

**Population Frequencies for Selected Genetic Variants in the Iowa Population
Genes Important in Pyloric Stenosis**

**A proposal to the Center for Congenital and Inherited Disorders,
Iowa Department of Public Health**

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Summary

Previously, we received approval to use residual newborn metabolic screening specimens to determine the carrier frequency for the most common Fukutin kinase-related protein gene (FKRP) variant, C826A, in the Iowa population. DNA was extracted and genotyped for the C826A variant from 4,115 randomly selected residual specimens from the Iowa newborn metabolic screening program. We also received approval to retain the extracted DNA and specimens to use for testing additional variants for future projects approved by the Center for Congenital and Inherited Disorders Advisory Committee (CIDAC). As such, we propose to identify population frequencies for variants in selected genes suggested to influence development of selected, major structural birth defects. Previous studies have been limited by use of small convenience samples, thereby limiting the accuracy and generalizability of identified frequencies. Use of a well-characterized, population-based sample will greatly facilitate our knowledge of the frequency of these individual variants and the selection of such variants to include in large-scale etiologic studies. Herein we describe our proposed use of Iowa residual newborn metabolic screening specimens to generate population frequencies for variants in selected genes thought to influence development of pyloric stenosis. Successful completion of this proposal will permit application of this approach to variants in genes thought to influence development of other birth defects.

Specific Aim

To use residual specimens, initially obtained to study the frequency of the FKRP C826A variant, to identify the Iowa population frequencies for variants in the following genes: Nitric Oxide Synthase 1 (NOS1); Smooth Muscle Myosin Heavy Chain 11 (MYH11); N-Methyl-D-Aspartate Receptor Subunit 2A (NMDAR2A); Transient Receptor Potential Cation Channel member 6 (TRPC6); Transient Receptor Potential Cation Channel member 5 (TRPC5); Motilin Preprotein (MLN); and Vitamin D [1,25-dihydroxyvitamin D3] receptor (VDR).

Background and Significance

Infantile hypertrophic pyloric stenosis, commonly referred to as pyloric stenosis, affects 1 to 8 deliveries per 1,000 live births. It is characterized by muscular hypertrophy of the pyloric sphincter, which causes obstruction of the gastric outlet in infants. This obstruction can produce projectile vomiting, dehydration, weight loss, electrolyte imbalance, and death in severe cases. Pyloric stenosis is typically diagnosed between 2 and 8 weeks after birth with pyloromyotomy being the standard method of treatment. It is the most common indication for surgery among infants.

Prevalence rates for pyloric stenosis have been found to be higher in teenage mothers, among male infants compared to female infants (4:1), and among Caucasians compared to African Americans (2:1); Asians and Native Americans tended to have the lowest prevalence rates. Among Caucasians worldwide, the prevalence rates of pyloric stenosis have differed over the past four decades. In the United States, relatively stable rates have been observed, whereas increased rates were observed in the United Kingdom and some parts of Europe in the 1970s and 1980s with stable or decreased rates observed in other parts of Europe. More recent estimates show a marked decline

in prevalence over the past two decades in Sweden, Denmark, and Scotland, which coincided with a decrease in sudden infant death syndrome (SIDS), and prompted some to hypothesize a dual benefit of the “back to sleep campaign”. Recent analyses of temporal trends of both pyloric stenosis and SIDS in Scotland failed to support this hypothesis.

Although a common condition among infants, the etiology of pyloric stenosis remains elusive. Several factors, such as preterm birth, birth weight (>4500 grams), neonatal exposure to selected medications (erythromycin and macrolides) as well as maternal pregnancy history, stress, smoking, and selected medications during pregnancy, have been linked to pyloric stenosis, but results have been inconsistent. The preponderance of affected males and Caucasians suggests a strong genetic component for pyloric stenosis. This thinking is further supported by the proportion (15%) of cases found to have a positive family history of pyloric stenosis; however, this defect does not demonstrate a recognized pattern of Mendelian inheritance as evidenced by recurrence estimates within sibships that tend to be considerably higher for males than females.

