collected by self-report data from questionnaires for many individuals used in this analysis. While it was possible to verify gestational ages from medical records in some cases, including all births delivered at the participating institutions, many gestational ages could not be verified and may be subject to reporting errors. Additionally, many individuals for whom we could not verify precise gestational ages

were reported as "full term" and assigned gestational age of 40 weeks. While this is the most likely gestational age for infants to be born [12], we may lose some variability in the overall distribution of gestational age by doing so. As modeling of trait variance is essential to segregation analysis, this approach also may have affected our results.

Our findings suggest that genetic influences on birth timing are important. Modeling for both mother and infant phenotypes indicate that a genetic component influences gestational age and is complex in nature. In analyses using either mother or infant as proband, monogenic Mendelian models were strongly rejected, suggesting that a single gene model cannot fully explain birth timing in these families. A number of genes probably contribute to the genetic influence on birth timing and PTB described. Analyses of gestational age attributed to the infant support a model in which mother's genome and/or maternally-inherited genes acting in the fetus are largely responsible for birth timing, with a smaller contribution from the paternally-inherited alleles in the fetal genome. Hence, considering the mother of a preterm delivery as proband may be more useful in identifying specific genetic contributions to PTB. Interestingly, results from the heterogeneity χ^2 test comparing race-stratified analyses suggest that genetic influences on birth timing may differ between Blacks and Whites. Hence, in future association studies, race-stratified analyses or population stratification corrections may improve our ability to identify specific genes associated with PTB. Overall, as multiple genes in the mother's genome may explain the bulk of genetic influences on birth timing, future studies to identify specific genes influencing PTB perhaps will be most fruitful by using large scale studies of mothers' genomes.

Material and Methods

Proband mother-infant pair was initially identified through premature birth of a live singleton fetus before 37 weeks of gestation [1] by review of delivery logs at university medical center hospitals at Washington University and University of Helsinki or by self-identification through the study's website from 2003 to October 2007. To avoid misclassification bias at borderline gestational ages, we defined PTB as <35 weeks in the US cohort and <36 weeks in the Finland cohort. Our gestational age criteria was less stringent in the Finland cohort due to the high number of early ultrasounds performed, leading to more accurate gestational ages in this cohort. To include only families with spontaneous onset of preterm singleton birth, the following mother-infant pairs were further excluded: elective deliveries without spontaneous onset of labor and deliveries in which either maternal (e.g. systemic infection) or fetal (e.g. malformation) disease with known predisposition to premature birth was indicated. Families were extended through affected individuals on both maternal and paternal sides until no additional first degree relatives were identified as either mother of infant of a preterm delivery. Families were recruited into the study only if two or more members were mothers and/or infants of preterm deliveries. 55 families were recruited from the US, of which 31 were Black and 24 were White. 41 White families were recruited from Finland. In the US cohort, families ranged from 9 to 36 individuals with phenotypic information with a median family size of 18 individuals. In the Finland cohort, families ranged from 8 to 73 individuals with phenotypic information with a median family size of 16 individuals. Informed consent was obtained from participants and the study was approved by the institutional review board of Washington University School of Medicine and the ethics committee of Helsinki University Central Hospital.

From the US cohort, an individual's gestational age was calculated based on his or her mother's self report of expected due dates and actual delivery dates for a pregnancy or of how many weeks early that family member was born. For all individuals born at the Washington University School of Medicine, gestational ages were verified from medical records. From the Finland cohort, an individual's gestational age was obtained from medical records. Individuals for whom pedigree information indicated that they were born <35 or <36 weeks without a specific gestational age designated were assigned a gestational age of 34 or 35 weeks, respectively. Similarly, those individuals indicated as "term" were designated 40 weeks. Infant's gestational age was treated as a quantitative trait, which was standardized to a normal distribution (μ=0, σ=1) prior to any analysis. A total of 1378 individuals had non-missing phenotypes, with a median of 13 individuals per family (range 3-80). The number of sibpairs with phenotypic information for this phenotype was 309, with a median of 3 sibpairs per family (range 1-7). The median number of generations with phenotypic information was 3 (range 1-5).

For both cohorts, a variable representing the average gestational age of all children born to a given mother was constructed. For mothers who had one or more children born before 37 weeks, this variable was calculated as the mean of the gestational ages for all children born to that woman. Mothers who gave birth to all of their children at term (>37 weeks) were assigned a value of 40 weeks. Mothers for whom one or more children had missing gestational ages were coded as missing. Additionally, all males and females who had not yet given birth were coded as missing. This phenotype was treated as a quantitative trait, which was standardized to a normal distribution (μ=0, σ=1) prior to any analysis. Univariate statistics and standardizations for each phenotype were

performed with SAS language v. 9.1.3 for Linux OS (SAS Institute, Cary, NC). A total of 404 individuals had non-missing phenotypes, with a median of 4 individuals per family (range 1-17). The number of sibpairs with phenotypic information for this phenotype was 309, with a median of 0 sibpairs per family (range 0-5). The median number of generations with phenotypic information was 2 (range 1-4).

We used the Pedigree Analysis Package (PAP), Version 5.0 [119] to perform segregation analysis. Under the mixed Mendelian model (model 1), the phenotype is influenced by a major gene, polygenic background and an untransmitted environmental component. The major gene is biallelic (A, a), with allele A, occurring at frequency p, associated with lower trait values. Mean values for the three genotypes (μ_{AA}, μ_{Aa}, μ_{aa}, where the order of the means is constrained to be $\mu_{AA} \leq \mu_{Aa} \leq \mu_{aa}$) and a common standard deviation for all genotypes are estimated. Parent-to-offspring transmission probabilities for the three genotypes (τ_{AA}, τ_{Aa}, and τ_{aa}) also are included in the model. τ_{AA}, τ_{Aa}, and τ_{aa} designate the probability of transmitting allele A for the genotypes AA, Aa, and aa, with Mendelian expectations of 1, 0.5, 0, respectively. When the τ's are set equal to p, there is no transmission of the major effect. Polygenic heritability (H) after accounting for the putative major gene effect was also estimated. For parent of origin models, heterozygotes who inherited A from their mother (Aa) and heterozygotes who inherited A from their father (aA) are distinguished from one another and allowed to have different means. Many of the free τ's models did not converge initially. In order to estimate these models, τ_{AA} and τ_{aa} were fixed to 1 and 0, respectively, and likelihoods calculated under these additional assumptions were used for the analysis.

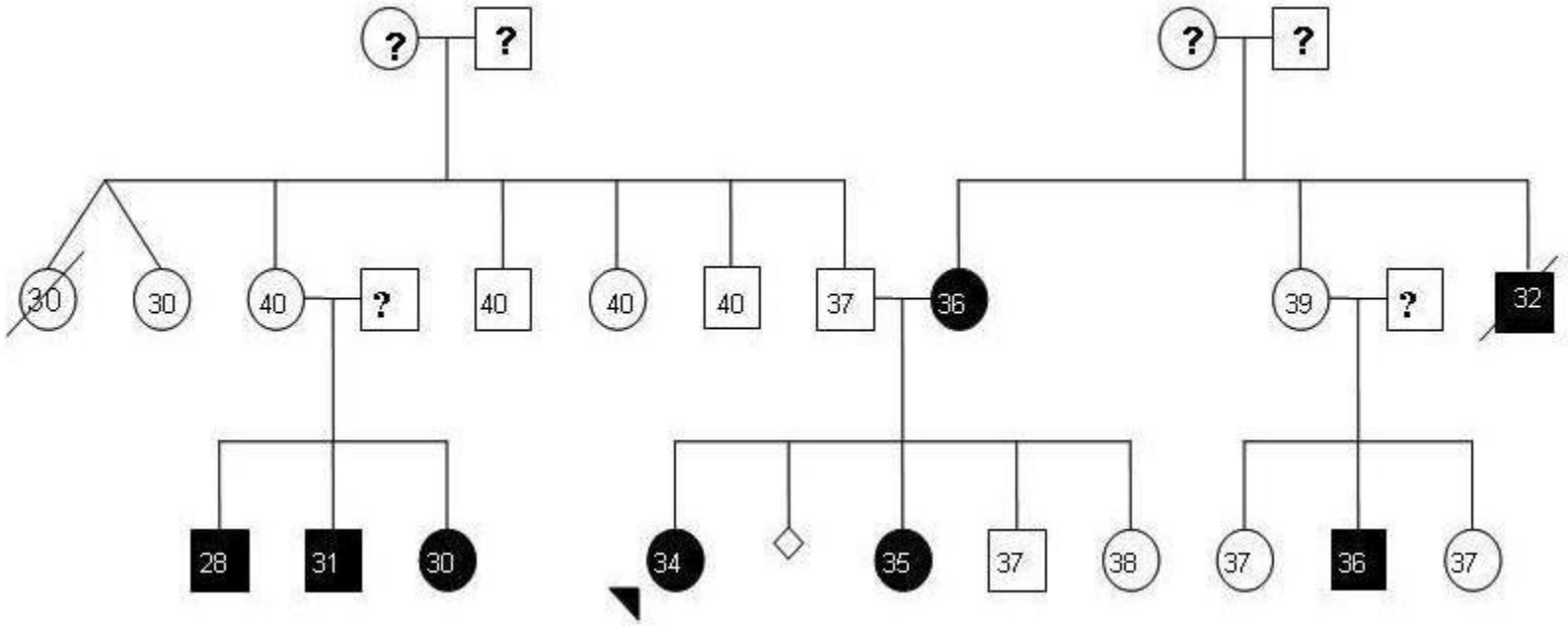
All parameters were estimated using a maximum likelihood method. Nested models representing null hypotheses were tested against a more general model using a LRT, in which the difference between negative twice the log-likelihood (-2 ln L) values for two models approximates a chi-square distribution with degrees of freedom equal to the number of independent parameter restrictions. The most parsimonious model of those not rejected by likelihood ratio test ($p>0.01$) was determined using AIC [120], which is computed as -2 ln L of the model plus twice the number of parameters estimated. The model with the lowest AIC indicates the most parsimonious fit to the observed data.

To account for the ascertainment of the families, the likelihood of each model was conditioned on the likelihood of the proband's phenotype under the model, an appropriate correction for the manner in which these families were extended [121]. While our criterion required 2 or more preterm first degree relatives for a family to be enrolled in the study, the ascertainment scheme is approximately equivalent to single ascertainment [122]. Because not all preterm deliveries in the metropolitan St. Louis or Finnish health care systems occurring during the study period were captured under our ascertainment scheme, the probability that a family was identified through multiple probands is small and should be proportional to the number of affected deliveries in a family, as expected under single ascertainment[123]. Under single ascertainment, conditioning on the proband's phenotype is sufficient to adjust for the ascertainment of families [123]. Hence, analyses for infant's gestational age were corrected for the proband infant's gestational age jointly with PTB (<37 weeks) affection status. Similarly, analyses attributing gestational age to the mother were corrected for the proband mother's average gestational age of children jointly with her PTB (<37 weeks) affection status.

To test genetic heterogeneity among races, we used the heterogeneity χ^2 test [110; 111] in which the -2lnL value under the best fitting model for the combined data is subtracted from the summation of -2lnL values for stratified analyses to obtain the test statistic. This test statistic approximates a χ^2 distribution with df equal to K*J-K where J is the number of subgroups and K is the number of parameters in the model.

Figure 3.1A: A representative pedigree of familial preterm birth with gestational age in weeks of each individual indicated.

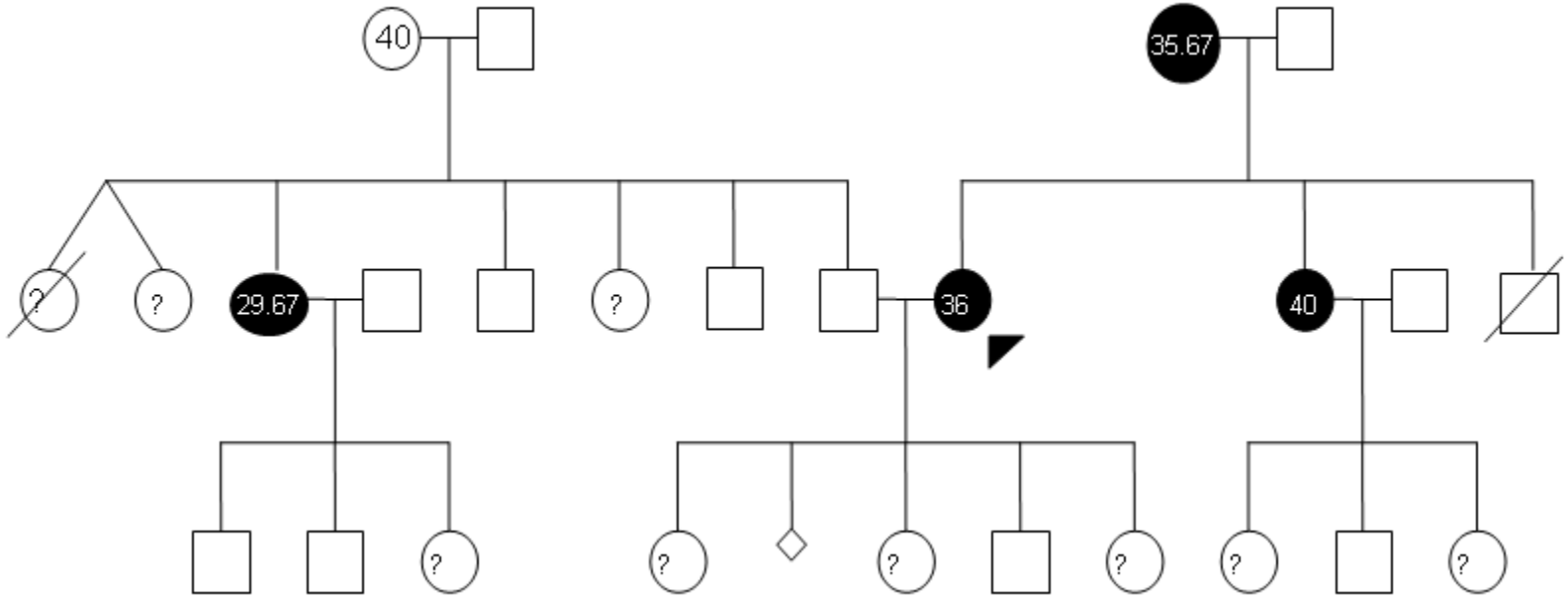
Preterm birth, infant as affected



Question marks indicate that the gestational age for that individual is unknown. Panel A depicts the pattern of affection status (<37 weeks) if the infant is considered proband of a preterm delivery. Multiple births are excluded due to the likelihood of singleton and multiple births having different mechanisms for early birth.

Figure 3.1B: A representative pedigree of familial preterm birth with gestational age in weeks of each individual indicated.

Preterm birth, mother as affected



Question marks indicate that the gestational age for that individual is unknown. Panel B depicts the pattern of affection status (<37 weeks) if the mother is considered proband of a preterm delivery. Multiple births are excluded due to the likelihood of singleton and multiple births having different mechanisms for early birth.

Table 3.1: General characteristics of the study subjects as a single cohort and stratified by race.

	Combined	Blacks	Whites
Infant's gestational age			
N (% <37 weeks)	1130 (33.63%)	301 (41.53%)	829 (30.76%)
Mean	37.12	36.12	37.49
Standard deviation	4.09	4.83	3.73
Kurtosis	1.73	0.24	2.47
Skewness	-1.45	-1.08	-1.58
Mother's average gestational age			
N (% <37 weeks)	191 (54.97%)	48 (70.83%)	143 (49.65%)
Mean	35.69	34.22	36.19
Standard deviation	4.24	4.45	4.07
Kurtosis	-0.24	-0.66	0.17
Skewness	-0.76	-0.32	-0.95

Table 3.2: Segregation analysis of infant's gestational age in 96 multiplex families (n=1224 nonfounders).

	Model	Estimated parameters	"-2lnL"	test	$\chi 2$	df	p-value	AIC
1	Mixed	6	2266.85					2278.85
2	Sporadic	2	2754.87	2 vs 1	488.02	4	$p < 0.001$	2758.87
3	Multifactorial	3	2690.72	3 vs 1	423.87	3	$p < 0.001$	2696.72
4	Additive	5	2303.33	4 vs 1	36.48	1	$p < 0.001$	2313.33
5	Recessive	4	2545.26	5 vs 1	278.41	2	$p < 0.001$	2553.26
6	Dominant	4	2305.64	6 vs 1	38.79	2	$p < 0.001$	2313.64
7	Mixed recessive	5	2544.08	7 vs 1	277.23	1	$p < 0.001$	2554.08
8	Mixed dominant	5	2270.77	8 vs 1	3.92	1	0.05	2280.77
9	Mixed free τ's	9	2100.61	1 vs 9	166.24	3	$p < 0.001$	2118.61
10	Mixed equal τ's	6	2354.73	10 vs 9	254.12	3	$p < 0.001$	2366.73
11	Recessive free τ's	8	2291.99	7 vs 11	252.09	3	$p < 0.001$	2307.99
12	Recessive equal τ's	5	2354.73	12 vs 11	62.74	3	$p < 0.001$	2364.73
13	Dominant free τ's	8	2106.20	8 vs 13	164.57	3	$p < 0.001$	2122.20
14	Dominant equal τ's	5	2354.73	14 vs 13	248.53	3	$p < 0.001$	2364.73
15	Parent of origin #	6	2078.97					2090.97
16	Maternal	4	2320.79	16 vs 15	260.83	3	$p < 0.001$	2328.79
17	Paternal	4	2356.23	17 vs 15	296.27	3	$p < 0.001$	2364.23

most parsimonious model

Table 3.3: Parameter estimates for segregation analysis of infant's gestational age in 96 multiplex families (n=1224 nonfounders).

		A allele frequency	Transmission probabilities				Mean values for genotypes				Common standard deviation	Heritability
	Model	P	τ_{AA}	τ_{Aa}	τ_{aA}	τ_{aa}	μ_{AA}	μ_{Aa}	μ_{aA}	μ_{aa}	σ	H
1	Mixed	0.27	[1]	[0.5]	[0.5]	[0]	-1.73	0.52[a]	[μ_{Aa}]	0.37[a]	0.56	0.38
2	Sporadic	[1]	[1]	[0.5]	[0.5]	[0]	0.01	[μ_{AA}]	[μ_{AA}]	[μ_{AA}]	0.92	[0]
3	Multi-factorial	[1]	[1]	[0.5]	[0.5]	[0]	0.24	[μ_{AA}]	[μ_{AA}]	[μ_{AA}]	0.91	0.33
4	Additive	0.27	[1]	[0.5]	[0.5]	[0]	-1.82	0.46[a]	[μ_{Aa}]	0.31[a]	0.56	[0]
5	Recessive	0.03	[1]	[0.5]	[0.5]	[0]	-1.55	[μ_{AA}]	[μ_{Aa}]	0.32	0.71	[0]
6	Dominant	0.26	[1]	[0.5]	[0.5]	[0]	-1.84	0.39	[μ_{Aa}]	[μ_{Aa}]	0.57	[0]
7	Mixed-recessive	0.03	[1]	[0.5]	[0.5]	[0]	-1.56	[μ_{AA}]	[μ_{Aa}]	0.33	0.71	0.05
8	Mixed-dominant	0.27	[1]	[0.5]	[0.5]	[0]	-1.75	0.45	[μ_{Aa}]	[μ_{Aa}]	0.56	0.36
9	Mixed- free τ's	0.05	1.00	0.99	[τ_{Aa}]	0.16	-1.60	0.42	[μ_{Aa}]	0.58	0.55	0.42
10	Mixed-equal τ's	0.33	[p]	[p]	[p]	[p]	-1.84	0.40	[μ_{Aa}]	0.40	0.58	0.28
11	Recessive free τ's	0.01	0.98	0.70	[τ_{Aa}]	0.04	-1.77	[μ_{AA}]	[μ_{Aa}]	0.42	0.57	0.25
12	Recessive equal τ's	0.06	[p]	[p]	[p]	[p]	-1.84	[μ_{AA}]	[μ_{Aa}]	0.40	0.58	0.28
13	Dominant free τ's	0.05	1.00	0.98	[τ_{Aa}]	0.16	-1.56	0.49	[μ_{Aa}]	[μ_{Aa}]	0.55	0.40
14	Dominant equal τ's	0.33	[p]	[p]	[p]	[p]	-1.84	0.40	[μ_{Aa}]	[μ_{Aa}]	0.58	0.28
15	Parent of origin #	0.20	[1]	[0.5]	[0.5]	[0]	-2.38	-0.69	0.60	0.58	0.37	[0]
16	Maternal	0.09	[1]	[0.5]	[0.5]	[0]	-1.71	[μ_{AA}]	0.40	[μ_{Aa}]	0.57	[0]
17	Paternal	0.12	[1]	[0.5]	[0.5]	[0]	-1.81	0.39	[μ_{AA}]	[μ_{Aa}]	0.57	[0]

Most parsimonious model. [ ] denotes that the parameter was set and not estimated.
[a] Parameter estimates are approximately equal within standard error.

Table 3.4: Segregation analysis of mother's average gestational age of children in 96 multiplex families (n=192 nonfounders).

	Model	Estimated parameters	"-2lnL"	test	$\chi 2$	df	p-value	AIC
1	Mixed	6	175.06					187.06
2	Sporadic	2	222.42	2 vs 1	47.36	4	$p < 0.001$	226.42
3	Multifactorial	3	216.37	3 vs 1	41.31	3	$p < 0.001$	222.37
4	Additive	5	181.57	4 vs 1	6.50	1	0.01	191.57
5	Recessive	4	187.88	5 vs 1	12.82	2	0.002	195.88
6	Dominant	4	182.62	6 vs 1	7.55	2	0.02	190.62
7	Mixed recessive	5	186.70	7 vs 1	11.63	1	$p < 0.001$	196.70
8	Mixed dominant	5	175.68	8 vs 1	0.62	1	0.43	185.68
9	Mixed free τ's * #	7	153.94	1 vs 9	21.13	3	$p < 0.001$	167.94
10	Mixed equal τ's	6	173.56	10 vs 9	19.62	3	$p < 0.001$	185.56
11	Recessive free τ's *	6	184.09	7 vs 11	2.60	3	0.46	196.09
12	Recessive equal τ's	5	173.56	12 vs 11	-10.54	3	N/A	183.56
13	Dominant free τ's *	6	157.15	8 vs 13	18.53	3	$p < 0.001$	169.15
14	Dominant equal τ's	5	173.56	14 vs 13	16.40	3	$p < 0.001$	183.56

most parsimonious model

* indicates the model did not converge on its own. τAA was set equal to 1 and τaa set equal to 0 in order to estimate the model.

Table 3.5: Parameter estimates for segregation analysis of mother's average gestational age of children in 96 multiplex families (n=192 nonfounders).

		A allele frequency	Transmission probabilities			Mean values for genotypes			Common standard deviation	Heritability
	Model	p	τ_{AA}	τ_{Aa}	τ_{aa}	μ_{AA}	μ_{Aa}	μ_{aa}	σ	H
1	Mixed	0.27	[1]	[0.5]	[0]	-1.02	0.89	0.99	0.45	0.58
2	Sporadic	[1]	[1]	[0.5]	[0]	0.55	[μ_{AA}]	[μ_{AA}]	0.87	[0]
3	Multifactorial	[1]	[1]	[0.5]	[0]	0.74	[μ_{AA}]	[μ_{AA}]	0.86	0.44
4	Additive	0.25	[1]	[0.5]	[0]	-1.28	0.72	0.94	0.48	[0]
5	Recessive	0.04	[1]	[0.5]	[0]	-0.94	[μ_{AA}]	0.89	0.49	[0]
6	Dominant	0.28	[1]	[0.5]	[0]	-1.08	0.87	[μ_{Aa}]	0.48	[0]
7	Mixed-recessive	0.04	[1]	[0.5]	[0]	-1.06	[μ_{AA}]	0.91	0.47	0.43
8	Mixed-dominant	0.28	[1]	[0.5]	[0]	-1.01	0.94	[μ_{Aa}]	0.45	0.59
9	Mixed free τ's * #	0.16	[1]	0.77	[0]	-1.01	0.87	1.08	0.44	0.52
10	Mixed equal τ's	0.40	[p]	[p]	[p]	-1.00	0.92	0.92	0.45	0.61
11	Recessive free τ's *	0.02	[1]	0.66	[0]	-0.85	[μ_{AA}]	0.91	0.49	0.02
12	Recessive equal τ's	0.08	[p]	[p]	[p]	-1.00	[μ_{AA}]	0.92	0.45	0.61
13	Dominant free τ's *	0.18	[1]	0.75	[0]	-0.96	0.96	[μ_{Aa}]	0.44	0.54
14	Dominant equal τ's	0.40	[p]	[p]	[p]	-1.00	0.92	[μ_{Aa}]	0.45	0.61

most parsimonious model

[ ] denotes that the parameter was set and not estimated.

* indicates the model did not converge on its own. τAA was set equal to 1 and τaa set equal to 0 in order to estimate the model.

Chapter 4: Considering rapidly evolving genes facilitates discovery of novel gene for PTB: follicle-stuimulating hormone receptor, *FSHR* [§]

Abstract

The signals initiating parturition in humans have remained elusive, due to divergence in physiological mechanisms between humans and model organisms typically studied. Because of relatively large human head size and narrow birth canal cross-sectional area compared to other primates, we hypothesized that genes involved in parturition have evolved rapidly along the human and/or higher primate phylogenetic lineages to decrease the length of gestation and alleviate complications arising from these constraints. Consistent with our hypothesis, many genes involved in reproduction show rapid evolution in their coding or adjacent noncoding regions. We screened >8,000 SNPs in 150 rapidly evolving genes in 165 Finnish preterm and 163 control mothers for association with PTB. A linkage disequilibrium block of SNPs in *FSHR*, rs11686474, rs11680730 and rs12473870, showed significant association across ethnically diverse populations. By considering rapid evolution, we identified a novel gene associated with PTB, *FSHR*. We anticipate other rapidly evolving genes will similarly be associated with PTB risk and elucidate essential pathways for human parturition.

Introduction

Because humans are born developmentally less mature than other mammals [124; 125], birth timing mechanisms may differ between humans and model organisms that have been typically studied [126]. Evidence suggests that parturition has changed along the human lineage in response to other uniquely human adaptations. The dramatic

[§] This chapter is adapted from: Plunkett J, et al. Evolution History of *FSHR* in Humans Predicts Role in Birth Timing. Submitted.

increase in brain size, along with the human pelvis becoming narrower to facilitate bipedalism, places unique constraints on birth in humans compared even with evolutionarily close relatives such as Neanderthals and chimpanzees [127; 128]. Given the historically high mortality rate associated with pregnancy, these human adaptations are likely to have generated a strong selective pressure to initiate parturition at a relatively earlier time in gestation compared to non-human primates to avoid cephalopelvic disproportion and arrested labor. High rates of human versus non-human primate divergence in human pregnancy-related genes, such as genes in the reproduction Gene Ontology (GO) category [129; 130] as well as GO categories related to fetal development, including transcription factors [131], nuclear hormone receptors [131], transcriptional regulation [132] and development [130], support the notion that human gestation length has been shortened to accommodate features unique to human pregnancy. As a result, the set of genes rapidly evolving on the human lineage likely includes genes that play important roles in regulating parturition and potentially influence PTB risk.

Genetic influences on birth timing in humans appear to be substantial; however, association studies using candidates selected from suspected pathways have not detected robust susceptibility variants for PTB. Genome-wide association studies (GWAS) are promising but will require large numbers of well-characterized subjects in order to overcome the challenge of multiple statistical comparisons. To address these limitations, we applied an evolutionarily-motivated filter to examine genes showing marked divergence between humans and other mammals, defined by relative nucleotide substitution rates in coding and highly conserved noncoding regions, for association with

PTB. Here we show that genes with the signature of rapid evolution in humans provide an informative group of candidates, and demonstrate as proof of concept that one rapidly evolving gene, follicle-stimulating hormone receptor (*FSHR*), has significant association with PTB.

Results and Discussion

Life history traits. Because of large human head size and narrow birth canal cross-section compared to other primates [127], we hypothesized that genes involved in parturition have evolved rapidly along the human phylogenetic lineage to decrease the length of gestation and alleviate the complications arising from these constraints. We performed a comparative analysis of life history traits in mammals to further evaluate whether the relative gestational period in humans has decreased compared to other primates and mammals. Data acquired by Sacher and Staffeldt [133] and reanalyzed by us show that both adult and neonatal primates have higher brain to body weight ratios compared to other mammals (Figure 4.1A, B). The decoupling of brain/body size ratios in primates makes it possible to ask whether gestation in primates is linked to brain size or body size. Primates and other mammals have equivalent gestational periods with respect to brain weight (Figure 4.1C). In contrast, the gestational period in primates is longer relative to the length of gestation in mammals with equivalent neonatal body weights (Figure 4.1D). This suggests that the length of gestation is expected to change with brain size but not body size.

Humans have evolved the highest adult brain to body weight ratio of any mammal [29]. In contrast to the evolution of brain/body ratios along the lineage leading to primates, the increase in the brain/body ratio along the lineage leading to humans is

present in adults but not neonates (Figure 4.1B). The simplest explanation is that adult brain/body ratios have changed independently of neonatal ratios. However, the ratio of brain/body weight is highest at birth and declines until adulthood. Thus, an alternative explanation is that both adult and neonatal brain/body ratios have increased in humans, as in primates, but that a concurrent decrease in the length of gestation lowered the neonatal brain/body ratio. This second possibility is supported by the relative immaturity of human neonates compared to other primates [124; 125] and that the length of human gestation, relative to either neonatal brain or body weight, is shorter than most other primates (Figure 4.1C,D).

To examine the evolution of gestation length relative to neonatal brain and body weight in primates, we inferred the evolution of these characters across a phylogenetic tree. For both gestation-neonatal body ratio (Figure 4.2A) and gestation-neonatal brain ratio (Figure 4.2B) there is a consistent trend of a relatively shorter length of gestation on branches leading to humans. Of note, human has the lowest gestation-neonatal body ratio (Figure 4.2A) or gestation-neonatal brain ratio (Figure 4.2B) of all the 20 primates. The gestation-neonatal brain ratio for humans is 69% that of gorilla and 45% that of chimpanzee. The gestation-neonatal body ratio of human is 49% that of gorilla and 50% that of chimpanzee.

Signature of rapid evolution. In light of this evidence for human adaptation for birth timing, we examined whether genes involved in parturition would display a signature of positive selection, an increased rate of amino acid altering to synonymous nucleotide substitutions (dN/dS; Figure 4.3). We found that, of 120 suggested candidate genes for PTB [134] that were included in ENSEMBL database, 7 showed statistically

significant increased rate acceleration (i.e. increased dN/dS; $p<0.05$) along the human lineage in comparison to the other lineages. Table 4.1 shows these 7 genes plus 2 accelerated human genes significantly rapidly evolving along the human-chimpanzee ancestor lineage (complete analysis of dN/dS reported in [135; 136]). Of these, common variants of *PGR* [137] and *MMP8* [138] have previously been found to contribute to PTB risk. Of note, this candidate gene list derived from the Institute of Medicine report on PTB [134], and information from the Gene Cards and Online Mendelian Inheritance in Man databases was relatively enriched for genes rapidly evolving along the human lineage compared to a normal distribution (3/120 expected; $p=0.02$) and exhibited a similar trend for enrichment in comparison to other human genes in the ENSEMBL database (408/10,440 observed with $p<0.05$; $p=0.14$ vs. suggested PTB candidates). Using criterion agnostic to possible involvement with PTB, and measuring genome-wide changes, we identified 175 genes either rapidly evolving along the human (40 genes) or on the human and human-chimpanzee ancestor lineages combined (135 genes) at a 5% false discovery rate (FDR) [139] from this analysis of protein-coding sequences.

Motivated by this evidence of protein coding region evolution for genes involved in parturition and that positive selection has also been found to act on noncoding regions, we developed a method to identify rapidly evolving noncoding sequences [132; 140]. We identified a total of 401 elements significant along the human lineage and 2103 elements significant along the human and human-chimpanzee ancestor lineages at a 5% FDR. To choose candidate genes, we calculated gene-wise p-values for each gene locus by assigning each conserved element to its nearest RefSeq gene [141] and a Fisher's combined p-value across the locus. This resulted in identification of a total of 279

candidate genes (complete analysis of rapidly evolving non-coding regions reported in [135; 136]). 150 of the genes identified as rapidly evolving in the protein-coding sequence and highly conserved noncoding elements screens, selected based on expression and functional information suggesting potential roles in parturition, were analyzed for association with PTB (Table 4.2).

Association analysis of rapidly evolving genes. Because recent data suggests that heritability of risk of PTB acts largely or exclusively through the maternal genome [29; 30; 32] and the Finnish have low environmental risk and high genetic homogeneity compared to other populations, we genotyped Finnish (165 preterm, 163 control) mothers for 8,490 SNPs in the gene regions of our prioritized list of 150 rapidly evolving genes (Figure 4.4). The most significant finding was rs6741370 ($p=9.28 \times 10^{-5}$) in the follicle-stimulating hormone (FSH) receptor gene (*FSHR*). 91 SNPs were significant at the $p<0.01$ level by allelic tests (Table 4.3). However, no SNPs were significant after correcting for 6,042 independent tests, considering relationships among markers, by the Bonferroni method ($p<8.27 \times 10^{-6}$). Of note, 8 of the 10 most statistically significant SNPs were located in *FSHR*. We identified *FSHR* as rapidly evolving in the noncoding analysis, with 40 changes in 4,218 bp of 17 conserved elements (human lineage $p = 5.4 \times 10^{-5}$). Moreover, *FSHR* was revealed as rapidly evolving in a study of noncoding conserved elements by Prabhakar and colleagues [140], which otherwise had limited overlap with our gene list (see Methods). *FSHR* also harbors SNPs with extreme iHS values in the Yoruban population, reflecting extended haplotype homozygosity and suggesting a recent selective sweep [142]. Bird and colleagues [143] identified a region less than 1 megabase downstream of the *FSHR* gene boundaries as rapidly evolving in

their study, further supporting rapid evolution of the locus. This information, together with the known importance of variation in human *FSHR* in subfertility [144; 145], a risk factor for preterm delivery independent of the use of assisted reproductive technologies [146; 147], and evidence suggesting its expression in uterus and cervix [148-150], motivated its specific study.

16 SNPs in *FSHR* showing potential association in the screening analysis ($p<0.1$) were genotyped in US White (147 preterm, 157 control), US Black (79 preterm, 164 controls) and US Hispanic (73 preterm, 292 control) mothers (Figure 4.5; Table 4.4). Several SNPs exhibited suggestive association ($p<0.01$) with PTB risk. One SNP in the US Blacks, rs12473815, was significant after correcting multiple testing (13 independent tests; $p<0.004$). The SNP rs12473815 is in high linkage disequilibrium (LD) with three nearby SNPs, rs11686474, rs11680730 and rs12473870 (Figure 4.5), all of which showed evidence of association across populations. Meta-analysis of these SNPs resulted in odds ratios ranging from 1.37 to 1.41 with a common 95% confidence interval of 1.26-1.50. Of note, when combining data from all populations tested by meta-analysis, p-values for rs11686474 ($p=0.0006$) and rs11680730 ($p=0.002$) are significant after a Bonferroni correction ($p<0.003$).

In *FSHR*, 4 SNPs in high LD show evidence of association with PTB across the populations studied. These SNPs lie within intron 2 of *FSHR* (Figure 4.5) and show little LD with variants outside of this intron, based on available information from the International HapMap Project database [151]. Variants in this intron may tag yet uncharacterized variants in coding regions or nearby regulatory sequences. Alternatively, an intronic variant in *FSHR* may affect risk directly by altering functional sequences

contained within the intron, such as microRNA binding sites, splice regulatory sites or transcription regulation sites. For instance, a variant in a splice enhancer site may change splicing patterns in favor of transcripts that promote PTB risk, as several alternatively spliced FSHR isoforms have been observed with altered function [152]. Risk-promoting variation in this gene likely contributes to birth timing, rather than size at birth, based on additional tests examining gestational age or birth-weight Z-score as a quantitative trait, rather than PTB affection status (Table 4.5). Hence, *FSHR* may represent a novel gene involved in birth timing and PTB risk.

FSHR encodes the follicle-stimulating hormone (FSH) receptor. FSH is secreted from the pituitary and, in females, acts primarily on receptors in the ovaries to stimulate follicle development and synthesis of estrogens. Investigators also have observed FSHR protein and mRNA expression in nongonadal tissues, including uterus and cervix [148-150]. In these tissues, FSHR may mediate uterine relaxation, as suggested by FSH's ability to modify electrical signaling in the myometrium, independent of estrogen and progesterone [148]. Padmanabhan and colleagues [153] noted a progressive rise in bioactive serum FSH levels during pregnancy. Because high levels of FSH are known to downregulate FSHR expression [154], increasing levels of FSH may lead to gradual desensitization to the hormone and resultant increase in contractility as term approaches. Additionally, evidence from the *FSHR* haploinsufficient mouse [155] suggests that FSHR levels affect the relative abundance of progesterone receptor isoforms A (PR-A) and B (PR-B). Increased PR-A: PR-B ratios, occurring in human pregnancy normally near term and observed in *FSHR* haploinsufficient mice in non-pregnant states, are correlated with

increased myometrium contractility. Hence, dysregulation of *FSHR* may contribute to early uterine contractility and promote PTB.

Aspects of our approach pose limitations on interpretation of this work. First, we assigned conserved elements to the nearest RefSeq gene to calculate gene-wise p-values; however, conserved elements may not be associated with the nearest gene *per se*, potentially affecting the accuracy of the estimated gene-wise p-values. Additionally, because we use adjacent genes to estimate expected synonymous and nonsynonymous rates for a given locus, rapidly evolving genes that are located physically nearby other genes undergoing rapid evolution, such as gene families with multiple members in the same region, may miss detection. The variability in number of probes represented on the Affymetrix Genome-Wide Human SNP Array 6.0 within the gene regions of the 150 rapidly evolving genes tested poses another limitation. Although the coverage is adequate for most rapidly evolving genes, there are some genes with too few probes tested to support or refute their potential association with PTB; as a result, this study may have failed to detect association between PTB and rapidly evolving genes underrepresented on this genotyping array. Lastly, while precedence exists for intronic variants affecting protein structure and function [156; 157], additional study is needed to determine whether any of the SNPs associated with PTB in this work has a functional effect.

Overall, *FSHR* represents a likely candidate for involvement in PTB. Even though *FSHR* has a plausible role in parturition physiology, it has not been considered in previous association studies or appeared on candidate gene lists like that in the Institute of Medicine report on PTB [134]. Thus, by considering rapid evolution, we identified a gene associated with PTB that otherwise would not have been revealed by current models

of parturition physiology. Moreover, our approach exploits an evolutionarily-motivated filter to more efficiently utilize currently available datasets for PTB, which are probably underpowered to detect variants of effect sizes reported in GWAS of other complex traits. Our approach represents an alternative method for *a priori* gene discovery in which fewer comparisons are made than in GWAS, thus potentially retaining more power to detect effect sizes typical for common variants. We anticipate that other rapidly evolving genes will similarly be associated with PTB risk and elucidate the essential pathways for human parturition.

Materials and Methods

Allometric Analysis. Data acquired by Sacher and Staffeldt [133] was used to examine the relationships among brain size, body size and gestation length among mammalian species. Specifically, we compared logarithm-transformed values for these traits between human, primate and non-primate mammals, using linear regression implemented in R [158]. Additionally, we used allometric data from this paper and the primate phylogeny delineated by Purvis [159] to trace the evolution of gestation-neonatal body size ratio, and gestation-neonatal brain size ratio, using Mesquite [160]. Given a phylogenic tree, the Mesquite method uses parsimony to reconstruct the ancestral states by assuming a squared change for a continuous character from state x to state y is $(x-y)^2$.

Coding sequence multiple sequence alignments. We obtained a set of 10,639 human gene predictions from the ENSEMBL database with one-to-one orthologs in the chimpanzee, macaque, mouse, rat, dog, and cow genomes (Release 46) [161]. We limited our analysis to only those proteins where the human, chimpanzee, macaque, and at least 75% of the mammalian genomes were present. To prevent spurious results arising from

comparing different isoforms from different species, we compared all of the human gene models against all of the chimpanzee gene models by BLAST searches, keeping the pair with the highest percent identity (and longest gene model in case of tie). We then compared this human gene model against all of the models from the other species, finding the best match among the gene models in the other species. We generated a multiple sequence alignment using the MUSCLE algorithm [162] and reverse translated these alignments to generate nucleotide alignments. We limited our analysis to only those proteins where the human, chimpanzee, macaque, and at least 75% of the mammalian genomes were present. Chi-squared analysis was used to determine the statistical significance of observed and expected genes with $p<0.05$ in suggested PTB candidate and overall human gene lists.

Noncoding sequence multiple sequence alignments. We obtained a set of highly conserved elements from UCSC Genome Browser [163]. In total, 443,061 noncoding sequences with a conservation score >=400 were tested. Of these elements, 34% overlapped coding sequence by at least one nucleotide and were excluded from the analysis. The remaining noncoding elements span 47 MB (approximately 1.5% of the genome). Therefore, these sequences represent only the most highly conserved noncoding sequences and not the entire 6% of the noncoding genome that is functionally constrained [164]. The median total branch length for these elements was 0.235, which is 1/4 the synonymous rate. Therefore, these are not perfectly conserved sequences, but they are evolving substantially slower than the neutral expectation. From the 17−way MultiZ alignments that are publicly available (downloaded March 2007) [165], we extracted the human, chimpanzee, macaque, mouse, rat, dog and cow sequences. We filtered this

alignment set using two criteria. First, any alignment that contained 2 or more human homoplasies (the human nucleotide was equal to a conserved nucleotide in the mammalian outgroups, but different from the nucleotide shared between macaque and chimpanzee) were removed. Second, if the human sequence had a paralog with a percent difference less than twice the percent difference of the human−chimpanzee orthologs, then that sequence was excluded (e.g. if the human−chimpanzee sequences were 98% identical, the human paralog had to be less than 96% identical). This filter reduced the chance that a rapidly evolving human sequence was actually a misaligned paralog.

Likelihood ratio tests. We used the phylogeny ((Human, Chimpanzee), Macaque), ((Mouse, Rat), (Dog, Cow))). The evolutionary models were implemented in the HYPHY package [166] and we used the Q−value software [139] to establish statistical thresholds to achieve 5% false discovery rates (p-value distributions and pi_0 values in Figure 4.6). HYPHY creates a molecular evolution programming language, enabling comparison of multiple evolutionary models and phylogenies. The source code and documentation of the HYPHY tests is available from the Fay Laboratory.

For both tests, the alternative model has one additional degree of freedom and the significance of the change in likelihood was determined using χ^2 statistics. Both models use adjacent coding or conserved noncoding sequences to estimate the expectation for a given sequence that accounts for variable mutation rates across the genome as well as lineage-specific differences in effective population size by allowing for branch-specific differences in selective constraint. For the coding sequence, we used 20 adjacent genes, ten upstream and downstream when possible, to estimate the expected synonymous rate and average constraints on each lineage. Twenty genes were used because the

synonymous rate does vary across the genome in windows of approximately 10 MB [167]. For noncoding sequences, we concatenated blocks of 25 kb of conserved noncoding sequence. These blocks typically spanned about 1 MB of the genome. Each element in the middle 50% of the window was tested against the expectation for that window to limit edge effect. The window was then advanced to the element at the 50% percentile of the window.

Previous studies of both coding [9, 46] and noncoding [11, 21] sequences identify regions evolving under positive selection by a rate of evolution faster than a neutral rate. However, we felt that this criterion is too restrictive since some genes may have an increased rate of evolution along the human lineage relative to other mammals, but not increased above the neutral rate. To include genes with a significantly increased rate in humans compared to other mammals for testing in a population association study, we identify genes as rapidly evolving by testing whether omega along the human (or human+human-chimpanzee ancestor) lineage is significantly higher than omega along the non-human lineages (or non-human+non-human-chimpanzee ancestor). Here, omega is dN/dS-adj or dNC/dNC-adj, where dNC is the noncoding rate and dS-adj and dNC-adj are the adjacent synonymous rates from the 10 upstream and 10 downstream genes and the adjacent noncoding rates from 25 kb of conserved noncoding sequences, respectively. Thus, we test whether the data is more likely under a model with 1 omega value or 2 omega values (Figure 4.3). The coding sequence model used the MG94xHKY85 [168] model of codon evolution. The noncoding sequences model used an HKY85 model. We calculated gene-wise p-values for each gene locus by assigning each conserved element to its nearest RefSeq gene [141] and a Fisher's combined p-value across the locus. Chi-

squared analysis was used to determine the statistical significance of observed and expected genes with $p<0.05$ in suggested PTB candidate and overall human gene lists.

Comparisons with published studies of rapid evolution. The following data was extracted from published studies for comparison with results from our coding analysis: genes with $p<0.01$ for test of $dN/dS>1$ from Nielson et al. [46]; genes with $p<0.01$ for test of $dN>dS$ on human lineage Clark et al. [9]; genes with adjusted $p<0.05$ for test of 1 omega versus 2 omegas (i.e. 1 on human lineage, 1 for other species) Arbiza et al [48]. For comparison with results from our noncoding analysis, we compared closest genes to human accelerated regions listed in Table S7 of Pollard et al. [11] and closest genes to regions in listed in Table S1 of Prabhakar et al [21]. Ensembl gene identification numbers and/or HUGO Gene Nomenclature Committee (HGNC) gene symbols, as available, were compared among studies to determine the degree of overlap. The Venny [169] online tool was used to visually represent the degree of overlap among studies as Venn diagrams (Figures 4.7 and 4.8).

Our list of rapidly evolving coding region gene list showed low overlap with previous studies that required for $dN/dS > 1$ in their analyses (6% with Clark et al. [130], 0% Nielson et al. [170]) and more overlap with Arbiza et al. [171] (26%), which considered rate acceleration on the human lineage by methods more similar to ours than those used by [130; 170] (Figure 4.7). For rapidly evolving conserved noncoding elements in humans, 22% of the elements we identified were in common with Prabhakar et al. [140]. Considering unique genes associated with rapidly evolving conserved noncoding elements in humans, 11% of our genes also were identified by Prabhakar et al.

[140], and 4% identified by Pollard et al. [132]. Similar to our study, 4% of unique genes in the Prabhakar study overlapped with those identified by Pollard et al. (Figure 4.8).

Candidate rapidly evolving gene list. To minimize the number of tests we would perform and thereby retain more power to detect small effects, we selected a subset of genes likely to be involved in parturition, based on expression and functional information, to use as candidate genes. A candidate gene list was developed using genes identified as rapidly evolving from following categories: 10% FDR human lineage from coding screen, 10% FDR human lineage from noncoding screen and 5% FDR human-chimpanzee lineage from coding screen. A total of 742 genes are included in this comprehensive list of rapidly evolving genes. To minimize the number of tests we would perform and thereby retain more power to detect small effects, we selected a subset of genes likely to be involved in parturition, based on expression and functional information, to use as candidate genes. Genes were included as candidates if at least 2 of 3 conditions met: had a GO term suggesting possible biological role in parturition (e.g. extracellular matrix, calcium ion, DNA-binding/transcription, intracellular signaling, cell fate/apoptosis, cell growth); were previously identified as candidate gene; had expression included relevant tissues (e.g. uterus, placenta, brain) documented in Unigene [172]. Duplicated genes from a list developed by Bailey and colleagues [173] that were identified as pregnancy, fetal, placental or hormone-related genes were also included as candidates. A total of 150 of genes were used as candidate genes in subsequent analysis (Table 4.2).

Human Subjects. Mothers of preterm or term infants were enrolled for genetic analysis by methods approved by Institutional Review Boards/Ethics Committees at each

participating institution. Informed consent was obtained for all participants. Mothers with PTB were included if the birth was spontaneous (non-iatrogenic), singleton, had no obvious precipitating stimulus (trauma, infection, drug use), and was less the 37 weeks (Yale University; New York University) or 36 weeks (University of Helsinki; University of Oulu; Centennial Hospital, Nashville, TN) of completed gestation. DNA from blood or saliva was prepared by standard methods. Race/ethnicity was assigned by self-report. For the US Black cohort, no differences in were found in the distribution of 24 ancestry informative markers selected across the genome comparing cases and controls (data not shown). All specimens were linked with demographic and medical data abstracted from maternal/neonatal records.

Genotyping. Initial genotyping of the Finnish cohort was performed using the Affymetrix® Genome-Wide Human SNP Array 6.0. Genotypes were called from cell intensity data by the birdseed v2 algorithm, implemented in Affymetrix® Genotyping Console 3.0. Of 428 SNP 6.0 arrays available for analysis, only 392 samples had their raw intensity data converted to genotype calls using the birdseed v2 algorithm in Affymetrix Genotype Console 3.0, after poor quality chips (i.e. "out of bounds" designation or XY gender call by Genotyping Console) were excluded. We selected SNPs within the gene regions, defined as 5 kilobases (kb) upstream to 5 kb downstream of the most inclusive gene boundaries between those listed for the longest transcript documented in the Ensembl database and those defined in our comparative genomic analysis, of our 171 rapidly evolving candidate genes for analysis. A total of 12,444 SNPs were located within our rapidly evolving genes. The gene coverage ranged from 0-900 SNPs/gene region, with a median of 13 SNPs/gene region. 11 genes had no SNPs in

the gene region represented on the SNP 6.0 array. Genotyping for additional analysis of the Finnish cohort and replication analysis was performed using the Sequenom iPLEX massARRAY technology (Sequenom, San Diego, CA).

Finnish cohort analysis. Data cleaning was performed with the Whole-genome Association Study Pipeline (WASP) software package [174] and PLINK [175]. An additional 6 individuals were excluded because of possible cryptic relatedness, as suggested by their presence in IBS distance-defined clusters far from the rest of the genotyped subjects. 58 individuals were removed due to a high genotype missing rate for the SNPs of interest (i.e. <95% call rate), leaving a total of 165 preterm and 163 control mothers in the final analysis. Of 12,444 SNPs selected, 9,610 SNPs were used in the final analysis after removing SNPs not in Hardy-Weinberg Equilibrium in controls ($p<0.001$), <95% genotype call rate, with minor allele frequency<0.05 or were duplicate probes. Allelic (χ^2, df 1) and genotypic (χ^2, df 2) tests for association, LD measures and odds ratios were estimated for each cohort using WASP and/or PLINK. Our primary analysis considered PTB affection status (i.e. delivery <36 weeks) as a binary trait, comparing allele and genotype frequencies between case and control groups by χ^2 test. We also examined gestational age and birth-weight Z-score as quantitative traits, standardized to normal distributions ($\mu=0$, $\sigma=1$) using a Wald test to compare the mean phenotype between different allele or genotype classes.

A variety of measures were taken to ensure that results from these test were explained by true associations. First, genomic control measures of population substructure ($\lambda=1.07$) indicated little inflation of statistics due to substructure. Correction for IBS clustering to bring λ to 1 resulted in the same SNPs being found as most

significantly associated, suggesting that whatever minor population substructure may exist does not explain the association findings. Confounding of the results due to genotyping batch or location effects was not observed. Furthermore, minor allele frequencies observed in controls were generally consistent with HapMap estimates for Caucasians. As some SNPs are expected to show significant association with PTB by chance due to the large number of tests we are performing, we corrected for multiple testing using the simpleM method [176], which estimates the number of independent tests, given the LD relationships among SNPs, used to adjust the significance level.

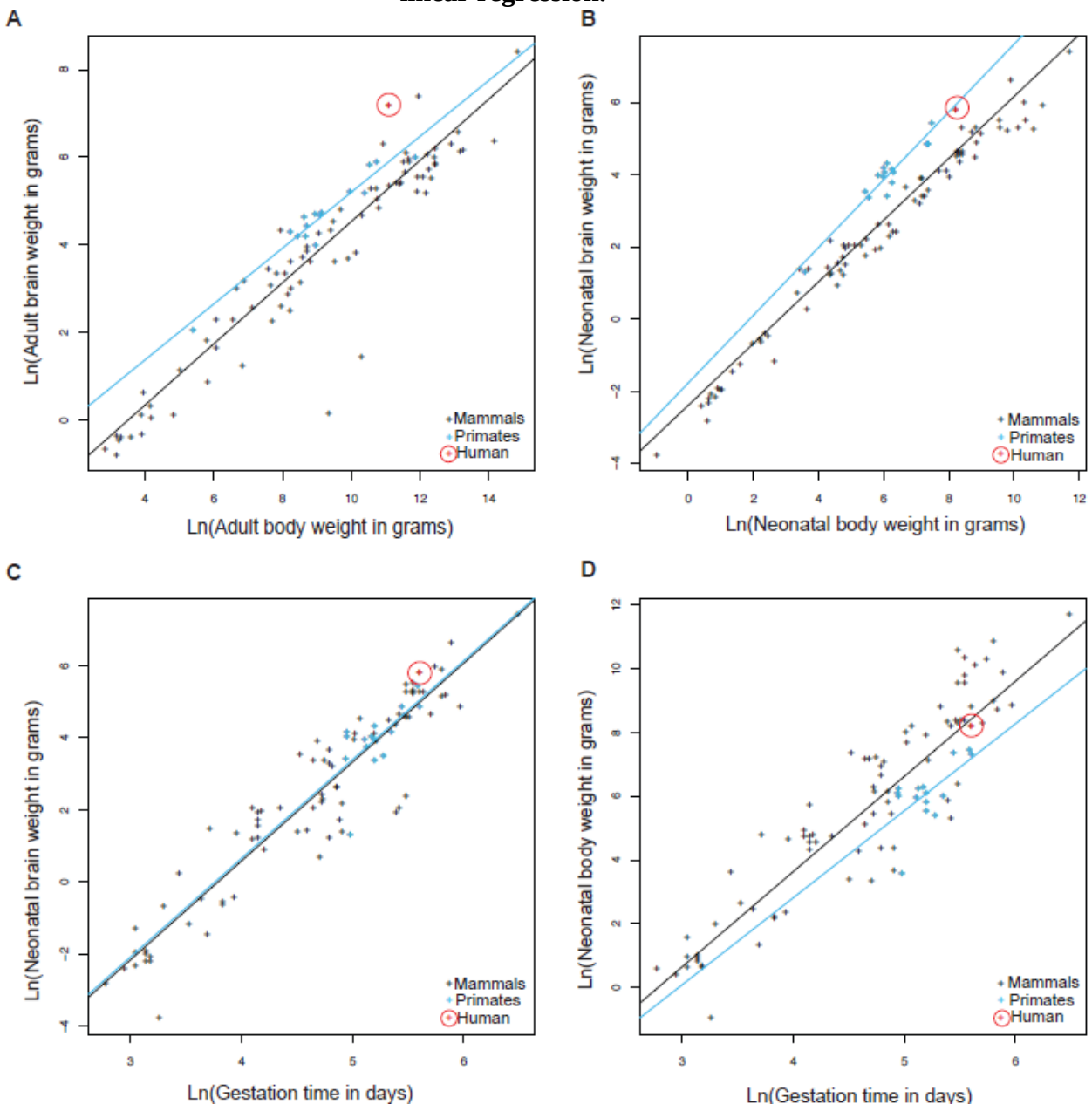
Extension of genotyping in Finnish cohort. Because *FSHR* showed evidence of enrichment of significant p-values as well as representing a plausible agent in parturition, we chose to examine the genes in greater depth. Of the 149 SNPs tested in *FSHR* in the Affymetrix analysis, 22 showed evidence of association ($p<0.01$) in the SNP 6.0 analysis and 9 were genotyped with the Sequenom technology for cross-platform validation. SNP genotypes showed high degree of concordance across platforms (~98%) and association results were consistent.