Despite evidence to suggest a genetic component for pyloric stenosis, to date, no major gene has been identified. Studies of extended pedigrees have identified autosomal-dominant, monogenetic forms of pyloric stenosis localized to regions 12q24.2-q24.31, 16p12-p13, 16q24, 11q14-q22, and Xq23. These regions, respectively, encode for aspects of control and regulation of gastrointestinal motility. For example, 12q24.2-q24.31 encodes for NOS1, which catalyzes nitric oxide formation in the peripheral nervous system that contributes to smooth muscle relaxation. Although promising, these findings have been generated using a rather small sample size (NOS1) or familial cases through studies of extended pedigrees (16p12-p13, 16q24, 11q14-q22, and Xq23). None have been replicated nor extrapolated to a population-based sample of nonfamilial, isolated cases. Further, a recent study of a small sample of pyloric stenosis patients and unaffected controls in Sweden that examined a coding in the motilin gene found no statistically significant association between the variant and occurrence of pyloric stenosis. This investigation was a follow-up to earlier reports of erythromycin as a risk factor for pyloric stenosis, as this drug acts as a motilin agonist.

Dr. Paul Romitti is a collaborator in the New York State Birth Defects Genomics Project, an intramurally-funded project of the National Institute of Child Health and Human Development. The goal of the project is to identify genetic variants important in the development of several major birth defects. As part of his role in the project, Dr. Romitti is taking the lead in investigating genetic risk factors for pyloric stenosis. As preparatory work for this analysis, it is necessary to establish frequencies for several non synonymous variants in genes that influence gastrointestinal motility using a sample population of European descent. The Appendix shows the information on genotype frequency available to date. Most information has been derived from the polymorphism discovery resource (PDR) panel and/or the Centre d'Etude du Polymorphisme Humain (CEPH) collection. The PDR panel includes samples from 90 individuals drawn from the United States population with European, African, Mexican, or Asian descent or who are Native Americans. The purpose of the panel was to discover human genetic

variation across ethnic subpopulations, but not to assess the frequency of variations in specific ethnic subpopulations. The CEPH-European collection contains 60 Utah residents with Northern and Western European ancestry and is one of the 11 ethnic subpopulations used in the International HapMap Project. Like the PDR panel, the CEPH collection was intended to identify genetic variation, but not frequency of such variation across racial/ethnic subpopulations. With the predominance of pyloric stenosis occurring among Caucasian populations, use of Iowa residual newborn screening specimens will provide an ideal population-based sample from which to generate frequencies of variants in the proposed genes.

Preliminary Studies

Dr. Romitti and Ms. Kristyn Rose have developed a rapid approach to screen for the presence of the C826A variant of FKRP and successfully applied this approach using Iowa residual newborn screening specimens. DNA was extracted from each of the 4,115 specimens and placed into 384-well microtitre plates. Genotyping was conducted using the 7900HT Fast Real-time PCR System and a TaqMan Assay-By-Design for the C826A variant from Applied Biosystems. Of the 4,115 specimens extracted, 4,092 (99%) were successfully genotyped.

Research Design and Methods

Multiple databases were used to identify relevant single nucleotide polymorphisms (SNPs) for investigation. The SNPs selected for each identified region were limited to nonsynonymous coding SNPs (i.e., nonsense, missense, or frame shift) identified in humans. Dr. Romitti and Ms. Rose have identified On-Demand Taqman Assays for 24 of the 50 variants listed in the Appendix; Assays-by-Design will be created for the remaining 26 variants. Per above, a portion of the extracted DNA will be placed into 384-well microtitre plates. Genotyping will be performed using the 7900HT Fast Real-time PCR System. The TaqMan system utilizes fluorescent tags to distinguish between the wild type and minor alleles.

With the approval of the Ms. Kim Piper, State Genetics Coordinator, Dr. Stan Berberich has provided information for sex, birth weight, gestational age, plurality, birth month and year and storage conditions (e.g., room temperature) to Dr. Romitti for the residual newborn screening specimens provided for the analysis of the presence of the FKRP C826A variant; information on race/ethnicity and birth defects was unavailable. We request that the birth certificate number for each provided residual specimen be linked to birth certificate information for maternal and paternal race/ethnicity to permit stratification of specimens into racial/ethnic subpopulations. We also request that the birth certificate number be linked to the Iowa Registry for Congenital and Inherited Disorders (IRCID) to exclude from analysis specimens of children with birth defects. We propose that the Dr. Berberich provide the birth certificate number of each specimen, delinked from the specimen identification number, to the IRCID to conduct the needed linkages. The IRCID will return codes for maternal and paternal race/ethnicity (white non-Hispanic, black non-Hispanic, other non-Hispanic, Hispanic) and birth defect (yes/no). Dr. Berberich will link the provide information to the specimen identification number and return the specimen identification number and race/ethnicity

and birth defect codes to Dr. Romitti. With only Dr. Berberich having the link between the birth certificate number and specimen identification number, this will retain the anonymity of the specimens for Dr. Romitti and his collaborators.