To increase coverage, we genotyped an additional 42 SNPs spanning the *FSHR* gene region in a subset of the Finnish cohort (n= 105 preterm, 95 control mothers) based on DNA availability and quality. For SNP selection, data from the HapMap CEU population was examined in the Haploview program [177], using tagger and haplotype block functions, to identify regions of high LD. We selected 1 SNP per haplotype block, defined using the D' confidence interval method [178], having the highest minor allele frequency (MAF) in the CEU population for genotyping. We also included coding SNPs and SNPs to improve coverage of conserved elements contributing to the gene's

designation as "rapidly evolving." This selection scheme resulted in approximately 20-30% coverage of the gene region at $r^2 \geq 0.8$. Data cleaning and analysis was conducted as described above. In total, 40 SNPs met quality control standards (Hardy-Weinberg Equilibrium in controls $p>0.001$, >95% call rate, MAF>0.01) and were analyzed. Of the SNPs genotyped to increase coverage, those that showed suggestive association ($p<0.1$; n=16) were examined further.

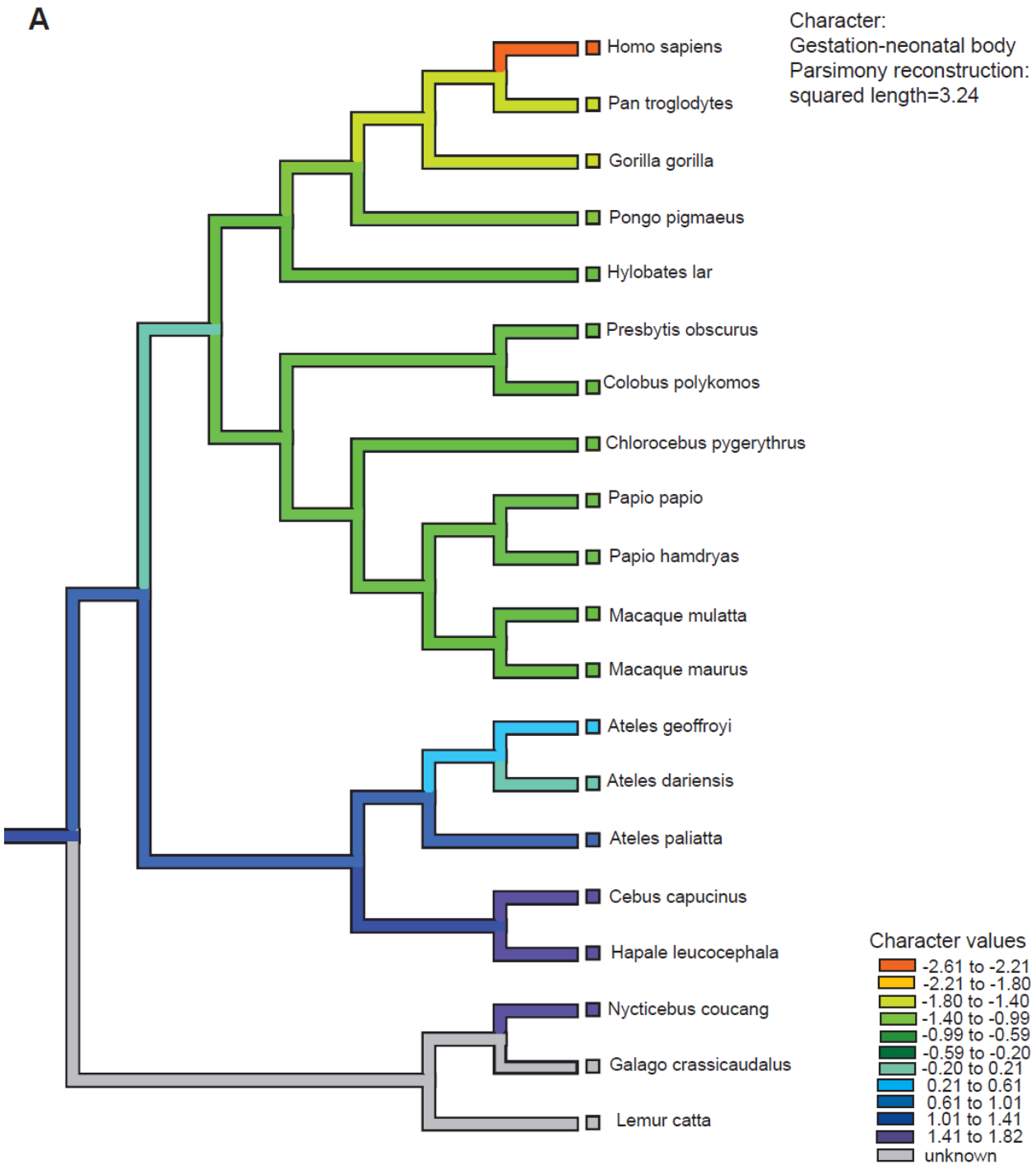
Replication Analysis. 16 SNPs in *FSHR* showing potential association in the screening analysis ($p<0.1$) were genotyped in US White (147 preterm, 157 control), US Black (79 preterm, 164 controls) and US Hispanic (73 preterm, 292 control) mothers (Figure 4.4; Table 4.4). Data cleaning and analysis was performed as described above. Meta-analysis of data for significant SNPs was done using the Mantel-Haenszel method, after successfully passing the test of homogeneity.

Figure 4.1. Allometric analysis of brain size, body size, and gestational length by linear regression.



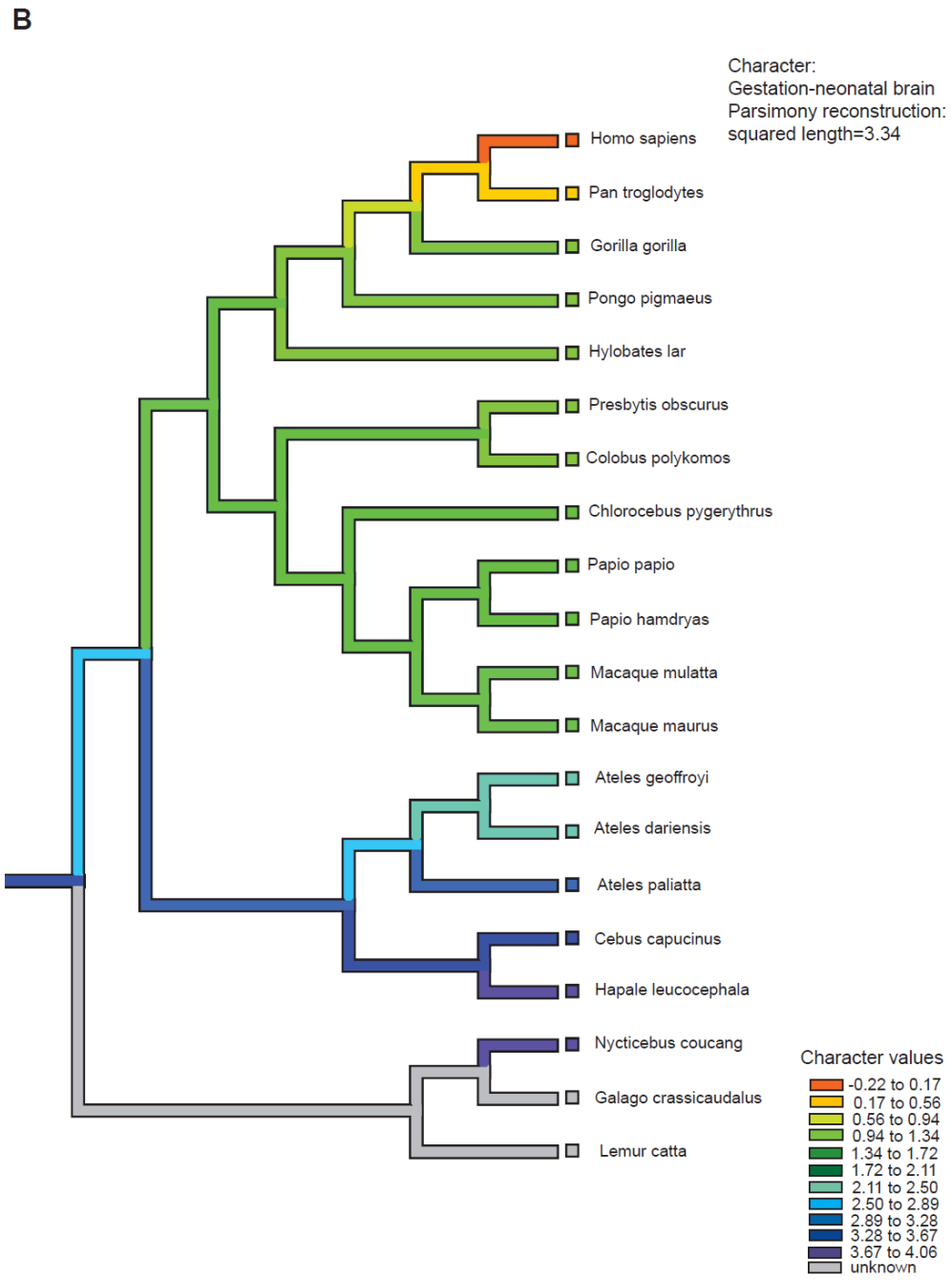
Brain to body weight ratios for adults (A) and neonates (B) are shown for humans (red), other primates (blue), and other mammals (black). The black line shows least squares fits to the 91 mammalian species. Neonatal brain (C) and body size (D) to gestational time ratios are displayed for the same species. The blue line shows least squares fits to 15 primate species. Allometric data was acquired by Sacher and Staffeldt (1974).

Figure 4.2A. Phylogenetic analysis of brain size, body size, and gestational length in primates.



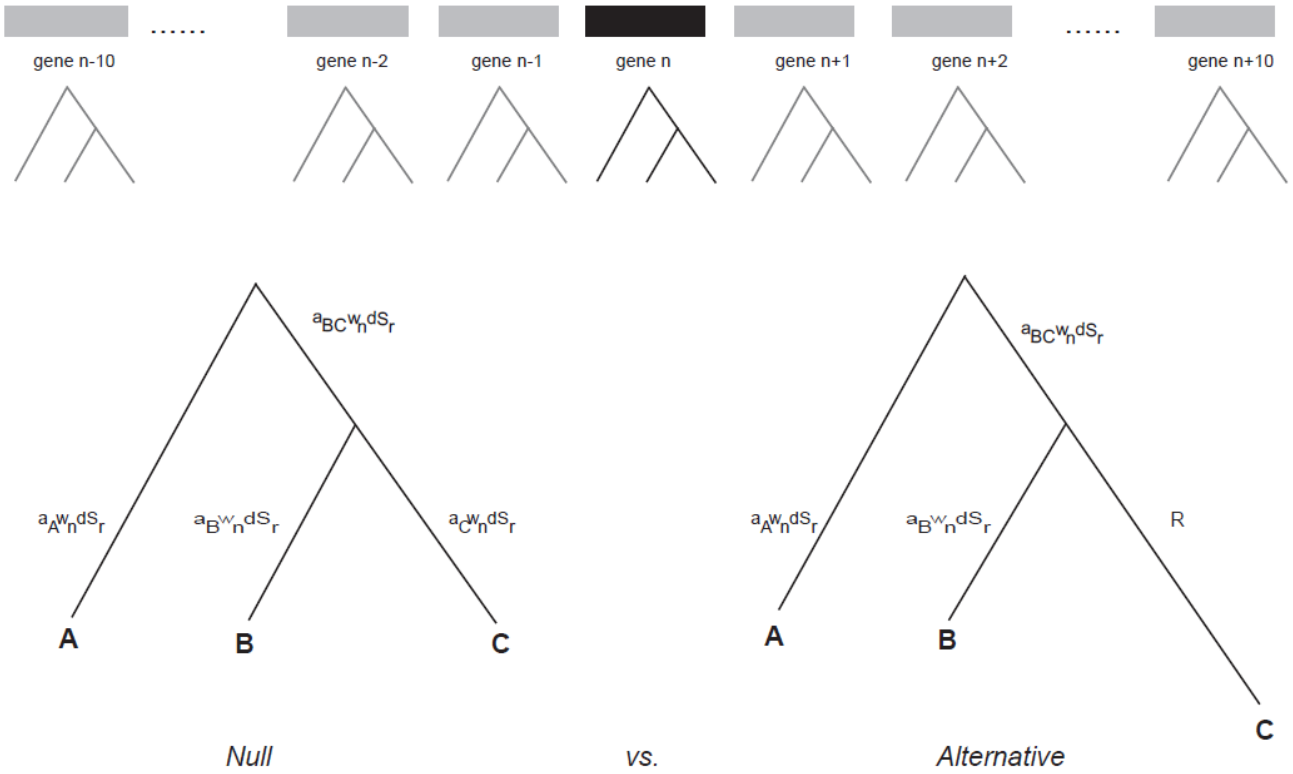
Gestational time to neonatal brain size natural logarithm-transformed ratios are shown for each species and color coded along each lineage as inferred by parsimony. Allometric data was acquired by Sacher and Staffeldt (1974) and phylogeny by Purvis (1995)

Figure 4.2B. Phylogenetic analysis of brain size, body size, and gestational length in primates.



Gestational time to neonatal body size natural logarithm-transformed ratios are shown for each species and color coded along each lineage as inferred by parsimony. Allometric data was acquired by Sacher and Staffeldt (1974) and phylogeny by Purvis (1995) .

Figure 4.3: A likelihood ratio test to identify lineage-specific constraints.



For each gene of interest, we use the ten upstream and downstream genes to estimate a regional synonymous rate (dSr) and the expected lineage-specific constraint scaling factors (a). These scaling factors take into account that the constraint on each lineage will vary due to the effective population size and other species-specific parameters. Using these regional parameters, a gene-specific dN/dS ratio (w) is estimated. In this case, the lineage of interest leads to extant species C. In the null model, the nonsynonymous substitution rate is estimated as aCwndSr. This is compared to the alternative model, where nonsynonymous branch length is set to a free parameter (R).

Figure 4.4. Flowchart representing study design for testing association of rapidly evolving genes with preterm birth.

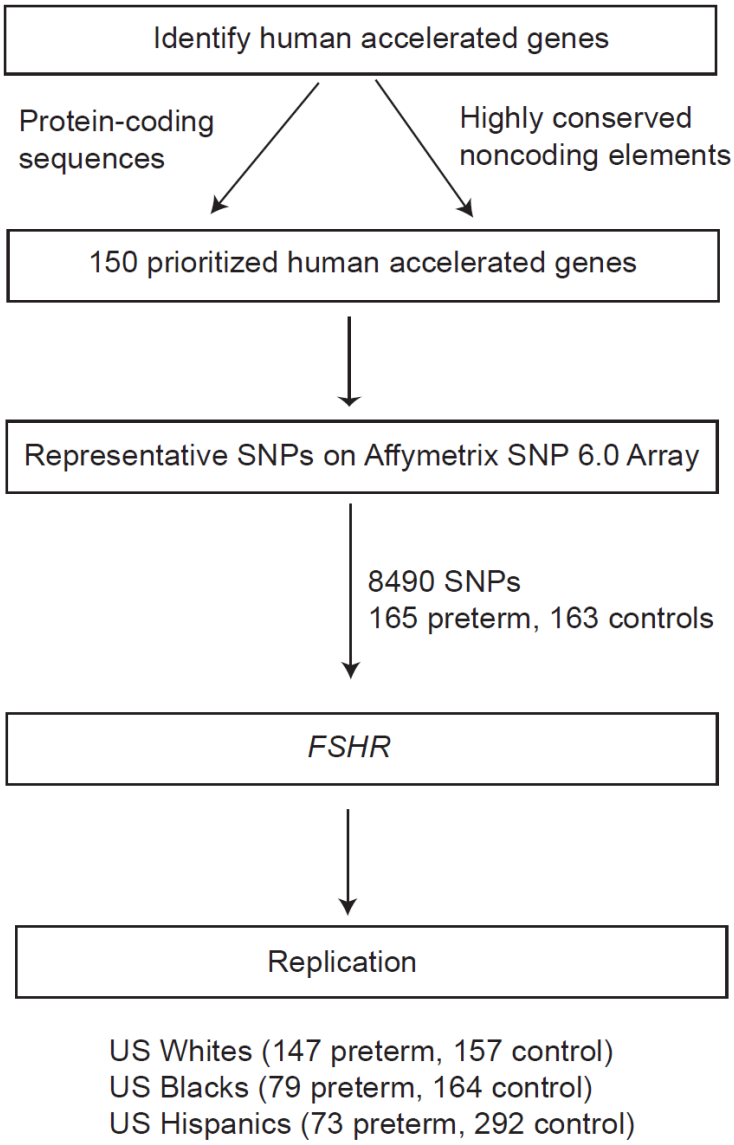
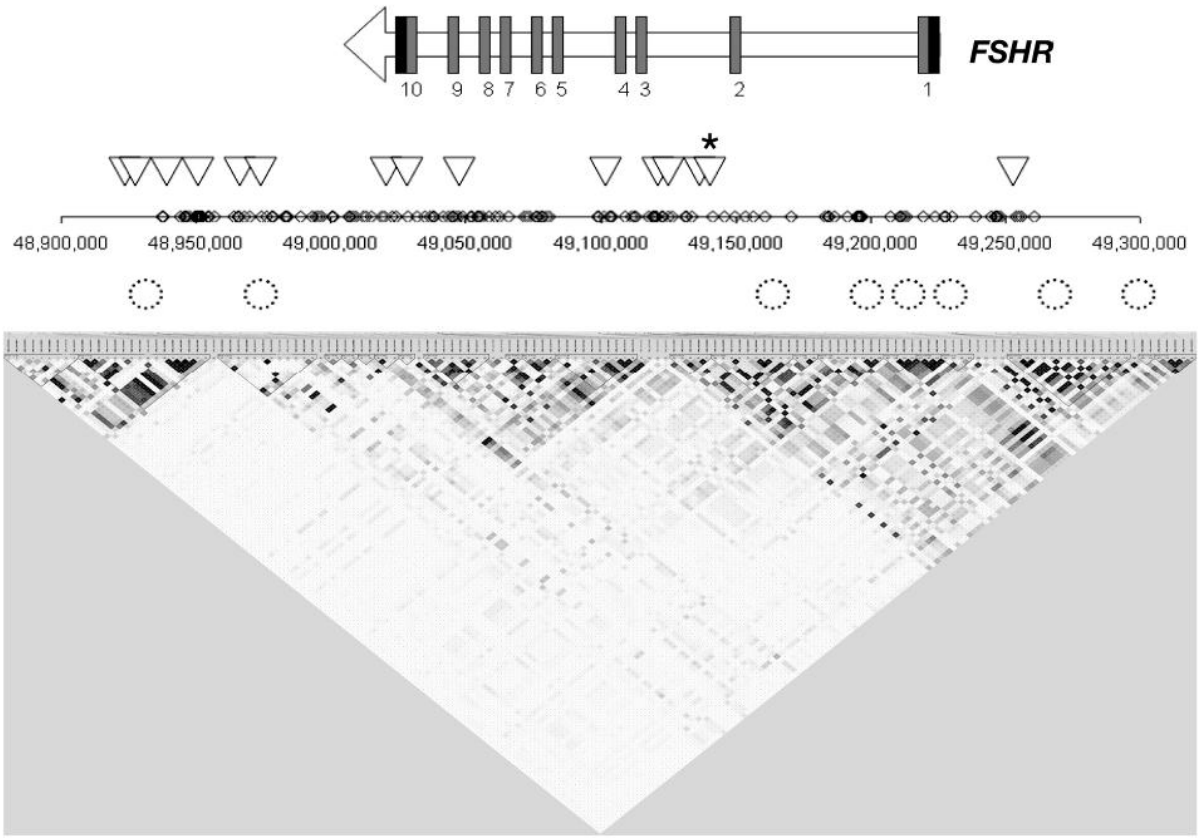
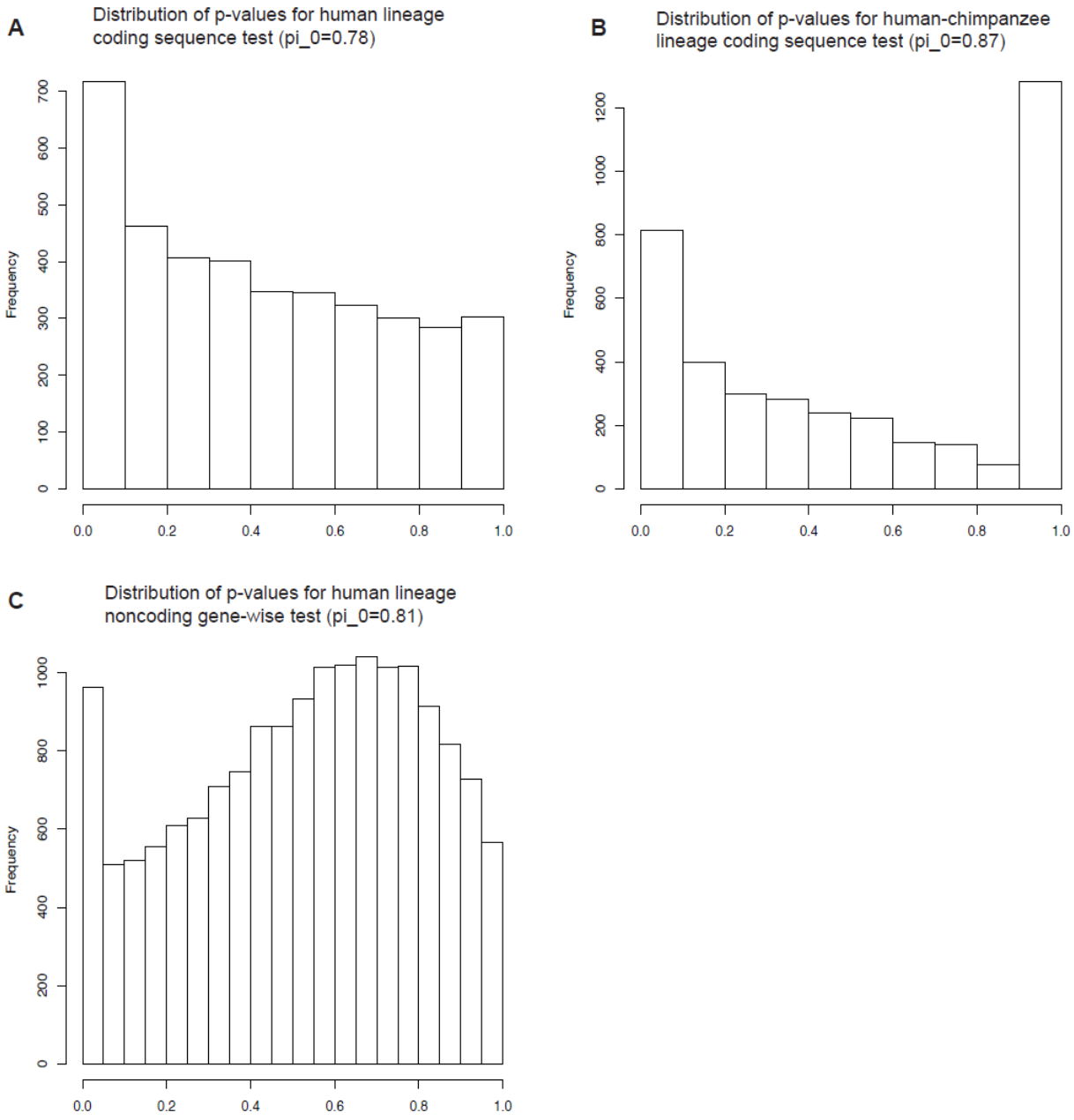


Figure 4.5. Overview of the SNPs tested in the *FSHR* gene region.



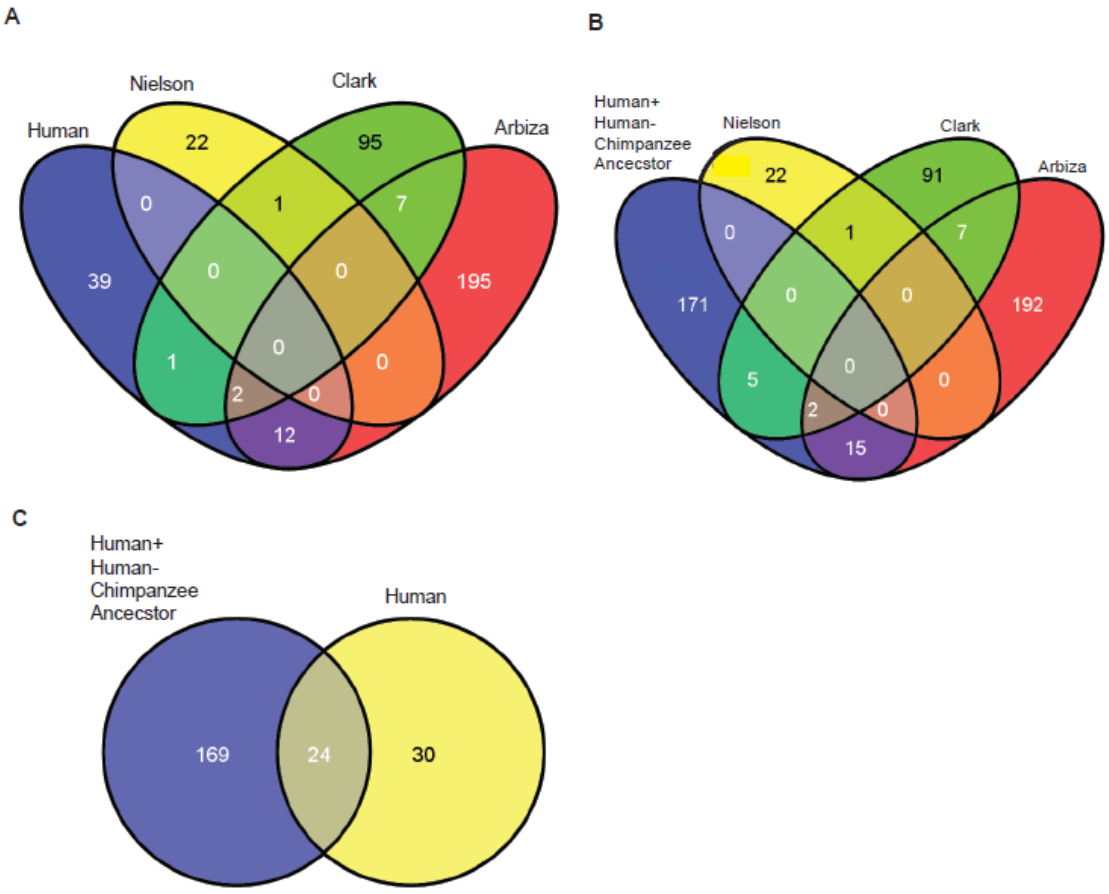
The gene structure for *FSHR* is represented by an arrow in which black rectangles designate 3' and 5' untranslated regions and dark grey rectangles designate coding exons. Diamonds represent SNPs on the Affymetrix SNP 6.0 array examined in the Finnish cohort. Triangles represent SNPs tested in the replication cohorts. A star indicates rs12473815, which is significant after multiple testing correction in US Blacks ($p<0.004$). Circles represent conserved elements examined in the region.

Figure 4.6: Distributions of p-values for coding and noncoding screens used to determine false discovery rate thresholds for significance.



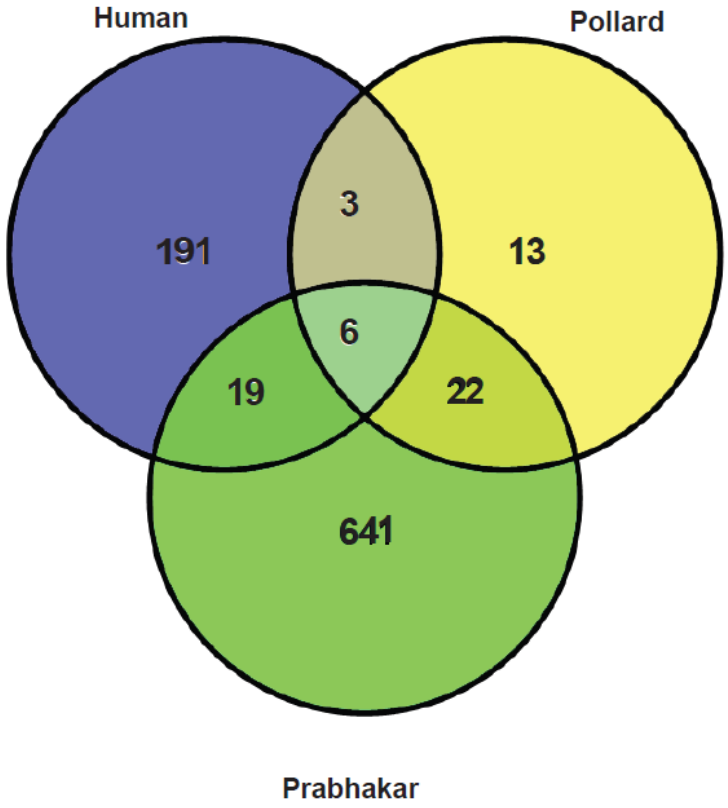
Panel A depicts the distribution of p-values for test for significant rate acceleration on human lineage compared to other mammalian lineages for coding sequences. Panel B depicts the distribution of p-values for test for significant rate acceleration on human-chimpanzee lineage compared to other mammalian lineages for coding sequences. Panel C depicts the distribution of gene-wise p-values for test for significant rate acceleration on human lineage compared to other mammalian lineages for noncoding sequences.

Figure 4.7: Venn diagram illustrating the overlap between the results of our coding analysis and similar studies.



Genes identified by Arbiza et al. (2006), Clark et al. (2003), Nielson et al. (2005) are compared to genes we identified as rapidly evolving on the human lineage (10% FDR, Panel A) or on the human+human-chimpanzee ancestor lineage (5% FDR, Panel B). Panel C depicts the overlap between genes we identified as rapidly evolving on the human lineage (10% FDR) or on the human+human-chimpanzee ancestor lineage (5% FDR).

Figure 4.8: Venn diagram illustrating the overlap between the results of our noncoding analysis and similar studies.



Unique genes identified by Pollard et al. (2006) and Prabhakar et al. (2006) are compared to genes we identified as rapidly evolving on the human lineage (10% FDR).

Table 4.1. Sample of candidate genes showing rapid coding region evolution in humans.

Gene		Human			Human-chimpanzee ancestor		
		Expected Ratio[a]	Observed Ratio	p-value[b]	Expected Ratio[a]	Observed Ratio	p-value[b]
OXT	Oxytocin-neurophysin 1 precursor	0.25	1.47	0.017	0.16	0.37	0.546
PTGER4[c]	Prostaglandin E2 receptor, EP4	0.49	1.10	0.018	0.33	0.33	0.539
ESR1	Estrogen receptor	0.22	0.55	0.020	0.15	0.13	0.216
NR2C1	Orphan nuclear receptor TR2	0.36	0.93	0.024	0.24	0.22	0.818
NTF3[d]	Neurotrophin-3 precursor	0.29	0.60	0.042	0.26	0.15	0.439
OXTR	Oxytocin receptor	0.13	0.43	0.048	0.16	0.20	0.168
PGR [d]	Progesterone receptor	0.24	0.68	0.048	0.27	0.31	0.127
PAPPA [d]	Pregnancy-associated plasma protein-A	0.30	0.29	0.099	0.22	0.34	1.79×10^{-8}
MMP8	Matrix metalloproteinase-8	0.51	0.67	0.230	0.54	0.83	3.94×10^{-4}

[a] The ratio reported is the ratio of the nonsynonymous to synonymous substitutions (dN/dS) for coding sequence.
[b] The p-value reported is from the likelihood ratio test comparing the rate on the human or the human plus the human-chimpanzee ancestral lineage to the expected rate from the background model.
[c] Gene identified as rapidly evolving by Arbiza and colleagues (2006).
[d] Gene also was identified as rapidly evolving by Clark and colleagues (2003).

Table 4.2: Candidate rapidly evolving genes examined for association with preterm birth.

Gene	Ensembl ID	Chromosome	Gene region start (bp)[A]	Gene region stop (bp)
ENSG00000187980	PLA2G2C	1	20,358,071	20,379,247
ENSG00000054118	THRAP3	1	36,457,626	36,547,744
ENSG00000197587	DMBX1	1	46,693,885	46,769,955
ENSG00000134245	WNT2B	1	112,804,363	112,872,035
ENSG00000163554	SPTA1	1	156,842,020	156,928,130
ENSG00000133055	MYBPH	1	201,398,562	201,416,565
ENSG00000133063	CHIT1	1	201,436,600	201,496,865
ENSG00000117335	CD46	1	205,987,025	206,040,481
ENSG00000162814	SPATA17	1	215,879,672	216,238,784
ENSG00000143771	CNIH4	1	222,606,183	222,638,777
ENSG00000183814	LIN9	1	224,480,481	224,577,619
ENSG00000084674	APOB	2	21,072,806	21,334,249
ENSG00000170820	FSHR	2	48,936,801	49,262,724
ENSG00000028116	VRK2	2	57,432,980	58,284,303
ENSG00000168758	SEMA4C	2	96,884,204	96,905,699
ENSG00000196228	SULT1C3	2	108,123,399	108,265,257
ENSG00000125571	IL1F7	2	113,382,017	113,412,506
ENSG00000183840	GPR39	2	132,885,617	133,125,602
ENSG00000169554	ZEB2	2	144,811,277	145,811,772
ENSG00000183091	NEB	2	152,045,110	152,304,406
ENSG00000138399	FASTKD1	2	170,089,515	170,147,595
ENSG00000138435	CHRNA1	2	175,316,097	175,359,048
ENSG00000064933	PMS1	2	190,352,355	190,508,863
ENSG00000013441	CLK1	2	201,411,164	201,442,667
ENSG00000116117	PARD3B	2	204,824,164	206,193,781
ENSG00000163283	ALPP	2	232,931,318	232,959,619
ENSG00000163286	ALPPL2	2	232,975,096	233,011,310
ENSG00000157985	CENTG2	2	235,971,127	236,715,338
ENSG00000168387	ASB14	3	57,252,242	57,297,334
ENSG00000189283	FHIT	3	59,361,681	60,575,887
ENSG00000196353	CPNE4	3	132,731,274	133,593,392
ENSG00000169744	LDB2	4	15,988,937	16,703,648
ENSG00000145241	CENPC1	4	68,015,584	68,104,114
ENSG00000083857	FAT	4	187,740,918	188,341,814
ENSG00000205096	DUX4_HUMAN	4	191,214,485	191,232,642
ENSG00000174358	SLC6A19	5	1,249,710	1,281,385
ENSG00000171540	OTP	5	76,948,651	76,999,618
ENSG00000164292	RHOBTB3	5	95,087,606	95,162,827
ENSG00000170482	SLC23A1	5	138,722,343	138,751,981
ENSG00000204956	PCDHGA1	5	140,685,388	140,698,003

ENSG00000173210	ABLIM3	5	148,496,326	148,625,192
ENSG00000065029	ZNF76	6	35,330,427	35,376,740
ENSG00000180872	DEFB112	6	50,131,694	50,461,861
ENSG00000135346	CGA	6	87,847,192	87,893,643
ENSG00000164520	RAET1E	6	150,246,014	150,258,863
ENSG00000048052	HDAC9	7	18,279,517	19,021,186
ENSG00000196335	STK31	7	23,711,404	23,843,843
ENSG00000105954	NPVF	7	25,213,311	25,256,794
ENSG00000091138	SLC26A3	7	107,188,393	107,275,261
ENSG00000178234	GALNT11	7	151,348,797	151,463,085
ENSG00000156006	NAT2	8	18,280,063	18,341,561
ENSG00000120907	ADRA1A	8	26,662,251	26,968,234
ENSG00000198363	ASPH	8	62,573,374	63,945,182
ENSG00000064218	DMRT3	9	961,964	986,732
ENSG00000153707	PTPRD	9	8,145,485	10,571,307
ENSG00000106829	TLE4	9	81,284,764	81,955,007
ENSG00000156345	CCRK	9	89,766,183	89,784,487
ENSG00000182752	PAPPA	9	117,840,886	118,209,421
ENSG00000167123	CEECAM1	9	130,201,775	130,253,724
ENSG00000165997	ARL5B	10	18,983,319	19,274,735
ENSG00000095794	CREM	10	35,450,807	35,546,892
ENSG00000165731	RET	10	42,796,962	42,950,850
ENSG00000095587	TLL2	10	98,109,356	98,268,658
ENSG00000166407	LMO1	11	8,197,433	8,345,763
ENSG00000166961	MGC35295	11	60,276,052	60,305,780
ENSG00000149021	SCGB1A1	11	61,938,099	62,054,461
ENSG00000173153	ESRRA	11	63,824,620	63,845,786
ENSG00000204571	KRTAP5-11	11	70,963,408	70,987,532
ENSG00000118113	MMP8	11	102,083,599	102,105,868
ENSG00000204403	CASP12	11	103,980,451	104,254,354
ENSG00000137713	PPP2R1B	11	111,097,898	111,156,373
ENSG00000064309	CDON	11	125,320,174	125,512,554
ENSG00000111266	DUSP16	12	12,513,420	12,632,500
ENSG00000123360	PDE1B	12	53,224,671	53,264,290
ENSG00000110958	PTGES3	12	55,338,379	55,373,318
ENSG00000151846	PABPC3	13	24,563,276	24,575,705
ENSG00000150893	FREM2	13	38,058,234	38,379,883
ENSG00000174126	ENSG00000174126	13	40,907,081	40,917,746
ENSG00000139842	CUL4A	13	112,906,151	112,984,825
ENSG00000092054	MYH7	14	22,947,820	22,987,727
ENSG00000196792	STRN3	14	30,427,761	30,570,340
ENSG00000151322	NPAS3	14	32,428,709	33,386,974
ENSG00000136352	NKX2-1	14	35,997,916	36,065,064
ENSG00000198807	PAX9	14	36,195,877	36,683,420
ENSG00000184302	SIX6	14	60,034,147	60,062,098

ENSG00000140009	ESR2	14	63,556,569	63,824,462
ENSG00000100815	TRIP11	14	91,500,614	91,581,139
ENSG00000182256	GABRG3	15	24,794,429	25,456,729
ENSG00000198838	RYR3	15	31,385,469	31,950,591
ENSG00000154237	LRRK1	15	99,271,983	99,432,838
ENSG00000131650	KREMEN2	16	2,949,218	2,963,381
ENSG00000183632	TP53TG3	16	32,546,984	32,574,764
ENSG00000102962	CCL22	16	55,930,968	55,962,600
ENSG00000050820	BCAR1	16	73,815,429	73,864,452
ENSG00000186153	WWOX	16	76,653,469	77,833,566
ENSG00000070444	MNT	17	2,229,115	2,256,834
ENSG00000006047	YBX2	17	7,127,322	7,143,598
ENSG00000133020	MYH8	17	10,229,495	10,271,188
ENSG00000141048	MYH4	17	10,280,658	10,318,846
ENSG00000125414	MYH2	17	10,360,323	10,433,169
ENSG00000176160	HSF5	17	53,847,530	53,925,744
ENSG00000213218	CSH3	17	59,298,106	59,309,848
ENSG00000136488	CSH2	17	59,298,288	59,332,647
ENSG00000136487	GH2	17	59,306,304	59,317,955
ENSG00000189162	CSH1	17	59,343,295	59,354,930
ENSG00000171634	BPTF	17	63,206,700	63,416,200
ENSG00000089685	BIRC5	17	73,714,361	73,738,310
ENSG00000181409	AATK	17	76,700,703	76,759,467
ENSG00000186765	FSCN2	17	77,105,153	77,119,582
ENSG00000101605	MYOM1	18	3,051,806	3,215,106
ENSG00000101489	BRUNOL4	18	33,072,000	34,124,249
ENSG00000133313	CNDP2	18	70,309,577	70,344,336
ENSG00000131196	NFATC1	18	75,266,605	75,440,665
ENSG00000174837	EMR1	19	6,833,582	6,908,102
ENSG00000132024	CC2D1A	19	13,873,014	13,907,691
ENSG00000127507	EMR2	19	14,699,205	14,755,353
ENSG00000189231	PSG3	19	47,912,635	47,941,508
ENSG00000131113	PSG1	19	48,044,852	48,080,711
ENSG00000170848	PSG6	19	48,093,080	48,118,883
ENSG00000170853	PSG11	19	48,198,649	48,227,471
ENSG00000124435	PSG2	19	48,255,202	48,283,665
ENSG00000204941	PSG4	19	48,358,736	48,406,630
ENSG00000008438	PGLYRP1	19	51,209,255	51,223,144
ENSG00000105499	PLA2G4C	19	53,237,916	53,310,865
ENSG00000104826	LHB	19	54,206,049	54,217,159
ENSG00000104827	CGHB_HUMAN	19	54,212,940	54,224,444
ENSG00000189052	CGHB_HUMAN	19	54,233,875	54,245,378
ENSG00000213030	CGB	19	54,237,709	54,249,212
ENSG00000196337	CGB7	19	54,244,344	54,258,929
ENSG00000171101	SIGLECP3	19	56,357,397	56,395,399

ENSG00000131848	ZSCAN5	19	61,415,347	61,576,564
ENSG00000125780	TGM3	20	2,216,426	2,274,202
ENSG00000101452	DHX35	20	37,019,406	37,243,569
ENSG00000064655	EYA2	20	44,941,086	45,255,897
ENSG00000101181	GTPBP5	20	60,186,496	60,216,218
ENSG00000060491	OGFR	20	60,901,622	60,920,797
ENSG00000154640	BTG3	21	17,882,811	17,949,761
ENSG00000157554	ERG	21	38,670,792	38,990,795
ENSG00000100302	RASD2	22	34,223,536	34,284,987
ENSG00000188677	PARVB	22	42,721,506	42,901,434

[A] Positions refer to NCBI36 (hg18, March 2006 assembly) build of the human genome.

Table 4.3: SNPs in the rapidly evolving gene regions tested with p-values <0.01 in the Finnish cohort (n=165 cases, 163 controls).

SNP Affymetrix Probe ID	Ensembl Gene ID	Gene Symbol	Chromosome	Location (bp) [A]	Allelic P-value	Genotypic P-value	Odds Ratio
SNP_A-8453479	ENSG00000170820	FSHR	2	48,937,400	0.0052	0.0078	0.47 (0.27-0.81)
SNP_A-2214277	ENSG00000170820	FSHR	2	48,945,764	0.0087	0.0079	0.59 (0.40-0.88)
SNP_A-8574083	ENSG00000170820	FSHR	2	48,946,762	0.0021	0.0036	0.54 (0.36-0.80)
SNP_A-1963108	ENSG00000170820	FSHR	2	48,947,479	0.0074	0.03	0.36 (0.16-0.78)
SNP_A-8393579	ENSG00000170820	FSHR	2	48,949,643	0.0034	0.0031	1.60 (1.17-2.20)
SNP_A-1925725	ENSG00000170820	FSHR	2	48,951,679	0.0020	0.0063	0.43 (0.25-0.75)
SNP_A-2259062	ENSG00000170820	FSHR	2	48,954,639	0.0045	0.0042	1.58 (1.15-2.17)
SNP_A-8693449	ENSG00000170820	FSHR	2	48,967,735	6.78×10^{-4}	0.0016	0.56 (0.40-0.78)
SNP_A-4232093	ENSG00000170820	FSHR	2	49,098,912	3.94x10-4	0.0015	2.36 (1.45-3.84)
SNP_A-8574095	ENSG00000170820	FSHR	2	49,119,336	8.08x10-5	6.44x10-4	2.35 (1.53-3.63)
SNP_A-1795584	ENSG00000170820	FSHR	2	49,124,921	0.0060	0.02	0.64 (0.47-0.88)
SNP_A-1963125	ENSG00000170820	FSHR	2	49,131,327	5.98×10^{-4}	0.0026	0.58 (0.43-0.79)

SNP_A-2119506	ENSG00000170820	FSHR	2	49,141,487	2.72×10^{-4}	9.92×10^{-4}	1.82 (1.32-2.52)
SNP_A-2187829	ENSG00000170820	FSHR	2	49,141,564	3.92×10^{-4}	0.0014	1.80 (1.30-2.49)
SNP_A-8694413	ENSG00000170820	FSHR	2	49,145,845	3.38×10^{-4}	0.0010	1.82 (1.31-2.52)
SNP_A-1872585	ENSG00000170820	FSHR	2	49,145,866	6.81×10^{-4}	0.0021	1.76 (1.27-2.45)
SNP_A-4275652	ENSG00000170820	FSHR	2	49,184,334	0.0011	0.0042	1.97 (1.31-2.97)
SNP_A-1963128	ENSG00000170820	FSHR	2	49,184,353	5.94×10^{-4}	0.0026	1.75 (1.27-2.41)
SNP_A-4261924	ENSG00000170820	FSHR	2	49,184,796	0.0011	0.0042	1.97 (1.31-2.98)
SNP_A-8574097	ENSG00000170820	FSHR	2	49,194,560	0.0062	0.0094	1.82 (1.18-2.82)
SNP_A-2171016	ENSG00000170820	FSHR	2	49,210,919	0.0062	0.02	1.82 (1.18-2.82)
SNP_A-2081721	ENSG00000170820	FSHR	2	49,219,551	0.0089	0.03	1.77 (1.15-2.73)
SNP_A-2248307	ENSG00000170820	FSHR	2	49,227,555	0.0054	0.02	1.86 (1.20-2.90)
SNP_A-8574895	ENSG00000028116	VRK2	2	57,597,195	0.0046	0.02	1.91 (1.21-3.00)
SNP_A-8399741	ENSG00000157985	CENTG2	2	236,112,093	0.0028	0.0071	1.73 (1.21-2.48)
SNP_A-2183124	ENSG00000157985	CENTG2	2	236,137,704	0.0019	0.0065	1.64 (1.20-2.24)
SNP_A-8437696	ENSG00000157985	CENTG2	2	236,156,777	0.0013	0.0057	1.67 (1.22-2.29)

SNP_A-8560303	ENSG00000189283	FHIT	3	59,763,565	0.0085	0.03	0.56 (0.37-0.87)
SNP_A-8624292	ENSG00000189283	FHIT	3	59,878,852	0.0082	0.04	2.76 (1.26-6.04)
SNP_A-8444000	ENSG00000189283	FHIT	3	59,879,851	0.0076	0.02	0.65 (0.48-0.89)
SNP_A-4242521	ENSG00000189283	FHIT	3	59,893,263	0.0064	0.0042	0.65 (0.48-0.89)
SNP_A-8383162	ENSG00000189283	FHIT	3	59,896,687	0.0039	0.0096	0.63 (0.47-0.86)
SNP_A-8558654	ENSG00000189283	FHIT	3	59,897,057	0.0050	0.01	0.64 (0.47-0.88)
SNP_A-1910513	ENSG00000189283	FHIT	3	59,966,929	0.0051	0.02	0.49 (0.29-0.81)
SNP_A-8498171	ENSG00000189283	FHIT	3	60,120,766	0.0073	0.03	1.53 (1.12-2.08)
SNP_A-8689355	ENSG00000189283	FHIT	3	60,136,687	0.0076	0.02	1.52 (1.12-2.07)
SNP_A-2298264	ENSG00000189283	FHIT	3	60,473,008	0.0053	0.0041	1.74 (1.18-2.58)
SNP_A-2095659	ENSG00000189283	FHIT	3	60,475,589	0.0097	0.0076	1.65 (1.13-2.41)
SNP_A-4203581	ENSG00000189283	FHIT	3	60,478,478	0.0014	0.0053	0.42 (0.24-0.72)
SNP_A-8694222	ENSG00000189283	FHIT	3	60,478,506	0.0021	0.0067	0.45 (0.27-0.76)
SNP_A-4251667	ENSG00000196353	CPNE4	3	133,390,589	0.0058	0.0033	1.55 (1.13-2.11)
SNP_A-1795008	ENSG00000196353	CPNE4	3	133,390,995	0.0030	0.0030	1.59 (1.17-2.17)

SNP_A-2247251	ENSG00000196353	CPNE4	3	133,398,962	0.0079	0.02	0.62 (0.44-0.89)
SNP_A-2144689	ENSG00000196353	CPNE4	3	133,404,646	0.0079	0.02	0.62 (0.44-0.89)
SNP_A-1932424	ENSG00000196353	CPNE4	3	133,406,698	0.0073	0.02	0.62 (0.44-0.88)
SNP_A-1906715	ENSG00000196353	CPNE4	3	133,421,585	0.0060	0.02	0.61 (0.43-0.87)
SNP_A-8412574	ENSG00000196353	CPNE4	3	133,450,246	0.0098	0.0056	1.50 (1.10-2.04)
SNP_A-8318381	ENSG00000169744	LDB2	4	16,007,023	0.0093	0.01	1.59 (1.12-2.25)
SNP_A-4281442	ENSG00000169744	LDB2	4	16,020,079	0.0071	0.01	1.62 (1.14-2.30)
SNP_A-8620026	ENSG00000169744	LDB2	4	16,027,754	0.0025	0.0091	1.63 (1.19-2.23)
SNP_A-8502613	ENSG00000169744	LDB2	4	16,086,960	0.0039	0.01	1.65 (1.17-2.31)
SNP_A-8672025	ENSG00000169744	LDB2	4	16,088,861	0.0048	0.01	1.61 (1.15-2.24)
SNP_A-8478172	ENSG00000169744	LDB2	4	16,104,049	0.0022	0.0071	1.67 (1.20-2.32)
SNP_A-1802900	ENSG00000169744	LDB2	4	16,128,817	0.0040	0.02	0.61 (0.43-0.85)
SNP_A-8620032	ENSG00000169744	LDB2	4	16,400,295	0.0048	0.0030	2.27 (1.27-4.08)
SNP_A-8582336	ENSG00000169744	LDB2	4	16,460,122	0.0090	0.03	0.65 (0.48-0.90)
SNP_A-1892692	ENSG00000164292	RHOBTB3	5	95,145,598	0.0071	0.02	0.56 (0.37-0.86)

SNP_A-2273250	ENSG00000164292	RHOBTB3	5	95,152,785	0.0071	0.02	0.56 (0.37-0.86)
SNP_A-1921353	ENSG00000135346	CGA	6	87,865,333	0.0029	0.0075	1.74 (1.21-2.51)
SNP_A-1874035	ENSG00000153707	PTPRD	9	8,522,140	5.19×10^{-4}	0.0041	0.42 (0.25-0.69)
SNP_A-1874540	ENSG00000153707	PTPRD	9	8,687,215	0.0053	0.0056	1.79 (1.18-2.70)
SNP_A-1996956	ENSG00000153707	PTPRD	9	81,635,796	0.0087	0.04	0.59 (0.40-0.88)
SNP_A-4213521	ENSG00000153707	PTPRD	9	81,636,214	3.70×10^{-4}	0.0023	0.56 (0.41-0.77)
SNP_A-4258746	ENSG00000153707	PTPRD	9	99,670,770	9.83×10^{-4}	0.0040	1.68 (1.23-2.29)
SNP_A-1840834	ENSG00000153707	PTPRD	9	99,677,160	9.83×10^{-4}	0.0040	1.68 (1.23-2.29)
SNP_A-2067220	ENSG00000166407	LMO1	11	8,285,210	0.0043	0.02	1.60 (1.16-2.22)
SNP_A-2031381	ENSG00000166407	LMO1	11	8,285,769	0.0033	0.02	1.63 (1.18-2.26)
SNP_A-1850261	ENSG00000166407	LMO1	11	8,286,194	0.0033	0.02	1.63 (1.18-2.26)
SNP_A-8519096	ENSG00000166407	LMO1	11	8,286,416	0.0045	0.02	1.60 (1.16-2.22)
SNP_A-1782188	ENSG00000111266	DUSP16	12	12,629,753	0.0014	0.0018	0.59 (0.43-0.82)
SNP_A-4291855	ENSG00000151322	NPAS3	14	32,907,061	0.0080	0.0061	1.61 (1.13-2.28)
SNP_A-4294654	ENSG00000198807	PAX9	14	36,499,281	0.0075	0.03	1.91 (1.18-3.07)

SNP_A-8513359	ENSG00000198807	PAX9	14	36,507,279	0.0020	0.0072	0.43 (0.25-0.75)
SNP_A-8382205	ENSG00000198807	PAX9	14	36,515,956	0.0079	0.03	2.30 (1.23-4.33)
SNP_A-2263150	ENSG00000182256	GABRG3	15	24,900,346	0.0071	0.0064	0.60 (0.41-0.87)
SNP_A-8488733	ENSG00000182256	GABRG3	15	24,905,945	0.0057	0.02	1.58 (1.14-2.19)
SNP_A-2192706	ENSG00000182256	GABRG3	15	24,955,101	0.0020	0.0045	1.68 (1.21-2.34)
SNP_A-8494398	ENSG00000182256	GABRG3	15	24,974,470	0.0035	0.0091	1.59 (1.16-2.16)
SNP_A-8686463	ENSG00000182256	GABRG3	15	25,000,416	0.0064	0.02	1.59 (1.14-2.22)
SNP_A-2305114	ENSG00000182256	GABRG3	15	25,325,462	0.0099	0.04	2.02 (1.17-3.46)
SNP_A-8606414	ENSG00000182256	GABRG3	15	25,403,913	0.0017	0.0063	0.60 (0.43-0.82)
SNP_A-2193067	ENSG00000186153	WWOX	16	77,109,437	0.0081	0.04	0.49 (0.29-0.84)
SNP_A-8366136	ENSG00000186153	WWOX	16	77,702,883	0.0060	0.03	2.85 (1.31-6.22)
SNP_A-8309208	ENSG00000186153	WWOX	16	77,729,566	0.0038	0.0013	1.70 (1.18-2.43)
SNP_A-8653985	ENSG00000186153	WWOX	16	77,748,399	0.0088	0.02	2.06 (1.19-3.57)
SNP_A-4246524	ENSG00000174837	EMR1	19	6,847,380	0.0017	0.0012	0.31 (0.14-0.67)
SNP_A-2019253	ENSG00000157554	ERG	21	38,967,270	0.0026	0.0051	1.88 (1.24-2.85)

SNP_A-2019255	ENSG00000157554	ERG	21	38,978,129	0.0027	0.0053	1.87 (1.24-2.83)
SNP_A-1876025	ENSG00000100302	RASD2	22	34,235,013	0.0030	0.0034	1.62 (1.18-2.24)
SNP_A-8549971	ENSG00000100302	RASD2	22	34,243,552	0.0037	0.0089	1.58 (1.16-2.15)

[A] Positions refer to NCBI36 (hg18, March 2006 assembly) build of the human genome.

Table 4.4: SNPs in the *FSHR* gene region tested across Finnish and 3 independent US populations.

SNP information			Finnish (n= 165 cases, 163 controls)		US Hispanics (73 cases, 292 controls)		US Blacks (n=79 cases, 164 controls)		US Whites (n= 147 cases, 157controls)	
SNP	Position (bp) [A]	Location within gene	Genotypic test	Allelic test	Genotypic test	Allelic test	Genotypic test	Allelic test	Genotypic test	Allelic test
rs10174620	48,945,764	3'	**0.008**[B]	**0.009**	0.94	0.80	0.60	0.65	0.50	0.58
rs10490128	48,946,762	3'	**0.004**	**0.002**[D]	0.99	0.91	0.66	0.64	0.35	0.49
rs1558604	48,949,643	3'	**0.003**[D]	**0.003**[D]	0.81	0.60	0.59	0.33	0.46	0.49
rs13418054	48,951,522	3'	0.15	0.05	0.66	0.34	NA[C]	NA[C]	0.48	0.76
rs10186748	48,954,639	3'	**0.004**	**0.004**	0.87	0.77	0.32	0.38	0.64	0.53
rs9789744	48,967,735	3'	**0.002**[D]	**6.78 x10^{-4}**[D]	0.65	0.34	0.17	0.83	0.85	0.69
rs9789406	48,968,088	3'	0.28	0.12	0.43	0.22	0.88	0.66	0.18	0.30
rs6166	49,043,425	exon 10	0.38	0.49	0.50	0.56	0.43	0.68	0.25	0.49
rs6165	49,044,545	exon 10	NA[C]	NA[C]	0.86	0.66	0.41	0.29	0.34	0.63
rs1007540	49,062,612	intron 8-9	0.54	0.37	0.05	0.04	0.61	0.69	0.25	0.49
rs3788982	49,098,912	intron 3-4	**0.001**[D]	**3.94 x10^{-4}**[D]	0.38[E]	0.36[E]	0.36[E]	0.17[E]	0.32	0.09
rs11686474	49,141,487	intron 2-3	**9.92x10^{-4}**[D]	**2.72 x10^{-4}**[D]	0.08[E]	0.25[E]	0.03[E]	**0.008**[E]	0.35	0.56
rs11680730	49,141,564	intron 2-3	**0.001**[D]	**3.92 x10^{-4}**[D]	0.06[E]	0.22[E]	0.02[E]	**0.004**[E]	0.66	0.85
rs12473870	49,145,845	intron 2-3	**0.001**[D]	**3.38 x10^{-4}**[D]	0.18[E]	0.12[E]	0.03[E]	**0.006**[E]	0.49[E]	0.77[E]
rs12473815	49,145,866	intron 2-3	**0.002**[D]	**6.81 x10^{-4}**[D]	0.05[E]	0.25[E]	0.02[E]	**0.003**[D, E]	0.60[E]	0.86[E]
rs12996690	49,578,470	5'	NA[C]	NA[C]	0.72	0.61	0.79	0.59	0.73	0.85

[A] Positions refer to NCBI36 (hg18, March 2006 assembly) build of the human genome.
[B]Bolded numbers indicate p-value < 0.01.
[C]Marker excluded for failing one or more of the following measures: Hardy-Weinberg Equilibrium failure in controls p<0.001, call rate <95%, MAF<0.05.
[D]Marker significant correcting for 13 M-effective number of tests (p<0.004).
[E]Same allele/genotype trends in same direction as Finnish risk-promoting allele.

Table 4.5: Comparison of association results for SNPs in the *FSHR* gene region in Finnish mothers for the binary phenotype preterm birth affection status and quantitative phenotypes gestational age and birthweight Z-score.

SNP information			Preterm birth affection status (n= 165 cases, 163 controls)			Gestational age (n=294)	Birthweight Z-score (n=208)
Affymetrix Probe ID	dbSNP ID	Location (bp) [A]	Allelic test p-value	Odds ratio (95% CI)	Genotypic test p-value	QT p-value	QT p-value
SNP_A-8453479	rs11680746	48,937,400	**0.005**[B]	0.47 (0.27-0.81)	**0.008**	0.04	0.33
SNP_A-8538681	rs13001105	48,937,752	0.45	1.13 (0.82-1.55)	0.73	0.69	0.95
SNP_A-2278783	rs17037665	48,943,898	0.62	0.87 (0.49-1.52)	0.36	0.23	0.25
SNP_A-2214277	rs10174620	48,945,764	**0.009**	0.59 (0.40-0.88)	**0.008**	0.01	0.10
SNP_A-4298227	rs10187173	48,945,942	0.03	0.71 (0.52-0.96)	0.07	**0.008**	0.37
SNP_A-8280065	rs12614293	48,946,413	0.41	0.85 (0.58-1.25)	0.61	0.62	0.83
SNP_A-8574083	rs10490128	48,946,762	**0.002**	0.54 (0.36-0.8)	**0.004**	**0.002**	0.12
SNP_A-1963108	rs17556008	48,947,479	**0.007**	0.36 (0.16-0.78)	0.03	0.03	0.04
SNP_A-8393579	rs1558604	48,949,643	**0.003**	1.60 (1.17-2.20)	**0.003**	**0.001**	0.57
SNP_A-1826650	rs17037685	48,949,886	0.32	0.77 (0.45-1.30)	0.21	0.06	0.24
SNP_A-8483677	rs733726	48,950,339	0.57	0.86 (0.52-1.44)	0.21	0.28	0.22
SNP_A-2040656	rs17037700	48,950,558	0.44	0.80 (0.45-1.42)	0.35	0.14	0.55
SNP_A-4297732	rs13418054	48,951,522	0.05	0.65 (0.42-1.01)	0.15	0.04	0.84
SNP_A-1925725	rs17037707	48,951,679	**0.002**	0.43 (0.25-0.75)	**0.006**	0.03	0.35
SNP_A-2050457	rs6708130	48,952,201	0.03	0.62 (0.40-0.96)	0.09	0.02	0.90
SNP_A-1963109	rs12477968	48,954,153	0.32	0.77 (0.45-1.30)	0.21	0.06	0.24
SNP_A-2259062	rs10186748	48,954,639	**0.004**	1.58 (1.15-2.17)	**0.004**	**0.004**	0.54
SNP_A-1963110	rs17037739	48,963,807	0.02	1.48 (1.05-2.09)	0.04	0.02	0.98
SNP_A-4208066	rs10490127	48,963,897	0.04	0.64 (0.42-0.99)	0.12	0.02	0.87
SNP_A-8429689	rs6755046	48,965,520	0.02	0.58 (0.37-0.90)	0.04	**0.003**	0.85
SNP_A-8653066	rs4605416	48,965,818	0.02	0.58 (0.37-0.91)	0.05	**0.003**	0.82
SNP_A-1801629	rs17037744	48,966,005	0.01	0.56 (0.36-0.89)	0.04	**0.003**	0.81

SNP_A-8574085	rs10490126	48,967,652	0.05	0.65 (0.42-1.00)	0.14	0.03	0.94
SNP_A-8693449	rs9789744	48,967,735	**6.78×10^{-4}**	0.56 (0.40-0.78)	**0.002**	**0.001**	0.75
SNP_A-8465993	rs9789406	48,968,088	0.12	1.33 (0.93-1.91)	0.28	0.01	0.88
SNP_A-2089520	rs2110571	48,969,725	0.19	0.81 (0.60-1.11)	0.40	0.26	0.35
SNP_A-2144280	rs2110570	48,969,746	0.13	1.32 (0.92-1.88)	0.32	0.04	0.41
SNP_A-8371345	rs981273	48,974,053	0.67	1.10 (0.70-1.73)	0.68	0.86	0.49
SNP_A-8294098	rs4953637	48,976,209	0.14	0.79 (0.58-1.08)	0.26	0.12	0.16
SNP_A-1963111	rs10490124	48,977,978	0.10	1.75 (0.89-3.44)	0.07	0.92	0.33
SNP_A-1963112	rs10490123	48,978,206	0.48	0.83 (0.50-1.39)	0.16	0.76	0.33
SNP_A-4261919	rs2215912	48,978,309	0.48	0.83 (0.50-1.39)	0.16	0.76	0.33
SNP_A-2234736	rs7563889	48,983,394	0.19	1.55 (0.80-3.03)	0.14	0.85	0.25
SNP_A-2031669	rs7565910	48,988,842	0.09	0.76 (0.56-1.04)	0.21	0.12	0.12
SNP_A-1899686	rs4952929	48,992,854	0.91	1.02 (0.74-1.40)	0.99	0.67	0.98
SNP_A-8610973	rs17038295	48,995,342	0.74	1.09 (0.66-1.79)	0.93	0.66	0.03
SNP_A-8498619	rs1922479	49,000,591	0.26	1.20 (0.87-1.65)	0.35	0.77	0.04
SNP_A-8424745	rs6545082	49,007,179	0.24	1.26 (0.86-1.84)	0.49	0.93	0.51
SNP_A-8702492	rs2349411	49,008,184	0.43	0.85 (0.57-1.27)	0.09	0.70	0.46
SNP_A-2209987	rs4953644	49,008,904	0.47	0.78 (0.40-1.53)	0.55	0.82	0.09
SNP_A-2095708	rs12991538	49,009,151	0.40	0.80 (0.48-1.35)	0.13	0.66	0.18
SNP_A-1949646	rs989373	49,011,829	0.55	1.10 (0.80-1.52)	0.80	0.90	0.23
SNP_A-8402315	rs7565565	49,018,596	0.82	0.97 (0.71-1.31)	0.23	0.75	0.90
SNP_A-8314084	rs1024777	49,019,982	0.93	1.01 (0.75-1.38)	0.14	0.61	0.89
SNP_A-8372663	rs17037887	49,022,712	0.33	0.78 (0.46-1.30)	0.11	0.58	0.18
SNP_A-8480826	rs6706144	49,024,081	0.26	0.76 (0.48-1.22)	0.27	0.23	0.53
SNP_A-8678552	rs4952932	49,028,586	0.67	0.93 (0.68-1.28)	0.76	0.73	0.36
SNP_A-1816022	rs2160149	49,031,882	0.26	1.22 (0.87-1.70)	0.51	0.07	0.90
SNP_A-8308066	rs6545085	49,034,975	0.98	1.00 (0.73-1.39)	0.91	0.74	0.05
SNP_A-2258053	rs4140979	49,036,913	0.39	1.16 (0.83-1.61)	0.07	0.86	0.09
AFFX-SNP_9110811	rs4140979	49,036,913	0.48	1.13 (0.81-1.56)	0.09	0.77	0.10

SNP_A-2117142	rs1861168	49,037,518	0.65	0.93 (0.68-1.27)	0.37	0.77	0.64
SNP_A-1963114	rs1882559	49,041,818	0.95	1.02 (0.63-1.64)	0.80	0.85	0.06
SNP_A-1849765	rs6166	49,043,425	0.49	0.90 (0.66-1.22)	0.38	0.74	0.61
SNP_A-8656117	rs2058595	49,045,094	0.96	1.01 (0.73-1.39)	0.07	0.87	0.02
SNP_A-8671693	rs4953650	49,045,243	0.97	0.99 (0.72-1.38)	0.09	0.65	0.03
SNP_A-8301277	rs8179692	49,046,208	0.74	0.95 (0.70-1.29)	0.68	0.72	0.58
SNP_A-2150730	rs2072489	49,048,913	1.00	1.00 (0.62-1.62)	0.82	0.81	0.04
SNP_A-8570896	rs6705106	49,051,684	0.19	1.25 (0.89-1.76)	0.40	0.13	0.57
SNP_A-8589739	rs4953652	49,052,016	0.60	0.87 (0.52-1.46)	0.80	0.57	0.03
SNP_A-1875185	rs2284674	49,054,317	0.79	0.95 (0.66-1.37)	0.57	0.95	0.49
SNP_A-2181014	rs7594937	49,054,636	0.35	0.80 (0.50-1.28)	0.05	0.17	0.65
SNP_A-8557127	rs2268363	49,054,832	0.21	0.76 (0.50-1.17)	0.02	0.04	0.93
SNP_A-8588997	rs6545091	49,054,957	0.70	1.08 (0.74-1.55)	0.38	0.54	0.61
SNP_A-8401244	rs2268361	49,055,116	0.43	0.88 (0.64-1.21)	0.69	0.34	0.90
SNP_A-4261920	rs989359	49,055,989	0.70	1.08 (0.74-1.55)	0.38	0.56	0.42
SNP_A-1963115	rs1922472	49,056,089	0.86	0.97 (0.68-1.38)	0.61	0.97	0.50
SNP_A-2131602	rs2268359	49,058,614	0.69	0.92 (0.60-1.41)	0.91	0.33	0.02
SNP_A-8574090	rs2300437	49,060,997	0.76	0.93 (0.57-1.51)	0.13	0.39	0.84
SNP_A-8574091	rs1007541	49,062,538	0.36	0.82 (0.54-1.25)	0.64	0.16	0.10
SNP_A-8574092	rs1007540	49,062,612	0.37	0.83 (0.56-1.25)	0.54	0.87	0.37
SNP_A-8294822	rs3788985	49,066,013	0.86	0.95 (0.56-1.62)	0.14	0.23	0.99
SNP_A-8632763	rs2072486	49,071,420	1.00	1.00 (0.74-1.36)	0.27	0.75	0.71
SNP_A-2191583	rs13002977	49,072,538	0.60	0.92 (0.67-1.26)	0.84	0.24	0.48
SNP_A-8687005	rs10186089	49,072,628	0.36	0.82 (0.54-1.25)	0.64	0.16	0.10
SNP_A-1916373	rs13031735	49,072,827	0.80	0.96 (0.69-1.32)	0.97	0.67	0.35
SNP_A-1963116	rs1922466	49,073,584	0.73	0.95 (0.69-1.30)	0.93	0.26	0.50
SNP_A-1963117	rs1922465	49,073,612	0.60	0.92 (0.67-1.26)	0.84	0.24	0.48
SNP_A-1963118	rs6746533	49,076,076	0.60	0.92 (0.67-1.26)	0.87	0.26	0.39
SNP_A-4261921	rs6732220	49,076,376	0.72	0.94 (0.66-1.33)	0.50	0.52	0.63
SNP_A-2242483	rs1922463	49,078,084	0.51	0.90 (0.65-1.23)	0.56	0.13	0.51

SNP_A-8437573	rs3788983	49,098,768	0.02	0.66 (0.48-0.93)	0.06	0.02	0.25
SNP_A-4232093	rs3788982	49,098,912	**3.94×10^{-4}**	2.36 (1.45-3.84)	**0.001**	**0.008**	0.57
SNP_A-1957741	rs3788981	49,099,065	0.04	0.72 (0.53-0.99)	0.09	0.06	0.20
SNP_A-8327608	rs1882558	49,101,241	0.88	1.02 (0.74-1.41)	0.87	0.64	0.02
SNP_A-8323502	rs2349415	49,101,336	0.40	0.87 (0.63-1.21)	0.03	0.25	0.28
SNP_A-8399688	rs1504187	49,103,279	0.20	0.81 (0.58-1.12)	0.29	0.12	0.98
SNP_A-1855471	rs4246578	49,103,424	0.06	0.58 (0.33-1.03)	0.19	0.39	0.54
SNP_A-8326035	rs12713033	49,104,325	0.97	1.01 (0.73-1.38)	0.99	0.97	0.006
SNP_A-1963120	rs10495962	49,108,935	0.13	1.35 (0.92-1.98)	0.29	0.09	0.38
SNP_A-1944026	rs17038087	49,111,262	0.10	1.38 (0.94-2.03)	0.24	0.07	0.48
SNP_A-8588395	rs10171892	49,111,371	0.35	0.86 (0.62-1.18)	0.25	0.28	0.22
SNP_A-8604497	rs13008999	49,112,242	0.65	1.08 (0.78-1.49)	0.78	0.80	0.01
SNP_A-1872622	rs13009588	49,112,333	0.65	1.08 (0.78-1.49)	0.78	0.80	0.01
SNP_A-8320178	rs13009434	49,112,485	0.57	1.10 (0.80-1.51)	0.78	0.71	0.01
SNP_A-8574093	rs17038094	49,112,894	0.46	1.15 (0.80-1.65)	0.43	0.30	0.43
SNP_A-8574094	rs1910566	49,117,774	0.73	1.06 (0.77-1.44)	0.88	0.74	0.19
SNP_A-8574095	rs6741370	49,119,336	**8.08×10^{-5}**	2.35 (1.53-3.63)	**6.44×10^{-4}**	**0.001**	0.07
SNP_A-8335818	rs6545092	49,119,389	0.21	0.82 (0.61-1.12)	0.19	0.48	0.02
SNP_A-1963123	rs6545094	49,119,818	0.28	0.84 (0.62-1.15)	0.22	0.56	0.03
SNP_A-8431533	rs17038105	49,120,251	0.08	0.61 (0.35-1.07)	0.24	0.46	0.45
SNP_A-2197501	rs1277459	49,120,697	0.08	0.71 (0.48-1.04)	0.19	0.04	0.35
SNP_A-2065169	rs10865238	49,122,308	0.04	0.71 (0.52-0.98)	0.02	0.03	0.46
SNP_A-1863973	rs12465332	49,122,889	0.43	1.14 (0.83-1.57)	0.60	0.67	0.04
SNP_A-2143695	rs17038116	49,122,975	0.01	0.52 (0.31-0.86)	0.04	0.11	0.75
SNP_A-1795584	rs12614817	49,124,921	**0.006**	0.64 (0.47-0.88)	0.02	**0.009**	0.48
SNP_A-2165293	rs3850344	49,126,316	0.07	0.74 (0.54-1.02)	0.06	0.05	0.30
SNP_A-1963125	rs1504175	49,131,327	**5.98×10^{-4}**	0.58 (0.43-0.79)	**0.003**	**0.006**	0.63
SNP_A-1963126	rs1857706	49,131,455	0.07	0.66 (0.43-1.04)	0.11	0.54	0.17
SNP_A-2119506	rs11686474	49,141,487	**2.72×10^{-4}**	1.82 (1.32-2.52)	**9.92×10^{-4}**	**0.004**	0.13
SNP_A-2187829	rs11680730	49,141,564	**3.92×10^{-4}**	1.80 (1.30-2.49)	**0.001**	**0.007**	0.16

SNP_A-8694413	rs12473870	49,145,845	**3.38 x10^{-4}**	1.82 (1.31-2.52)	**0.001**	**0.006**	0.27
SNP_A-1872585	rs12473815	49,145,866	**6.81 x10^{-4}**	1.76 (1.27-2.45)	**0.002**	0.01	0.29
SNP_A-2006361	rs6724851	49,149,900	0.05	1.36 (1.00-1.86)	0.09	0.05	0.15
SNP_A-8536246	rs12622212	49,153,738	0.17	1.31 (0.89-1.94)	0.38	0.57	0.05
SNP_A-1783559	rs1604821	49,156,576	0.08	1.33 (0.97-1.81)	0.17	0.10	0.14
SNP_A-2132897	rs2349711	49,160,796	0.12	1.28 (0.94-1.74)	0.26	0.14	0.03
SNP_A-2226105	rs12052611	49,170,652	0.04	1.38 (1.01-1.87)	0.12	0.04	0.45
SNP_A-4275652	rs974895	49,184,334	**0.001**	1.97 (1.31-2.97)	**0.004**	**0.004**	0.24
SNP_A-1963128	rs974896	49,184,353	**5.94 x10^{-4}**	1.75 (1.27-2.41)	**0.003**	**0.005**	0.64
SNP_A-4261924	rs17772297	49,184,796	**0.001**	1.97 (1.31-2.98)	**0.004**	**0.004**	0.32
SNP_A-8574096	rs1504174	49,186,728	0.13	0.71 (0.46-1.10)	0.31	0.41	0.69
SNP_A-8574097	rs6760923	49,194,560	**0.006**	1.82 (1.18-2.82)	**0.009**	**0.010**	0.50
SNP_A-4208068	rs10495964	49,195,505	0.65	0.92 (0.65-1.31)	0.85	0.64	0.38
SNP_A-8574098	rs9309159	49,195,556	0.44	1.14 (0.82-1.57)	0.57	0.69	0.45
SNP_A-4208069	rs17038275	49,195,570	0.22	0.71 (0.41-1.23)	0.49	0.55	0.67
SNP_A-4208070	rs10495965	49,196,308	0.79	0.95 (0.67-1.35)	0.94	0.71	0.38
SNP_A-1963130	rs10495966	49,196,425	0.72	0.94 (0.66-1.33)	0.91	0.71	0.38
SNP_A-1963131	rs17038285	49,196,505	0.72	0.94 (0.66-1.33)	0.91	0.71	0.38
SNP_A-2199691	rs4971642	49,196,676	0.02	1.70 (1.10-2.63)	0.04	0.02	0.43
SNP_A-4197527	rs10199118	49,207,442	0.72	0.94 (0.66-1.33)	0.91	0.71	0.38
SNP_A-1808513	rs10211458	49,207,498	0.65	0.92 (0.65-1.31)	0.85	0.65	0.38
SNP_A-2171016	rs17038315	49,210,919	**0.006**	1.82 (1.18-2.82)	0.02	**0.007**	0.50
SNP_A-1929434	rs12477795	49,211,178	0.96	1.01 (0.70-1.45)	0.99	0.94	0.92
SNP_A-8316753	rs1553474	49,213,180	0.19	1.33 (0.87-2.04)	0.40	0.65	0.59
SNP_A-2081721	rs17038320	49,219,551	**0.009**	1.77 (1.15-2.73)	0.03	0.01	0.43
SNP_A-1963132	rs1157876	49,223,862	0.85	0.97 (0.69-1.36)	0.81	0.98	0.67
SNP_A-4261925	rs1504182	49,227,443	0.09	1.30 (0.96-1.77)	0.18	0.11	0.18
SNP_A-2248307	rs1504183	49,227,555	**0.005**	1.86 (1.20-2.90)	0.02	0.02	0.49
SNP_A-1963134	rs953547	49,230,415	0.02	1.45 (1.06-2.00)	0.03	0.04	0.40
SNP_A-2132947	rs4500983	49,239,003	0.11	1.29 (0.94-1.75)	0.22	0.14	0.15

SNP_A-1963135	rs10495963	49,244,517	0.33	1.21 (0.83-1.76)	0.62	0.45	0.46
SNP_A-8608826	rs6761392	49,246,886	0.29	1.18 (0.87-1.61)	0.53	0.16	0.47
SNP_A-8466163	rs13428062	49,248,239	0.58	0.89 (0.59-1.34)	0.59	0.55	0.81
SNP_A-1963136	rs1032838	49,253,850	0.22	0.81 (0.58-1.14)	0.48	0.16	0.61
SNP_A-8584561	rs13019040	49,254,126	0.26	0.83 (0.61-1.14)	0.32	0.06	0.44
SNP_A-1963137	rs972557	49,254,706	0.28	0.84 (0.62-1.15)	0.51	0.04	0.21
SNP_A-1963138	rs1504155	49,255,754	0.21	0.82 (0.61-1.12)	0.31	0.04	0.16
SNP_A-8430734	rs11125217	49,260,940	0.31	0.84 (0.61-1.17)	0.57	0.12	0.48

[A] Positions refer to NCBI36 (hg18, March 2006 assembly) build of the human genome.
[B] Bolded numbers indicate p-value < 0.01.

Chapter 5: Association of cystolic phospholipase A2 gamma, *PLA2G4C*, with PTB risk[**]

Abstract

Correlating differences in genomic sequences with differences in reproductive physiology across species may led to new insights into mechanisms underlying birth timing. *PLA2G4C*, a phospholipase A isoform involved in prostaglandin synthesis, emerged from a comparative genomics screen of highly conserved noncoding elements as rapidly evolving in humans. Detailed structural and phylogenic analysis of *PLA2G4C* suggested a short genomic element within the gene duplicated from a paralogous highly conserved element on chromosome 1 specifically in primates. To examine whether this gene demonstrating primate-specific evolution was associated with birth timing, we genotyped common variation in *PLA2G4C* in US Hispanic (n= 73 preterm, 292 control), US White (n= 147 preterm, 157 control) and US Black (n= 79 preterm, 166 control) mothers. SNPs rs8110925 and rs2307276 in US Hispanics and rs11564620 in US Whites were significant after correcting for multiple tests ($p<0.004$). Additionally, rs11564620 (Thr360Pro) was associated with increased metabolite levels of the prostaglandin thromboxane in healthy individuals ($p=0.02$), suggesting this variant may affect *PLA2G4C* activity. Association findings suggest variation in *PLA2G4C* gene may influence PTB risk by increasing levels of prostaglandins, which are known to regulate labor.

[**] This chapter is adapted from: Plunkett J, et al. Primate-specific evolution of noncoding element insertion into *PLA2G4C* and human preterm birth. In preparation.

Introduction

A growing body of evidence supports genetic influences on PTB risk; however, few genes have been consistently associated with the disorder [179; 180]. Investigators have typically focused on candidate genes selected based on predicted parturition physiology; however, this approach may be limited by the divergence in physiological mechanisms between humans and model organisms that have been typically studied. For example, while a rapid decline in progesterone plays a prominent role in initiating parturition in rodents and sheep, this signal does not seem to precede human labor [181]. Other parturition-related traits, such as placental morphology and source of progesterone, also differ importantly in humans compared to model organisms typically studied and may limit what generalizations can be made [181].

Differences in parturition physiology between apes, including humans, and other mammals may have developed in response to uniquely human adaptations including relatively large human head size and narrow birth canal cross-sectional area [127]. Genes involved in parturition likely have evolved differentially along the human and/or higher primate phylogenetic lineages to decrease the length of gestation and alleviate the complications arising from these constraints. As a result, the set of genes rapidly evolving on the human and/or higher primate lineage likely includes genes that play important roles in regulating parturition and potentially influence PTB risk. Consistent with our hypothesis, we identified *FSHR* as rapidly evolving by nucleotide substitution and as associated with PTB risk across independent populations [136].

In addition to nucleotide substitution, genomic rearrangements account for an important portion of genomic divergence among species. For example, Frazer et al. [182]

and Wetterbom et al. [183] observed insertions and deletions frequently when comparing genome sequences among humans, chimpanzees and other primate species. Moreover, these rearrangements may account for a larger fraction of genomic divergence than nucleotide substitutions [183]. Rearrangements can lead to loss or acquisition of exons, splice sites and promoters, facilitating differences in expression patterns, such as those observed for transcript variants of *CHRM3* and *SFTPB* with differing transposable element insertion events [184; 185]. Hence, genomic rearrangement may contribute to rapid evolution along the human and/or higher primate lineages in response to unique physiological constraints.

We hypothesize that genes with genomic rearrangements from the ancestral state occurring on the human and/or higher primate lineages may play important roles in birth timing and preterm delivery. Here, we examine common variants in a gene, *PLA2G4C,* in which we have identified a primate-specific insertion event and whose expression in the uterus [186] and role in prostaglandin synthesis suggest a potential role in parturition, for association with PTB.

Results

Evolutionary history of a primate-specific *PLA2G4C* noncoding element. We identified genes showing evidence of rapid evolution along the human lineage, based on evidence from a comparative genomic screen of highly conserved noncoding elements as described in [136]. Among the rapidly evolving genes emerging from our noncoding screen, *PLA2G4C* was identified as the most statistically significant gene (human lineage $p=2.2x10^{-7}$, significant at 10% False Discovery Rate threshold) that was also included in

a list of PTB candidate genes [134]. Because the reported deletion of *PLA2G4C* in cattle [187] contrasted with its presence in the 17-way MultiZ alignments [165] we used to identify the gene as rapidly evolving (Figure 5.1A), we examined the history of this region in greater depth. We compared sequence surrounding the 130 basepair (bp) highly conserved noncoding element in intron 14 of *PLA2G4C* on chromosome 19q13.3, which strongly motivated the gene's designation as rapidly evolving along the human lineage, to other mammalian and primate genomes. From such comparisons, we determined that this 130 bp element on human chromosome 19 was highly similar to a highly conserved noncoding element on human chromosome 1 (BLASTN 114/130 bp identical (87%), BLAST expect value= $5x10^{-38}$; Figure 5.1B). Subsequent analysis showed the MultiZ alignments we used in our comparative genomics screen had misaligned the human chromosome 19 element with sequences in other mammals which were orthologous to human chromosome 1. When appropriate alignments were examined, we observed that the human chromosome 19 element was nearly identical in higher primate species (chimpanzee, gorilla, orangutan, macaque) examined, but, absent in syntenic sequences in lower primates (lemur, bushbaby, tarsier) and other mammalian species. Chromosome 1 elements from higher primates are more similar to lower primates and other mammalian species than chromosome 19 elements (Figure 5.2). The chromosome 1 element occurs in the 5' untranslated region of *RNF11*, a gene involved in inflammatory signaling, as it does in mouse. Thus, a duplication of chromosome 1 noncoding element to chromosome 19 likely occurred before the last common ancestor between apes and macaque. A phylogenetic tree of coding sequences for *PLA2G4C* follows the expected mammalian phylogeny (Figure 5.3), suggesting that the duplication did not include

coding sequences. Together these results suggest that neither element would qualify as rapidly evolving along the human lineage due to nucleotide substitution, but, the chromosome 19 element may represent a primate-specific change meriting further study.

Association with preterm birth. Because of recent data that suggests heritability of risk of PTB acts largely or exclusively through the maternal genome [29; 30; 32], we genotyped US Hispanic (73 preterm, 292 control), US Whites (n= 147 preterm, 157 control) and US Blacks (n= 79 preterm, 166 control) mothers for 14 SNPs in the *PLA2G4C* gene region (Table 5.1). The results from these analyses, reported in Table 5.2, include two SNPs, rs8110925 and rs2307276, in the US Hispanics and one in the US Whites, rs11564620, that were significant after correcting for 14 tests ($p<0.004$). The direction of effect was generally consistent across populations for these SNPs, as noted in Table 5.2. Meta-analysis p-values for SNPs rs8110925, rs2307276, and rs11564620 were also statistically significant after correcting for 14 tests ($p<0.004$; Figure 5.4).

Additionally, 2, 3 and 4 SNP haplotypes containing SNPs rs8110925 and rs2307276 were significant in the US Hispanics after correcting for 18 haplotype comparisons ($p<0.003$), although not more significant than single SNP association findings (Table 5.3). 2 SNP haplotypes containing rs11564620 were moderately significant ($p<0.05$) in US Whites (Table 5.3). Linkage disequilibrium (LD) among SNPs rs8110925, rs2307276, and rs11564620 was very low ($r^2<0.1$) in the three populations studied (Figure 5.5), suggesting multiple independent associations were observed.

Association with prostaglandin concentrations. To test the potential functional effect of associated *PLA2G4C* variants on prostaglandin metabolism, we compared levels of metabolites of prostaglandin E2 (PGE), prostaglandin I2 (PGI) and thromboxane (11-

DTXB2) among genotype classes for associated SNPs rs8110925, rs2307276, and rs11564620 in healthy individuals using a two-sided Wald test (Table 5.4). Of note, rs11564620, a nonsynonymous coding polymorphism, is associated with 11-DTXB2 levels (p=0.04) despite the limited sample size available. The minor allele of rs11564620, present at approximately 10% frequency in US Whites, is associated with both risk for PTB and higher 11-DTXB2 levels (Wilcoxon one-sided p=0.02; Figure 5.6).

Discussion

Genomic analysis of *PLA2G4C* indicated that the conserved element which brought the gene to our attention was highly similar to a conserved element on human chromosome 1. Further study suggested that the conserved element on chromosome 1 was duplicated to chromosome 19 along the primate lineage. As a result, human chromosome 19 noncoding element in intron 14 of *PLA2G4C* seems to represent a primate-specific change involving amplification and subsequent divergence rather than a region of increased nucleotide substitution, *per se*. We propose this duplicated element insertion represents a primate-specific change with a potential regulatory role in human parturition.

Single SNP and haplotype association results implicate the role of SNPs rs8110925, rs2307276, and rs11564620 in conferring PTB risk (Tables 5.2 and 5.3). The associated SNPs are located in an 8 kilobase (kb) region of the 3' end of *PLA2G4C*, near the genomic element of interest (Figure 5.7), but show little LD with each other (Figure 5.5) or other SNPs in *PLA2G4C* documented in the International HapMap Project database [151]. Of note, Polyphen [188] and SIFT [189] programs predict rs11564620, a

nonsynonymous polymorphism in exon 13 resulting in a change in amino acid 360 from threonine to proline, to be possibly damaging to the protein structure. This 8 kb region also includes coding sequence for aspartic acid 385, one of the three amino acids that make up the putative active site of the enzyme [190], such that the proline substitution may alter the active site's physical conformation. Supporting the potential functional effect of rs11564620, this polymorphism is associated with 11-DTXB2 levels in healthy individuals ($p=0.02$; Figure 5.6), with proline allele carriers having elevated thromboxane metabolite levels, compared to threonine homozygotes.

PLA2G4C encodes cytosolic phospholipase A2 gamma, which hydrolizes phospholipids from the cellular membrane to free arachidonic acid, from which prostaglandins, including prostaglandins D, E, F, I2 (also known as prostacyclin), and thromboxane A2 are generated. Prostaglandins likely play an important role in parturition. Pharmacologically, prostaglandins are used to induce abortion, for cervical ripening and labor induction, and drugs inhibiting prostaglandin synthesis are successful in preventing preterm labor [191]. Levels of prostaglandins, including thromboxane A2, are elevated in pregnant compared to non-pregnant women, and in later (36 weeks) compared to earlier (20, 30 weeks) gestation [192], suggesting a link between prostaglandin abundance and parturition timing. Prostaglandins may facilitate labor by several mechanisms. These hormones are known uterotonic agents and also promote luteolysis and the onset of labor in species that exhibit progesterone withdrawal prior to birth [193]. Prostaglandins may also facilitate delivery by affecting placenta function, since thromboxane A2 induces platelet aggregation and acts as a vasoconstrictor [192].

Hence, higher prostaglandin levels than expected may initiate parturition prematurely and lead to preterm delivery.

The PLA2G4C enzyme is the only cytosolic phospholipase A2 family member that is constitutively associated with the cellular membrane, the site of prostaglandin synthesis, rather than translocating to the membrane in response to calcium signaling [186]. Hence, dysregulation of *PLA2G4C* may alter prostaglandins levels independent of other parturition signals, such as oxytocin [194], that act via intracellular calcium signaling. For example, rs11564620 may contribute to a conformational change in the enzyme's active site, rendering it more active than usual and leading to increased synthesis of prostaglandins, as demonstrated by our observation of higher levels of thromboxane A2 in minor allele carriers for this polymorphism. Moreover, multiple splice isoforms of *PLA2G4C* exist, differing in transcript length, presence of certain exons and overlapping exons with different boundaries (AceView, NCBI, http://www.ncbi.nlm.nih.gov/IEB/Research/Acembly/). As a result, variation in *PLA2G4C* may contribute to differences in tissue-specific expression or relative abundance of various *PLA2G4C* isoforms, potentially altering function. Further study of the region encompassing these SNPs, including the genomic element of interest, is needed to examine the mechanism by which variation in *PLA2G4C* influences birth timing.

Specialization within multi-gene families, like the large phospholipase A2 gene family, can create individualized functions among paralogous genes. For example, *PLA2G4C* has a continuous association with the cellular membrane, unlike other phospholipase A2 genes, potentially differentiating its role in prostaglandin synthesis

from those of other family members. Genomic variation, such as the element insertion observed in *PLA2G4C,* may contribute to gene specialization, as demonstrated by divergence in *PLA2G4C* expression patterns in humans versus mice, who lack the element insertion and express *PLA2G4C* only in ovary and oocytes [195]. Specialized genes are potentially better therapeutic targets than gene products with multiple roles within cell, since pharmaceutically targeting such genes may lead to fewer side effects. As a result, *PLA2G4C* may be a useful target for designing novel therapies to prolong pregnancy and reduce the incidence of PTB.

Material and Methods

Genomic alignments to investigate evolutionary history of *PLA2G4C*. Noting the deletion of *PLA2G4C* reported in the Taurine Cattle genome [187] contrasted with its presence in the 17-way MultiZ alignments [165] we used to identify the gene as rapidly evolving (analysis conducted Spring 2007 and presented in [136]), we examined the history of this region in greater depth. We extracted sequence surrounding the 130 bp highly conserved noncoding element (human chromosome 19: 48,560,500 -48,560,630; hg19 genome build) which largely contributed to our designation of *PLA2G4C* as rapid evolving along the human lineage. A BLASTN search of the element revealed highly identical conserved noncoding elements on human chromosomes 1 (87% identity) and 2 (85% identity) (Figure 5.1B). We compared the human chromosome 19 and chromosome 1 sequences to 31 eutherian mammalian genomes using Ensembl Genomic alignments (accessed September 2009), and ClustalW alignment, and to specific primate genomes using BLASTN searches of human, chimpanzee, gorilla, orangutan, macaque, and

bushbaby genomes (accessed September 2009). We then reconstructed history of the element by creating phylogenies using the neighbor joining with Kimura distances and maximum likelihood methods with sequences homologous to the human chromosome 19 element (Figures 5.2) and coding sequences homologous to human *PLA2G4C* (Figure 5.3).

Human subjects. Study subjects were enrolled for genetic analysis by methods approved by Institutional Review Boards/Ethics Committees at each participating institution. Informed consent was obtained for all participants. Mothers with PTB were included if the birth was spontaneous (non-iatrogenic), singleton, had no obvious precipitating stimulus (trauma, infection, drug use), and was less than 37 weeks (Yale University; New York University) or 36 weeks (University of Helsinki; University of Oulu; Centennial Hospital, Nashville, TN) of completed gestation. Control mothers were included if they delivered two or more children at 37 weeks or later spontaneously. Healthy volunteers were recruited at Vanderbilt University for studies of prostaglandin metabolism. DNA from blood or saliva was prepared by standard methods. Race/ethnicity was assigned by self-report. All specimens were linked with demographic and medical data abstracted from maternal/neonatal records. DNA from blood or saliva was prepared by standard methods.

Prostaglandin metabolite levels. For individuals enrolled in the prostaglandin study, urine was collected by standard methods. Levels of the urinary metabolites of prostaglandin E (PGE), prostaglandin I (PGI) and thromboxane (11-DTXB2) were quantified by mass spectrometry and normalized to creatinine levels, an indicator of renal function, in 44 healthy control individuals of Black, Hispanic or White race (median age

29, 60% male, 77% White).

Genotyping. We genotyped 14 SNPs spanning the *PLA2G4C* gene region (Table 5.1) on human chromosome 19 in cohorts of US Hispanics (n= 73 preterm, 292 control mothers), US Whites (n= 147 preterm, 157 control mothers) and US Blacks (n= 79 preterm, 166 control mothers). For SNP selection, data from the HapMap CEU population was examined in the Haploview program [177], using tagger and haplotype block functions, to identify regions of high LD. We selected 1 SNP per haplotype block, defined using the D' confidence interval method [178], having the highest minor allele frequency (MAF) in the CEU population for genotyping. We also included coding SNPs and SNPs to improve coverage of conserved elements contributing to the gene's designation as "rapidly evolving." This selection scheme resulted in approximately 35% coverage of the gene region at $r^2 \geq 0.8$. SNPs showing evidence of association in one or more cohort ($p<0.01$; n=4) were then genotyped in healthy individuals on whom data on their concentrations of several prostaglandin metabolites was available to examine potential functional effects of the variants. All SNPs were genotyped using the Sequenom iPLEX massARRAY technology (Sequenom, San Diego, CA).

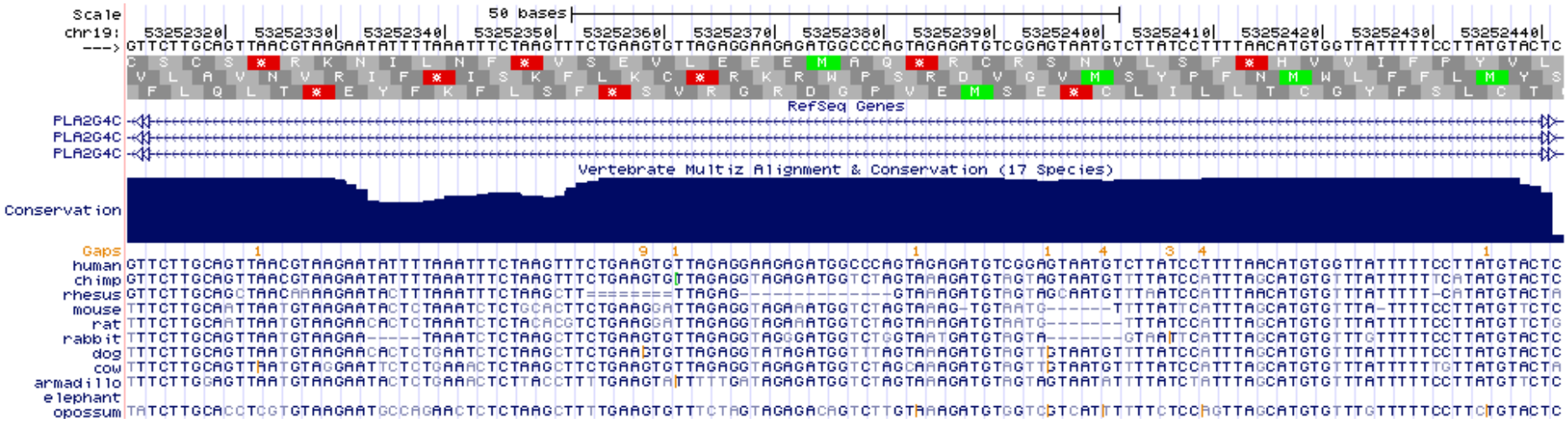
Data Analysis. Data cleaning and analysis was performed with Whole-genome Association Study Pipeline (WASP) [174] and PLINK [175]. We excluded individuals based on genotyping quality (<90% call rate) and possible cryptic relatedness and SNPs based on the following criteria: not in Hardy-Weinberg Equilibrium in controls ($p<0.001$ χ^2 test), <90% genotype call rate, MAF<0.01). Linkage disequilibrium among SNPs tested was determined using the Haploview program [177].

Our analysis considered PTB affection status (i.e. delivery <37 weeks) as a binary trait, comparing frequencies between case and control groups of alleles and genotypes by χ^2 test. Sliding windows of 2,3 and 4 SNP haplotypes also were compared between cases and controls [175]. Meta-analysis of data for significant SNPs was done using the Mantel-Haenszel method. We corrected for multiple testing using the simpleM method [176], which estimates the number of independent tests, given the LD relationships among SNPs, used to obtain a Bonferroni-corrected critical value.

To test the potential functional effect of associated *PLA2G4C* variants on prostaglandin metabolism, we examined the levels of PGE, PGI, and 11-DTXB2, standardized to normal distributions (μ=0, σ=1), as quantitative traits. A Wald test was performed to compare the mean phenotype between different allele or genotype classes for associated SNPs. We also tested whether rs11564620 risk-allele carriers had higher prostaglandin levels than noncarriers, by comparing the 11-DTXB2 value distribution among genotype classes with box plots and one-sided Wilcoxon nonparametric test performed in R [158].

Figure 5.1A: Genomic alignments suggest *PLA2G4C* element duplicated from another chromosome.

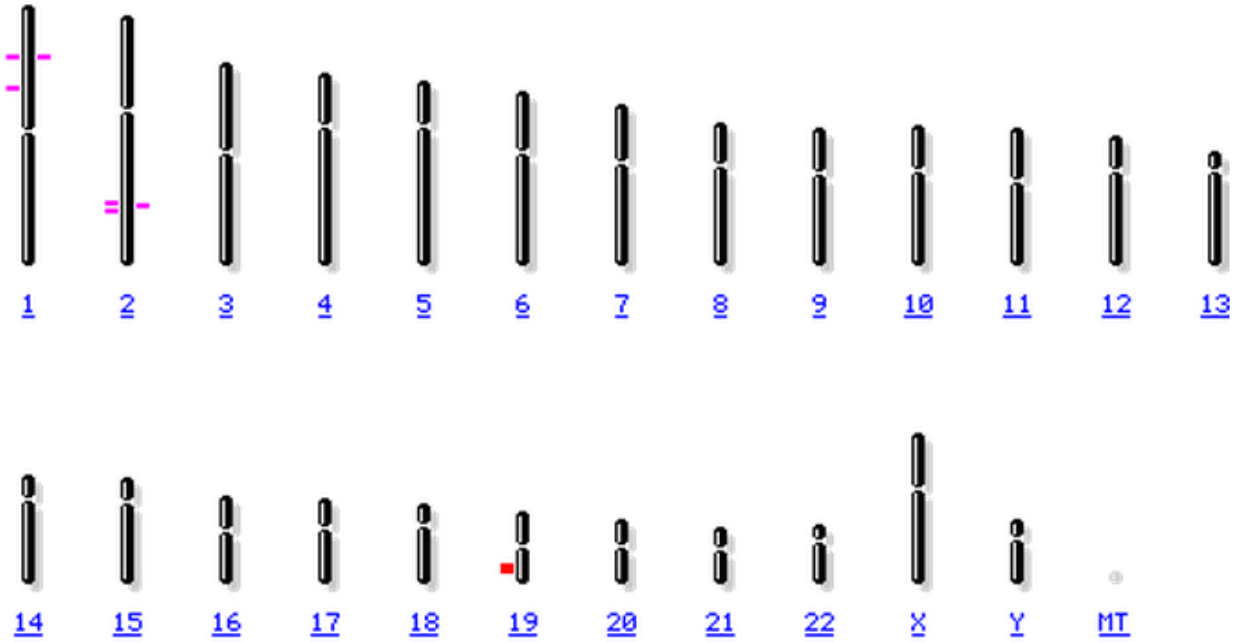
A



MultiZ alignments used in the noncoding analysis from which we initially identified *PLA2G4C* as rapidly evolving include sequence for lower mammals, including cow, in which the gene is absent (A).

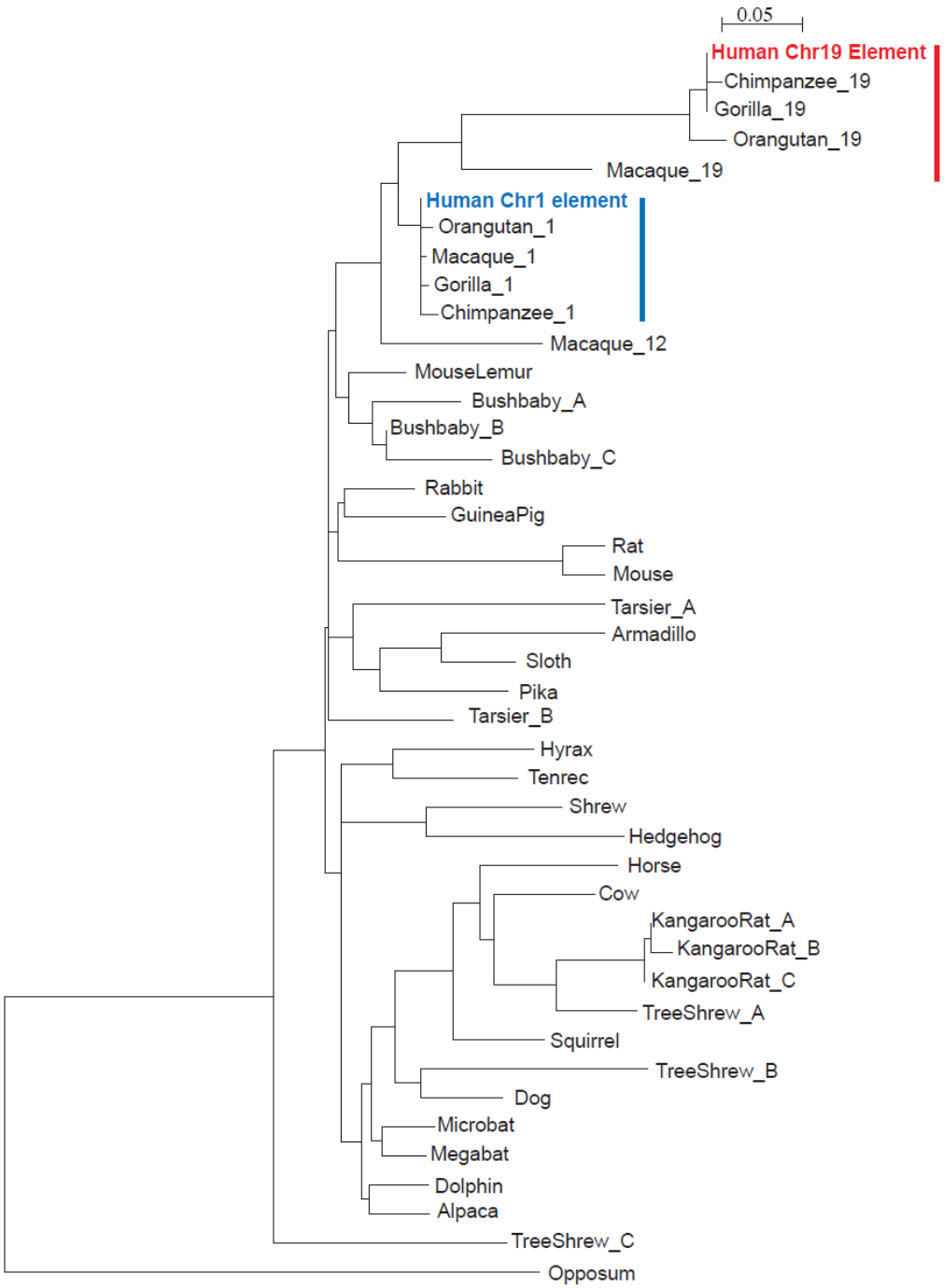
Figure 5.1B: Genomic alignments suggest *PLA2G4C* element duplicated from another chromosome.

B



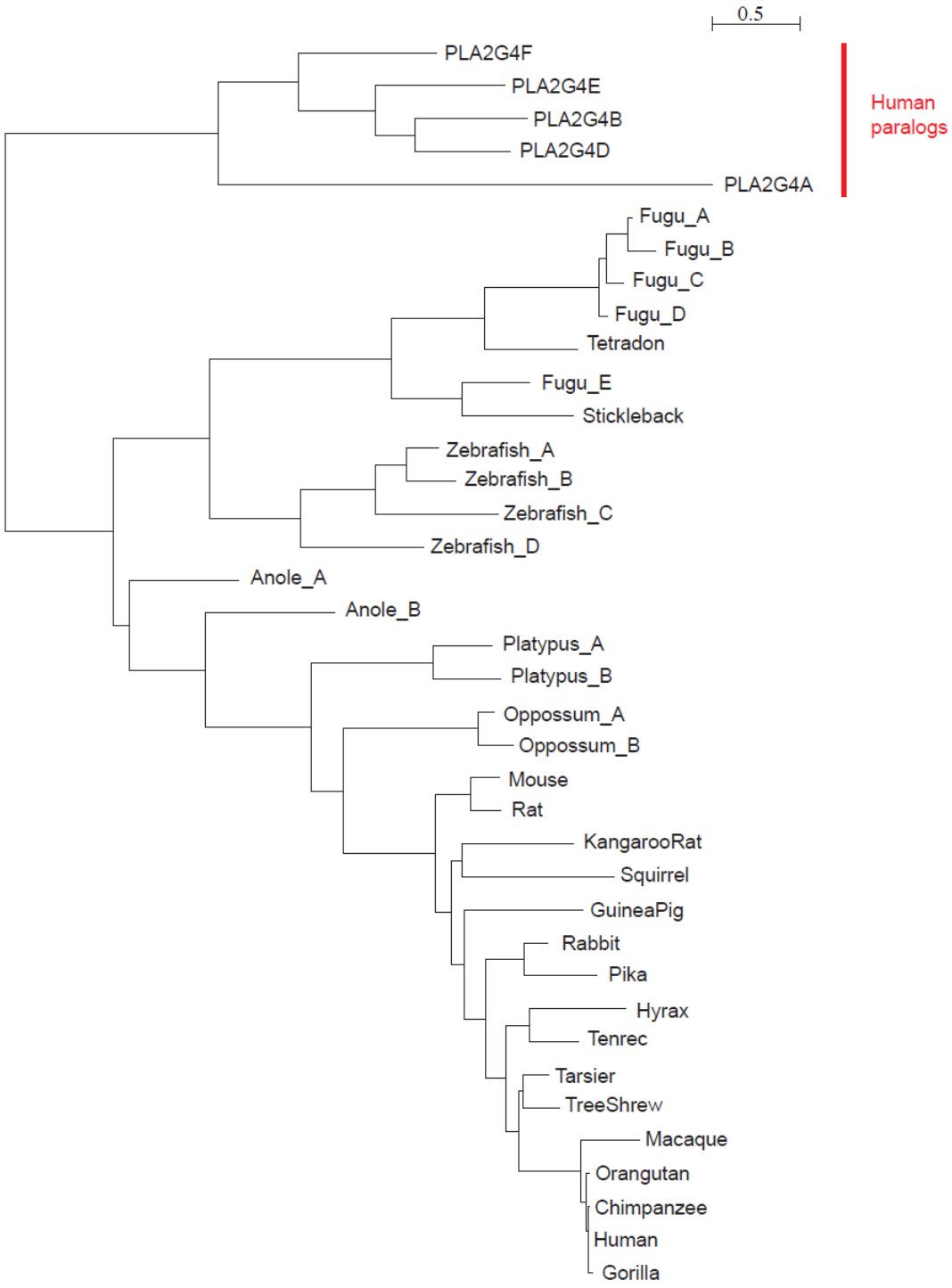
A BLASTN search of the element located in *PLA2G4C* intron 14 (chromosome 19) that led to the gene's designation as rapidly evolving by nucleotide substitution revealed highly identical conserved noncoding elements on human chromosomes 1 and 2 (B).

Figure 5.2: Phylogeny with sequences homologous to human chromosomes 19 noncoding element.



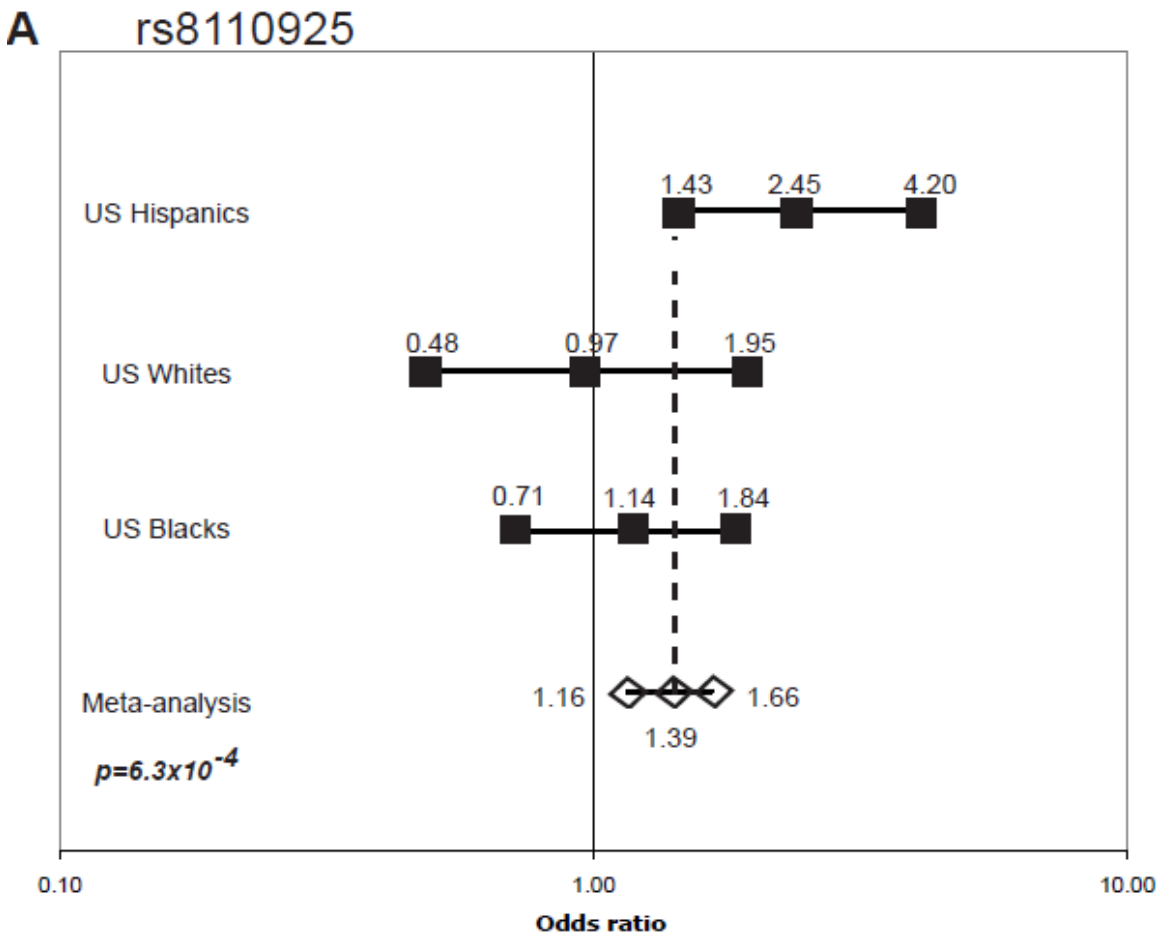
Species name followed by a number indicates from which chromosome the sequence is derived or by a letter indicates that multiple copies homologous to the human chromosome 19 noncoding element were identified for that species. Sequences from lower primates and other mammalian species are more similar to higher primate sequences orthologous to human chromosome 1noncoding element (indicated in blue) than sequences orthologous to human chromosome 19 noncoding element (indicated in red). A duplication of chromosome 1 noncoding element to chromosome 19 likely occurred before the last common ancestor between apes and macaque.

Figure 5.3: Phylogeny with coding sequences homologous to human *PLA2G4C*.



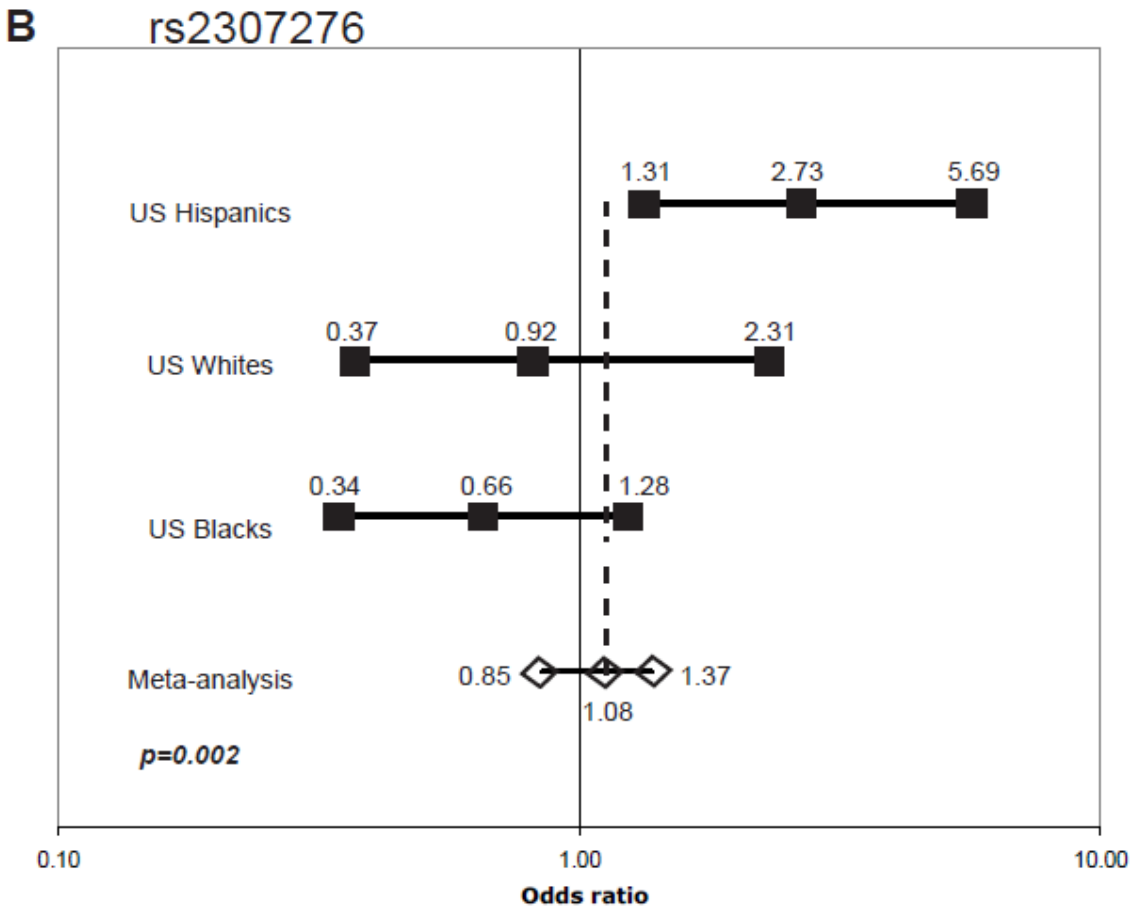
Species name followed by a letter indicates that multiple copies homologous to human *PLA2G4C* were identified for that species. Phylogenetic tree of coding sequences follows expected relationships between species, suggesting that the duplication event of chromosome 1sequence to chromosome 19 did not include coding sequence.

Figure 5.4A: Meta-analysis of significantly associated SNPs in *PLA2G4C*.



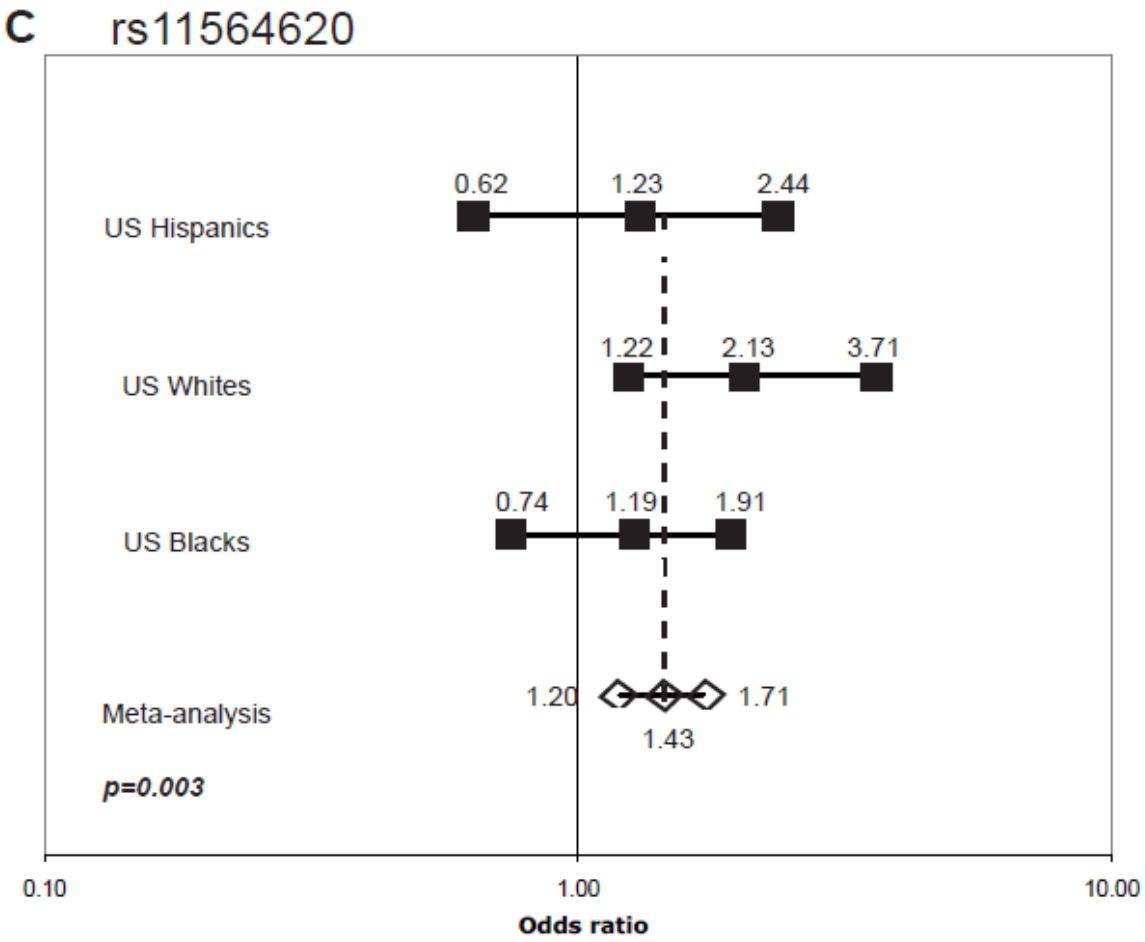
Meta-analysis for US Hispanic, US White and US Black SNP association results for rs8110925.

Figure 5.4B: Meta-analysis of significantly associated SNPs in *PLA2G4C*.



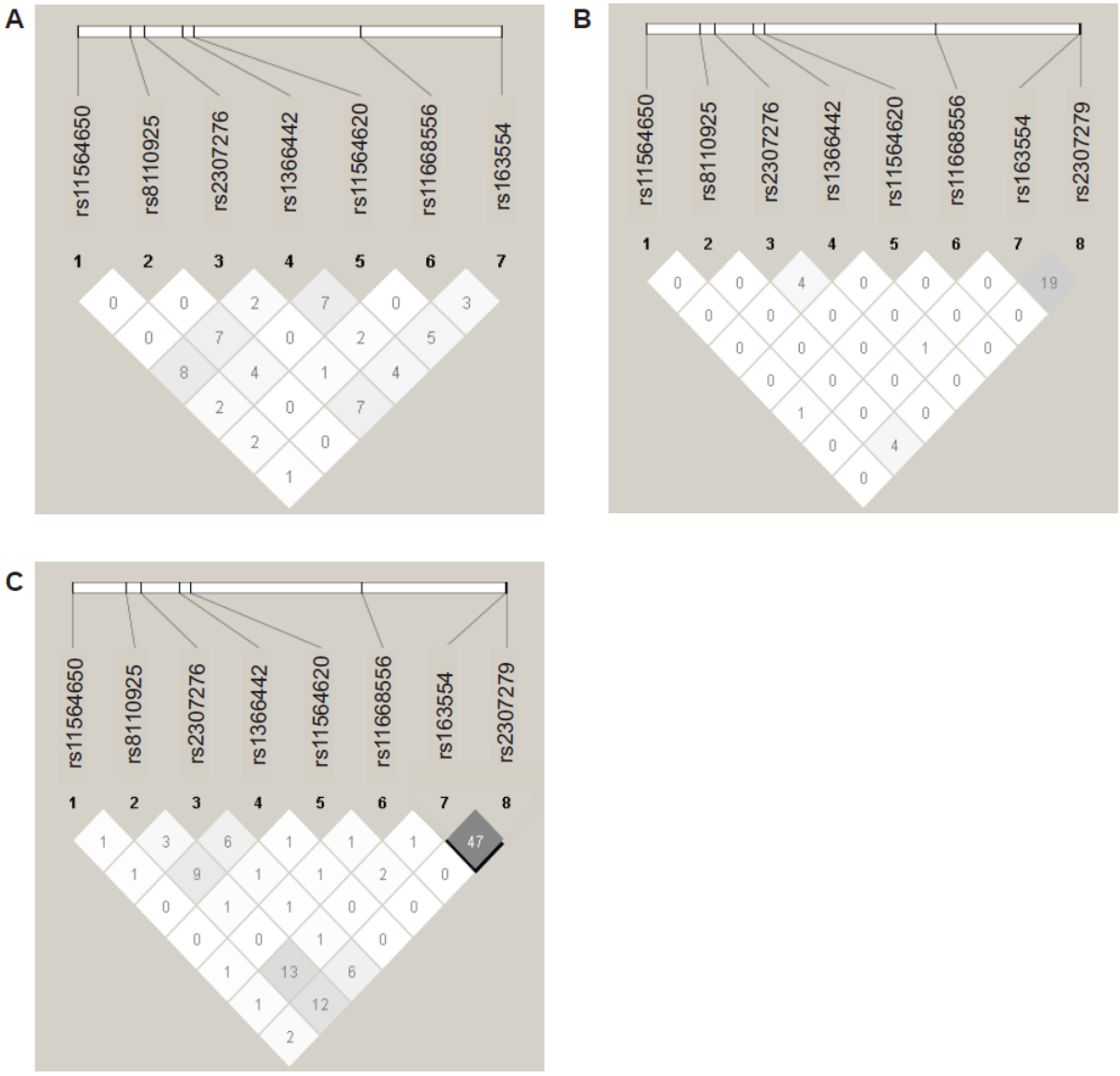
Meta-analysis for US Hispanic, US White and US Black SNP association results for rs2307276.

Figure 5.4C: Meta-analysis of significantly associated SNPs in *PLA2G4C*.



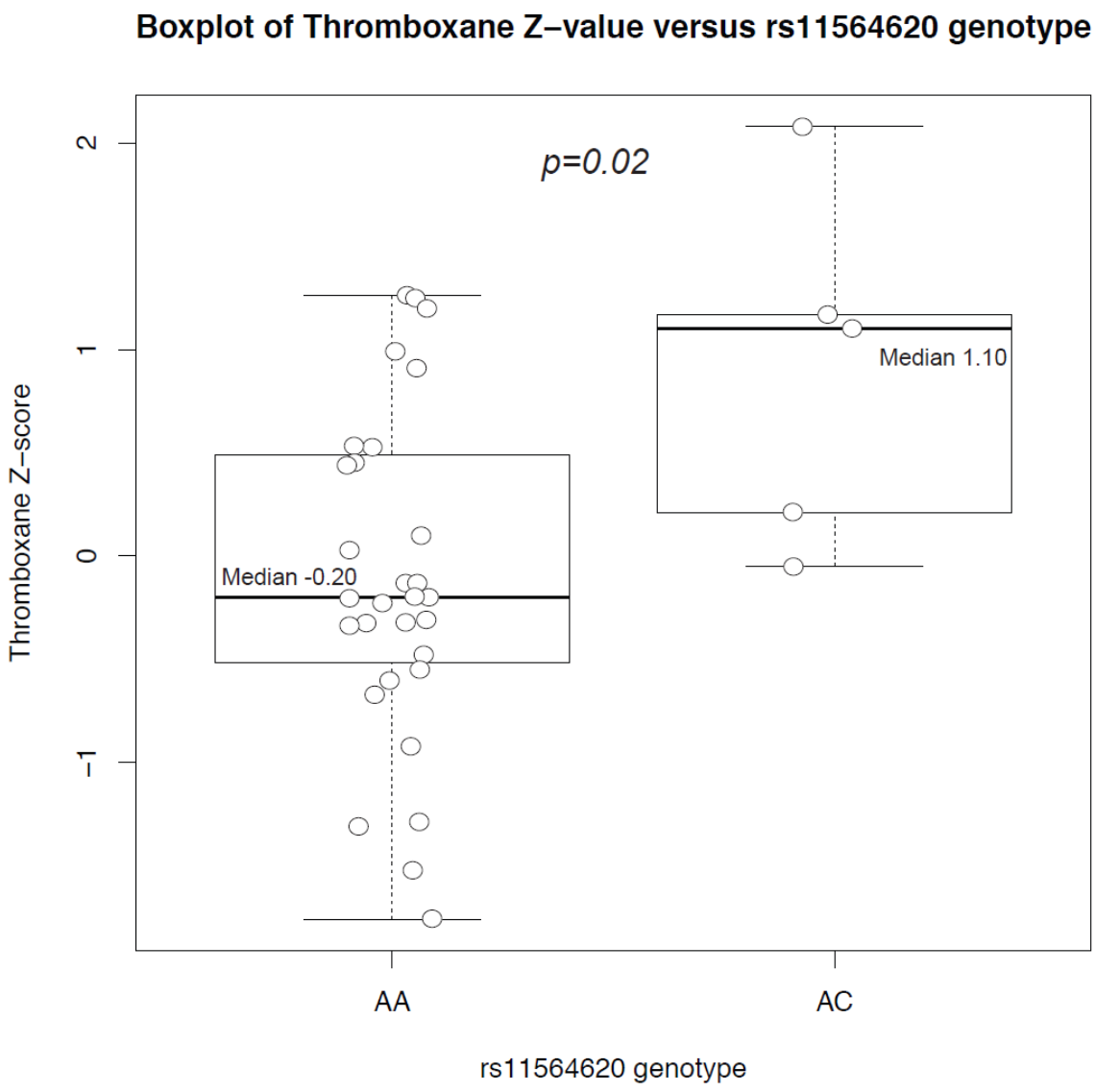
Meta-analysis for US Hispanic, US White and US Black SNP association results for rs11564620.

Figure 5.5: Linkage Disequilibrium among SNPs tested in *PLA2G4C*.



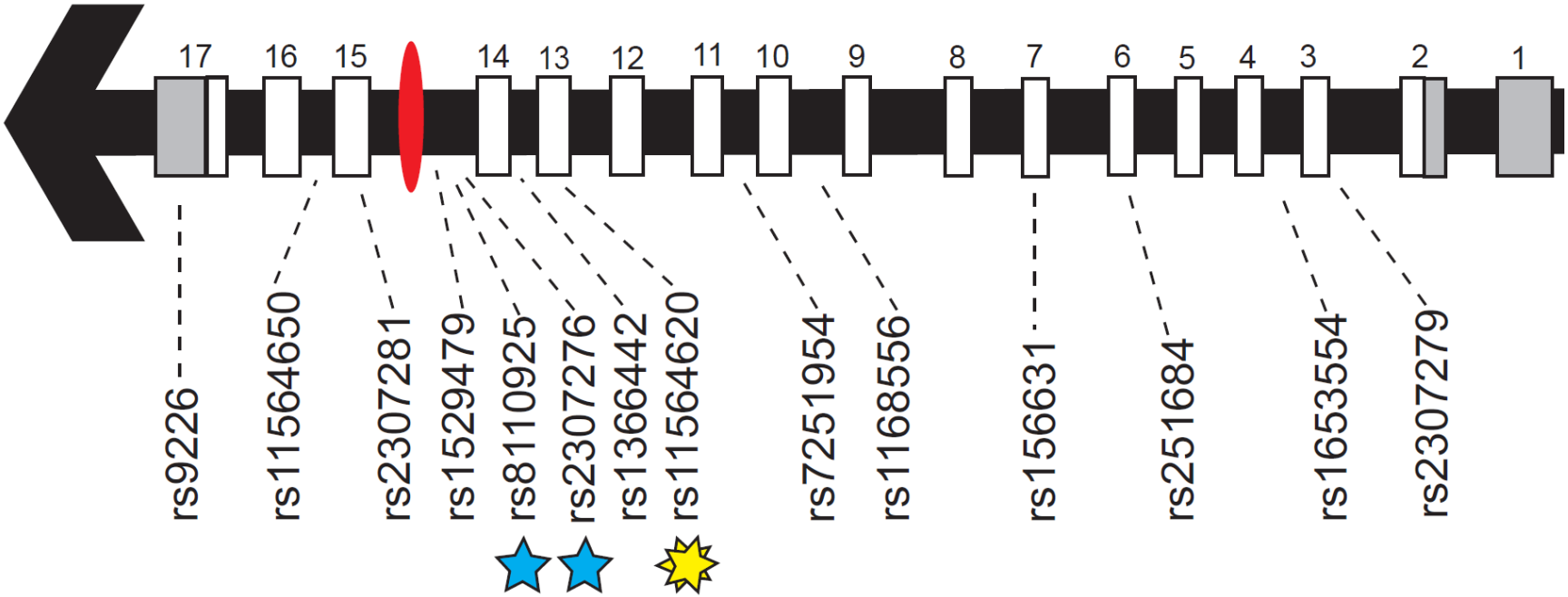
Linkage disequilibrium among SNPs tested in *PLA2G4C* for US Hispanics (A), US Whites (B), and US Blacks (C).

Figure 5.6: Comparison of thromboxane metabolite levels among rs11564620 genotype classes in healthy control population.



Median thromboxane metabolite levels are significantly greater among risk-allele carriers, by Wilcoxon one-sided test.

Figure 5.7: Overview of the SNPs tested in the *PLA2G4C* gene region.



The gene structure for *PLA2G4C* is represented by an arrow in which grey rectangles designate 3' and 5' untranslated regions and white rectangles designate coding exons. A red ellipse represents the primate-specific element of interest. Blue stars indicate rs2307276 and rs8110925, and yellow starburst indicates rs11564620, which is significant after multiple testing correction ($p<0.004$) in US Hispanics and US Whites, respectively.

Table 5.1: SNPs in the *PLA2G4C* gene region tested in all cohorts.

SNP	Position [a]	Location within gene
rs9226	48,551,546	Exon 17 - 3' UTR
rs11564650	48,556,979	Intron 15
rs2307281	48,558,286	Exon 15 - Asp426Asp
rs1529479	48,558,388	Intron 14
rs8110925	48,563,432	Intron 14
rs2307276	48,565,207	Intron 14
rs1366442	48,569,709	Intron 13
rs11564620	48,571,072	Exon 13 - Thr360Pro
rs7251954	48,583,239	Intron 10
rs11668556	48,591,380	Intron 9
rs156631	48,598,823	Exon 7- Ser203Pro
rs251684	48,601,454	Exon 6 - Pro170Pro
rs1653554	48,608,472	Intron 3
rs2307279	48,608,598	Exon 3 - Ala38Pro

[a] Positions refer to hg19 build of the human genome.

Table 5.2: Case-control association results for SNPs in the *PLA2G4C* gene region tested across 4 independent populations.

	US Hispanics		US White		US Blacks	
	(n= 73 cases, 292 controls)		(n= 147 cases, 157 controls)		(n= 79 cases, 166 controls)	
SNP	Allele p-value	Genotype p-value	Allele p-value	Genotype p-value	Allele p-value	Genotype p-value
rs9226	NA[b]	NA[b]	NA[b]	NA[b]	NA[b]	NA[b]
rs11564650	0.98	0.55	0.14	0.19	0.20	0.26
rs2307281	NA[b]	NA[b]	NA[b]	NA[b]	NA[b]	NA[b]
rs1529479	NA[b]	NA[b]	NA[b]	NA[b]	NA[b]	NA[b]
rs8110925	**7.92×10^{-4} [a,c]**	**5.66×10^{-5} [c]**	0.92	0.59	0.58 [d]	0.83 [d]
rs2307276	**5.45×10^{-3} [c]**	0.01	0.87	0.86	0.22	0.49
rs1366442	**0.01**	**0.03**	0.50	**0.02**	0.92 [d]	0.94 [d]
rs11564620	0.55 [e]	0.63 [e]	**6.98×10^{-3}**	**1.03×10^{-3} [c]**	0.47 [e]	0.36 [e]
rs7251954	NA[b]	NA[b]	NA[b]	NA[b]	NA[b]	NA[b]
rs11668556	0.32	0.28	0.78	0.85	0.55	0.38
rs156631	NA[b]	NA[b]	NA[b]	NA[b]	NA[b]	NA[b]
rs251684	NA[b]	NA[b]	NA[b]	NA[b]	NA[b]	NA[b]
rs1653554	0.84	0.30	0.37	0.51	0.44	0.43
rs2307279	NA[b]	NA[b]	0.59	0.12	0.83	0.89

[a]Bolded numbers indicate p-value <0.05.
[b]Marker excluded for failing one or more of the following measures: Hardy-Weinberg Equilibrium failure in controls p<0.001, <90% call rate, MAF<0.01.
[c]Marker significant correcting for 14 tests (p<0.004).
[d]Same allele trends in same direction as US Hispanics
[e]Same allele trends in same direction as US Whites

Table 5.3: Case-control association results for 2, 3 and 4 SNP haplotypes in the *PLA2G4C* gene region tested across 3 independent US populations.

	US Hispanics p-value	US White p-value	US Blacks p-value
2-SNP Haplotypes			
rs11564650-rs8110925	**$9.54x10^{-3}$** [a]	0.10	0.59
rs8110925-rs2307276	**$6.98x10^{-5}$** [b]	0.98	0.24
rs2307276-rs1366442	**$8.82x10^{-3}$**	0.64	0.64
rs1366442-rs11564620	**0.04**	**0.03**	0.90
rs11564620-rs11668556	0.25	**0.03**	0.63
rs11668556-rs1653554	0.49	0.82	0.42
rs1653554-rs2307279	NA[c]	0.47	0.68
3-SNP Haplotypes			
rs11564650-rs8110925-rs2307276	**$9.60x10^{-4}$** [b]	0.19	0.39
rs8110925-rs2307276-rs1366442	**$5.79x10^{-4}$** [b]	0.84	0.50
rs2307276-rs1366442-rs11564620	**0.03**	0.06	0.79
rs1366442-rs11564620-rs11668556	0.06	0.08	0.92
rs11564620-rs11668556-rs1653554	0.31	0.09	0.30
rs11668556-rs1653554-rs2307279	NA[c]	0.91	0.89
4-SNP Haplotypes			
rs11564650-rs8110925-rs2307276-rs1366442	**$3.26x10^{-3}$**	0.24	0.67
rs8110925-rs2307276-rs1366442-rs11564620	**$1.25x10^{-3}$** [b]	0.13	0.46
rs2307276-rs1366442-rs11564620-rs11668556	0.08	0.17	0.93
rs1366442-rs11564620-rs11668556-rs1653554	0.08	0.25	0.47
rs11564620-rs11668556-rs1653554-rs2307279	NA[c]	0.11	0.54

[a]Bolded numbers indicate p-value <0.05.
[b]Haplotype significant correcting for 18 comparisons ($p< 2.78x10^{-3}$).
[c] One or more marker excluded for failing one or more of the following measures: Hardy-Weinberg Equilibrium failure in controls p<0.001, <90% call rate, MAF<0.01.

Table 5.4: Association results for associated SNPs (p≤0.01) in the *PLA2G4C* gene region for the quantitative phenotypes of PGE, PGI, and TXB2 metabolite levels examined in healthy individuals (n=44).

SNP	PGE p-value	PGI p-value	TXB2 p-value
rs8110925	0.44	0.42	0.51
rs2307276	0.50	0.75	0.80
rs11564620	0.57	0.15	**0.04**[a]

[a]Bolded numbers indicate p-value <0.05.

Chapter 6: Conclusions and future directions

State of preterm birth genetics prior to this work

Despite the important public health consequences of PTB [3; 4], determinants of human parturition remain largely uncharacterized, making prediction and prevention of PTB difficult. Genetic studies are one way in which we can attempt to better understand this disorder. Prior to this work, little work had been done to characterize genetic influences on PTB comprehensively. For example, while a variety of evidence suggested that PTB was influenced by complex genetic and environmental factors, no study had tested genetic and non-genetic models to support this notion explicitly. Additionally, few specific genes were associated with PTB at the initiation of this work. Importantly, few studies examined genes outside of the inflammation and infection pathways, limiting the potential of genetic studies to identify new biology. As a result, we began this work to describe genetic influences on PTB in greater depth and identify novel genes associated with this disorder.

Dissertation specific aims

Because the etiology of PTB is complex and likely involves both genetic and environmental risk factors, developing a model for the genetic influences on PTB may facilitate gene discovery. As little work had been done to systematically identify a genetic model for PTB,we used sibling risk estimates and segregation analyses to identify one.

We examined two standard measures of familial aggregation, the sibling risk ratio, λs, and the sibling-sibling odds ratio (sib-sib OR) to test whether siblings of preterm infants were at higher risk for preterm delivery themselves. Risk to siblings of an affected individual was elevated above the population prevalence of a given disorder, as

indicated by λ_S (λ_S (95% CI): 4.3 (4.0-4.6)), and above that of siblings of unaffected individuals, as indicated by the sib-sib OR (sib-sib OR adjusted for known risk factors (95% CI): 4.2 (3.9-4.5)). These results suggest that the PTB aggregates in families, which may be explained in part by genetics.

Additionally, we performed segregation analyses to identify the best fitting genetic model for gestational age, a quantitative proxy for PTB. We performed segregation analysis for gestational age as a quantitative trait either attributed to the infant, infant's gestational age, or to the mother, by averaging the gestational ages at which her children were delivered, using 96 multiplex preterm families. Additionally, as pregnancies in which either the mother [10; 13] or father [6; 7] is Black are at increased risk for preterm delivery, we performed segregation analysis for each phenotype in the total sample, as well as stratified by Black and White race, to test for heterogeneity between these two groups. Results from our segregation analyses lend further support to a genetic component contributing to birth timing since sporadic (i.e. no familial resemblance) and nontransmission (i.e. environmental factors alone contribute to gestational age) models are strongly rejected. Moreover, these results corroborate the conceptualization of PTB as a complex diseases are influenced by a variety of factors, none necessary and sufficient to cause the disorder itself, in contrast to Mendelian disorders in which alterations of a single gene can lead to disease. Analyses of gestational age attributed to the infant support a model in which mother's genome and/or maternally-inherited genes acting in the fetus are largely responsible for birth timing, with a smaller contribution from the paternally-inherited alleles in the fetal genome. Additionally, results from a heterogeneity χ^2 test comparing race-stratified analyses suggest that

genetic influences on birth timing may differ between Blacks and Whites. Overall, as multiple genes in the mother's genome may explain the bulk of genetic influences on birth timing and heterogeneity exists among racial groups, future studies to identify specific genes influencing PTB perhaps will be most fruitful by using large scale studies of mothers' genomes and by considering racial composition of the study samples carefully in their statistical analysis.

We also aimed to discover specific genes associated with PTB using an *a priori* method, a screen of genes selected based on an evolutionary-motivated filter, rather than predicted parturition physiology. Because humans are born developmentally less mature than other mammals [124; 125], birth timing mechanisms may differ between humans and model organisms that have been typically studied [126]; as a result,we screened 150 genes, selected because of their rapid evolution along the human lineage, compared to other mammalian species, rather than our current understanding of parturition. An initial screen of over 8000 SNPs in 165 preterm and 163 Finnish mothers identified an enrichment of variants in *FSHR* associated with PTB and prompted further study of the gene. Additionally, the phospholipase gene, *PLA2G4C*, was examined in greater depth, as it was identified as the gene with the most statistically significant evidence for rapid evolution that was also included in a list of PTB candidate genes [134]. Several variants in *PLA2G4C* showed potential association and were considered for follow-up analysis.

To validate our initial findings,we examined 74 variants showing moderate levels of association in Finnish mothers in three additional US populations. Three SNPs in *PLA2G4C* and one SNP in *FSHR* were statistically significant after correcting for the number of independent tests performed for each gene. Meta-analysis p-values for these

variants were also significant correcting for multiple comparisons, supporting the role of *PLA2G4C* and *FSHR* in PTB.

Additional work to identify variants in these genes with functional effects was also initiated. First, we compared metabolite levels of three important downstream targets of *PLA2G4C* among genotype classes for significantly associated SNPs in healthy controls. The nonsynonymous SNP rs11564620 (Thr360Pro) was associated with levels of the prostaglandin thromboxane, suggesting this variant may affect *PLA2G4C* activity. Secondly, because the robustly associated SNP in *FSHR* was intronic and unlikely to have a direct effect on function, we initiated sequencing of coding and highly conserved non-coding regions in the gene in Finnish preterm and control mothers to identify additional variants, which may have functional effects.

Together, these experiments better characterize the nature of genetic influences on PTB and provide evidence for novel genes involved in this disorder.

Future directions

While two novel genes have been associated with PTB as the result of this dissertation project, additional work will be needed to dissect the genetic underpinnings of PTB robustly and apply this new knowledge towards improved patient care. Such work may include identifying additional variants in *FSHR* and *PLA2G4C* and determining the ability of highly associated SNPs to predict PTB. Because this work has examined only common variants, investigating rare variants may be particularly enlightening. Rare variants tend to have stronger effects on phenotype and may underlie associations attributed to common variants [196]. A single rare variant or a combination of multiple rare variants in *FSHR, PLA2G4C* or other genes may contribute to PTB risk, as has been shown for other common diseases, like type 1 diabetes and obesity [197];

thus, whole exome or targeted sequencing may identify risk-promoting variants in *FSHR* and *PLA2G4C,* and additional genes involved in PTB. For genetic variants of interest, gravid women may be followed prospectively to compare the pregnancy histories between women with high risk and low risk genotypes. Further study of the proteins encoded by *FSHR* and *PLA2G4C* will also be important to understand the biological mechanism by which these genes influence PTB risk. In addition to extensions of our work on PTB, the evolutionarily-motivated approach we used to identify *FSHR* and *PLA2G4C* also may be applied to other traits that differ between humans and other mammalian species, such as neurological and language-related traits, to assist gene discovery.

Alternative approaches may also be used to identify additional genes associated with PTB. Unbiased genome-wide screens, such as the case-control screen conducted on a Danish cohort as part of the National Institutes of Health Gene Environment Association Studies (GENEVA) program, may identify novel genes and pathways for birth timing, as for other complex disorders like type 2 diabetes [198]. Additionally, non-additive genetic effects, such as copy number or structural variation, may be important avenues for future research. Moreover, because complex disorders likely depend on a number of interacting factors, including genetic, epigenetic and environmental risk factors [31], some polymorphisms may only increase risk in the context of other genetic polymorphisms or certain environmental factors; as a result, considering polymorphisms in multiple genes or gene-environment interactions may increase our power to detect genetic effects. Investigators have applied this approach in candidate gene studies of PTB, including two studies of *IL6* found association with the gene and disorder only in

the presence of bacterial vaginosis [35] or microbial invasion of intra-amniotic cavity [37]. Similarly, a study found interactive effects for *TNF* x *IL6* x *IL6R*, but no main effects for any of the genes [34]. As a result, testing for gene-gene and gene-environment interactions globally may improve our ability to discover novel PTB genes. Such approaches may enable investigators to identify novel genes and pathways involved in birth timing with important clinical applications.

Overall, future studies to understand genetic influences on PTB likely will include identifying additional genes and specific variants associated with risk and followed with work to examine biological mechanisms for their involvement in birth timing. In the same way that the study of other complex disorders has affected the field of PTB genetics, the evolutionarily-motivated approach for prioritizing genes for inclusion in association studies we used to identify *FSHR* and *PLA2G4C* may be extended to other complex traits.

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181. Mitchell BF, Taggart MJ (2009) Are animal models relevant to key aspects of human parturition? Am J Physiol Regul Integr Comp Physiol 297:R525-545.
182. Frazer KA, et al. (2003) Genomic DNA insertions and deletions occur frequently between humans and nonhuman primates. Genome Res 13:341-346.
183. Wetterbom A, et al. (2006) Comparative genomic analysis of human and chimpanzee indicates a key role for indels in primate evolution. J Mol Evol 63:682-690.
184. Huh JW, et al. (2009) Gain of new exons and promoters by lineage-specific transposable elements-integration and conservation event on CHRM3 gene. Mol Cells 28:111-117.
185. Lee JR, et al. (2009) Lineage specific evolutionary events on SFTPB gene: Alu recombination-mediated deletion (ARMD), exonization, and alternative splicing events. Gene 435:29-35.
186. Tithof PK, et al. (2007) Distinct phospholipase A2 enzymes regulate prostaglandin E2 and F2alpha production by bovine endometrial epithelial cells. Reprod Biol Endocrinol 5:16.
187. Elsik CG, et al. (2009) The genome sequence of taurine cattle: a window to ruminant biology and evolution. Science 324:522-528.
188. Ramensky V, Bork P, Sunyaev S (2002) Human non-synonymous SNPs: server and survey. Nucleic Acids Research 30:3894-3900.
189. Ng PC, Henikoff S (2003) SIFT: predicting amino acid changes that affect protein function. Nucleic Acids Research 31:3812-3814.

190. Underwood KW, et al. (1998) A novel calcium-independent phospholipase A2, cPLA2-gamma, that is prenylated and contains homology to cPLA2. J Biol Chem 273:21926-21932.
191. Khan AH, Carson RJ, Nelson SM (2008) Prostaglandins in labor--a translational approach. Front Biosci 13:5794-5809.
192. Hayashi M, et al. (2002) The levels of five markers of hemostasis and endothelial status at different stages of normotensive pregnancy. Acta Obstet Gynecol Scand 81:208-213.
193. Gross G, et al. (2000) Inhibition of cyclooxygenase-2 prevents inflammation-mediated preterm labor in the mouse. Am J Physiol Regul Integr Comp Physiol 278:R1415-1423.
194. Burns PD, et al. (2001) Cellular mechanisms by which oxytocin mediates ovine endometrial prostaglandin F2alpha synthesis: role of G(i) proteins and mitogen-activated protein kinases. Biol Reprod 65:1150-1155.
195. Vitale A, et al. (2005) Mouse cPLA2[gamma], a novel oocyte and early embryo-abundant phospholipase A2 gamma-like protein, is targeted to the nuclear envelope during germinal vesicle breakdown. Developmental Biology 282:374-384.
196. Dickson SP, et al. Rare Variants Create Synthetic Genome-Wide Associations. PLoS Biol 8:e1000294.
197. Schork NJ, et al. (2009) Common vs. rare allele hypotheses for complex diseases. Current Opinion in Genetics & Development 19:212-219.
198. Grant SF, et al. (2006) Variant of transcription factor 7-like 2 (TCF7L2) gene confers risk of type 2 diabetes. Nat Genet 38:320-323.

Curriculum Vitae

Jevon Plunkett

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1343 Melia Street, Camarillo, California 93010
(805) 987-4743

RESEARCH INTERESTS

My main research interest is the genetic etiology of adverse pregnancy outcomes. I am particularly interested in exploring the complexity of pathways to disease, incorporating genetic information with physiological and environmental data to create a holistic view of disease. I am also interested in using genetic information in pharmacogenetic studies and public health screenings related to obstetrics.

EDUCATION

Washington University, Saint Louis, Missouri

- **Doctor of Philosophy in Human and Statistical Genetics,** August 2010

Dissertation: Genetics Influences on Preterm Birth
Mentors: Louis Muglia, M.D., Ph.D. and Ingrid Borecki, Ph.D.

- **Bachelor of Arts summa cum laude** in biology, with minor in psychology, May 2006

Honors thesis: Complexities in the Genetics of Alcohol Dependence: The Chromosome 5 GABA-A Receptor Gene Cluster and Alcohol-Related Behaviors in the Collaborative Study on the Genetics of Alcoholism
Mentor: Danielle Dick, Ph.D.

RESEARCH EXPERIENCE

- **Doctoral student**, 2006-2010

Washington University (2006-2008), Vanderbilt University (2009-2010)
Mentor: Louis Muglia, M.D., Ph.D.
Sibling risk estimates, segregation analysis, and case-control association tests to detect genetic effects in preterm birth.
**Fellowship support from: Mr. and Mrs. Spencer T. Olin Fellowship for Women in Graduate Study at Washington University in St. Louis, 2006-2010; National Institute of General Medical Science training grant (T32 GM081739) for interdisciplinary training of graduate students in psychology, neuroscience and genetics at Washington University, 2007-2009

- **Undergraduate honors student**, 2005-2006

Washington University Mentor: Danielle Dick, Ph.D.
Family-based SNP and haplotype association tests, genotype-environment interaction tests in Collaborative Study on Genetics of Alcoholism (COGA) sample.
**Summer funding from: Washington University Hoopes Undergraduate Research Award, 2005; Washington University/Howard Hughes Medical Institute (HHMI) Summer Undergraduate Research Fellowship, 2005
**Travel Award: Hoopes Undergraduate Research Travel Award, Washington University, 2006; Washington University/HHMI Undergraduate Research Travel Award, 2006

- **Undergraduate researcher**, 2002-2004

Washington University Mentor: Alan Shiels, Ph.D.
Parametric linkage analysis reactions and direct sequencing for family studies of hereditary eye disorders
**Summer funding from Washington University/HHMI Summer Undergraduate Research Fellowship, 2004; Washington University/HHMI Summer Scholars Program in Biology and Biomedical Research Award, 2002

TEACHING EXPERIENCE
Vanderbilt University, Nashville, Tennessee
- **Mentored graduate student's rotation project**, 2010
- **Mentored high school student's summer project**, 2009, 2010
- **Guest lecturer,** Research Internship Program, 2009

Washington University, Saint Louis, Missouri
- **Course Instructor,** Problem-based Learning (lower division undergraduate), Fall 2008
- **Course Instructor,** Summer Focus program Writing Course (rising high school seniors in summer research program), Summer 2008
- **Teaching Assistant,** Human Genetics (upper division undergraduate), Spring 2008
- **Teaching Assistant,** Fundamentals of Mammalian Genetics (graduate), Fall 2007
- **Teaching Assistant,** Principles of Biology II: Genetics (lower division undergraduate), Fall 2004
- **Tutor**, Principles of Biology courses, 2005-2006

OTHER PROFESSIONAL EXPERIENCE
- **Ad hoc reviewer**, Journal of Women's Health 2009, American Journal of Epidemiology 2008
- Professional Affiliations with: **American Society for Human Genetics** (ASHG) 2007-present; **International Genetic Epidemiology Society** 2009; **American Medical Writers Association** 2009; **Association for Women in Science**, 2006; **Behavior Genetics Association**, 2006
- **Shadowed Maternal-Fetal Medicine specialist**, Lavinda Carpenter, M.D., Vanderbilt University, Nashville, Tennessee, 2010
- **Clinic observer and volunteer at the student-run Shade Tree Family Clinic**, Vanderbilt University, Nashville, Tennessee, 2010
- **Participation in teaching development workshops** hosted by: American Society of Microbiology Conference on Undergraduate Education, Beverly, Massachusetts, 2008; Vanderbilt University Center for Teaching, Nashville, Tennessee, 2009-2010; Washington University Teaching Center, Saint Louis, Missouri, 2007-2008

UNIVERSITY SERVICE
Vanderbilt University, Nashville, Tennessee
- **Human Genetics Student Association member**, 2009-2010
- **Editor's Club member**, 2009-2010
- **Annals of Biomedical Student/Trainee Research and Current Trends (A.B.S.T.R.A.C.T.) publication contributor**, 2009

Washington University, Saint Louis, Missouri
- **Washington University Future Educators member and group facilitator**, 2006-2008
- **Human and Statistical Genetics program website development committee**, 2008
- **Graduate School of Arts and Sciences Peer Mentor**, 2007-2008
- **Human and Statistical Genetics program recruitment**, 2007-2008
- **Olin Fellowship Conference planning committee**, 2006-2008

PUBLICATIONS

Submitted / in preparation

1. **Plunkett J**, Doniger S, Orabona G, Morgan T, Haatajae R, Hallmane M, Puttonenf H, Menon R, Kuczynskii E, Norwitzi E, Snegovskikhi V, Palotie A, Peltonen L, Fellman V, DeFranco EA, Chaudhari BP, Teramo K, Borecki I, Fay J, Muglia L. Rapid evolution of *FSHR* in humans predicts role in birth timing. Submitted.
2. Haataja R, Karjalainen MK, Luukkonen A, Teramo K, Puttonen H, Ojaniemi M, Varilo T, Chaudhari BP, **Plunkett J**, Murray JC, McCarroll SA, Peltonen L, Muglia LJ, Palotie A, Hallman M. Mapping a new spontaneous preterm birth susceptibility gene *IGF1R* using linkage, haplotype sharing and association analysis. Submitted.
3. **Plunkett J**, Doniger S, Orabona G, Morgan T, Haatajae R, Hallmane M, Puttonenf H, Menon R, Kuczynskii E, Norwitzi E, Snegovskikhi V, Palotie A, Peltonen L, Fellman V, DeFranco EA, Chaudhari BP, Teramo K, Borecki I, Fay J, Muglia L. Primate-specific evolution of conserved noncoding element insertion into *PLA2G4C* and human preterm birth. In preparation.

Original research

1. Daw EW, **Plunkett J**, Feitosa M, Gao X, Van Brunt A, Ma D, Czajkowski J, Province MA, Borecki I. (2009) A framework for analyzing both linkage and association: an analysis of Genetic Analysis Workshop 16 simulated data. BMC Proc. 3 Suppl 7:S98.
2. **Plunkett J**, Feitosa MF, Trusgnich M, Wangler M, Palomar L, Kistka ZAF, DeFranco EA, Shen T, Stormo A, Puttonen H, Hallman M, Haataja R, Fellman V, Peltonen L, Palotie A, Daw EW , An P, Teramo K, Borecki I, Muglia LJ. (2009) Mother's genome or maternally-inherited genes acting in the fetus influence gestational age in familial preterm birth. Hum Hered 68:209-219.
3. **Plunkett J**, Borecki I, Morgan T, Stamilio D, Muglia LJ. (2008) Population-based estimate of sibling risk for adverse pregnancy outcomes. BMC Genetics, 9:44.
4. Kistka ZA, DeFranco EA, Ligthart L, Willemsen G, **Plunkett J**, Muglia LJ, Boomsma DI. Heritability of parturition timing: an extended twin design analysis. (2008) Am J Obstet Gynecol., 199 (1): 43 e1-5.
5. Dick DM, Wang JC, **Plunkett J**, Aliev F, Hinrichs A, Bertelsen S, Budde JP, Goldstein EL, Kaplan D, Edenberg HJ, Nurnberger J, Hesselbrock V, Schuckit M, Kuperman S, Tischfield J, Porjesz B, Begleiter H, Bierut LJ, & Goate A. (2007) Family-based association analyses of alcohol dependence phenotypes across DRD2 and neighboring gene ANKK1. Alcohol Clin Exp Res., 31(10): 1645-53.
6. Dick DM, **Plunkett J**, Hamlin D, Nurnberger J, Kuperman S, Schuckit M, Hesselbrock V, Edenberg H, Bierut L. (2007) Association analyses of the serotonin transporter gene with lifetime depression and alcohol dependence in the Collaborative Study on the Genetics of Alcoholism (COGA) sample. Psychiatric Genet., 17(1): 35-8.
7. Dick DM, **Plunkett J**, Wetherill LF, Xuei X, Goate A, Hesselbrock V, Schuckit M, Crowe R, Edenberg HJ, Foroud T. (2006) Association between GABRA1 and drinking behaviors in the collaborative study on the genetics of alcoholism sample. Alcohol Clin Exp Res., 30(7): 1101-10.

Invited Review

1. **Plunkett J** & Muglia LJ. (2008) Genetic Contributions to Preterm Birth: Implications from Epidemiological and Genetic Association Studies. Ann Med., 40(3):167-95.
2. Chaudhari BP, **Plunkett J**, Ratajczak CK, Shen TT, DeFranco EA, Muglia LJ. (2008) The genetics of birth timing: insights into a fundamental component of human development. Clin Genet., 74(6):493-501.

ABSTRACTS AND PRESENTATIONS

Abstracts

- **Plunkett J**, Muglia L. (2007) Sibling risk for adverse pregnancy outcomes. Am J Obstet Gynecol., 197 (6): S186.
- Defranco E, Kistka Z, Ligthart L, Willemsen G, **Plunkett J**, Muglia L, Boomsma DI. (2007) Heritability of parturition timing: An extended twin design analysis. Am J Obstet Gynecol., 197 (6): S199.

Presentations

- Rapidly evolving genes as candidate genes for preterm birth (2009) Platform presentation at American Society for Human Genetics Annual Meeting, Honolulu, Hawaii.
- Mother's genome or maternally-inherited genes acting in the fetus influence gestational age in familial preterm birth (2008) Platform presentation at American Society for Human Genetics Annual Meeting. Philadelphia, Pennsylvania.
- Sibling Risk for Adverse Pregnancy Outcomes (2008) Poster presentation at Society for Maternal-Fetal Medicine Annual Meeting, Dallas, Texas.
- Association between GABRA1 and Drinking Behaviors in the Collaborative Study on the Genetics of Alcoholism Sample (2006) Poster presentation at Behavior Genetics Association Annual Meeting, Storrs, Connecticut.

HONORS AND AWARDS

- **Semifinalist**, American Society of Human Genetics (ASHG) Trainee Award, Predoctoral Clinical category, 2009
- **Finalist**, ASHG Trainee Award, Predoctoral Clinical category, 2008
- **ASHG travel award** to attend American Society of Microbiology Conference on Undergraduate Education, 2008
- **Teaching citation**, Washington University, 2008
- **Phi Beta Kappa** Honor Society, 2006
- **Sigma Xi**, the Scientific Research Society, 2006
- Washington University College of Arts and Sciences **Dean's List**, 2002-2006

COMMUNITY INVOLVEMENT

Science outreach

- **Scientist on Site program participant**, Adventure Science Center, Nashville, Tennessee, 2009-2010
- **Graduate student mentor for Research Internship Program**, Vanderbilt University Center for Science Outreach, Nashville, Tennessee, 2009
- **Young Scientist Program**, Saint Louis Missouri, 2006-2008
    - Summer Focus program tutor, 2007, 2008
    - Teacher-Researcher Partnership member, 2008
    - Steering Committee member, 2006-2008
    - Forensics Teaching Team Member, 2006-2008
- **Science Fair Judge,** 2008-2009
    - Head Magnet Middle School, Nashville, Tennessee
    - Jackson Park Elementary School, University City, Missouri
- **Washington University Science Outreach**, Saint Louis Missouri, 2003-2006
    - Science Club for Webster Middle School, 2005-2006
    - Genetics Teaching Team Member 2003-2005

Service

- **Mentor for MentorNet e-mentoring program**, 2006-2010
- **First Church Unity**, Nashville, Tennessee, 2009-2010
    - Co-organizer, Creating Connections social networking group
    - Leader for teen programming
- **Breast Health Educator for Siloam Family Health Center**, Nashville, Tennessee, 2009
- **Girls on the Run International**, Nashville, Tennessee, 2009-2010
    - Coach and running buddy
    - Fundraising through SoleMates program
- **Team in Training**, The Leukemia & Lymphoma Society (LLS), Nashville, Tennessee, 2009
    - Team member**-** raised $1835 while training for the 2009 Country Music Half Marathon
    - Team captain - fundraising and training support for new team members
- **Restore St. Louis**, Saint Louis, Missouri, 2007-2008
    - Tutor, Firm Foundations Tutoring Program
    - Volunteer and crew leader, Work Day ministry

<u>Hobbies and personal interests</u>

- Endurance running
    - completed 2 half marathons to date, training for full marathon in 2010
- Ballroom dancing
    - Bronze level dancer
- Member of Toastmasters International, 2007-2008