We propose to successfully genotype at least 500 residual specimens for each of the variants listed in the Appendix. For minor allele frequencies of 1%, generation of 500 genotypes per variant will permit identification of approximately 5 specimens with the minor allele. Specimens will be selected from those previously provided and extracted for the identification of the presence of the FKRP C826A variant. Specimens selected will be comprised of singleton births with a birth weight of 2,500 grams or more, gestational age greater than 37 weeks, race/ethnicity of non-Hispanic white and sex distribution of 51% females and 49% males, which is proportional to the sex ratio of live births in Iowa averaged over the past ten years.

We expect this work to be completed within a one-year period of time. Funding will be provided by the New York State Department of Health. Results of the study will be disseminated via direct communication with investigators in the fellow Birth Defects Genomics Projects researchers and will be included in selected publications developed by the investigators.

Personnel

Paul Romitti, Ph.D., is a reproductive molecular epidemiologist, an Associate Professor in the UI Department of Epidemiology, and the Director of the Iowa Registry for Congenital and Inherited Disorders. He has multiple years of experience in supervising molecular epidemiologic studies and conducting data analyses of environmental and genetic factors and gene-environment interactions in studies of birth defects.

Jeffrey Murray, M.D., is a human molecular and clinical geneticist and Professor of Pediatrics in the UI Carver College of Medicine. Dr. Murray currently directs the UI Craniofacial Anomalies Research Center. He is an internationally-recognized molecular geneticist with broad experience in gene discovery and studies of gene-environment interaction effects, with particular expertise in craniofacial disorders.

Kristyn Rose, M.S., has a master's degree in epidemiology and has six years experience with coordinating population-based sample collection and laboratory analysis for studies of congenital and inherited disorders. Ms. Rose also coordinates Dr. Romitti's laboratory work and will conduct sample extraction and genotyping.

Rhinda Goedken, B.A., Operations Coordinator/Genetic Analyst, has a bachelor's degree in biology and has nineteen years experience with analysis of genetic data. Ms. Goedken coordinates all operations for Dr. Romitti's research program and will conduct analysis of genotype data.

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Appendix

Chromosome 12q24.2-q24.31: NOS1

ref SNP ID	Function	ref SNP Allele	Protein Change	Minor Allele Frequency	Test Population
rs56308341	Missense	C/T	Arg/His	NA	NA
rs56266548	Missense	A/G	Ala/Val	NA	NA
rs56219642	Missense	C/T	Val/Met	NA	NA
rs55922940	Missense	A/G	Leu/Pro	NA	NA
rs41514244	Missense	A/G	Ser/Leu	NA	NA
rs41474747	Missense	A/G	Pro/Ser	NA	NA
rs41356652	Missense	C/T	Gly/Ser	NA	NA
rs41340250	Missense	C/T	Gly/Ser	NA	NA
rs9658482	missense	A/G	Gln/Arg	G=0.01	PDR90
rs9658445	missense	A/G	Gly/Asp	G=0.01 G=0.01	PDR90 HapMap-CEU
rs9658403	missense	A/G	Asn/Asp	G=0.01	PDR90
rs9658356	missense	A/C	Asp/Ala	C=0.01 C=0.50	HapMap-CEU HapMap-CEU
rs9658279	missense	C/T	Pro/Ser	T=0.01	PDR90
rs4519169	missense	G/A	Pro/Leu	A=0.00	HapMap-CEU
rs1879417		T/C		C=0.43	HapMap-CEU
rs35873722		-/T			NA
rs36077311		-/T			NA
rs96558256		C/T			NA

Chromosome 16p12-p13: MYH11

ref SNP ID	Function	ref SNP Allele	Protein Change	Minor Allele Frequency	Test Population
rs35176378	missense	T/C	Met/Val	C=0.50	HapMap-CEU
rs35035518	missense	G/A	Ser/Leu	NA	NA
rs34321232	missense	T/G	Lys/Gln	NA	NA
rs34263860	missense	C/T	Ala/Thr	T=0.00	HapMap-CEU
rs16967510	missense	T/C	Val/Ala	C=0.06	HapMap-CEU
rs16967494	missense	C/T	Ala/Thr	T=0.36	HapMap-CEU
rs12149651	missense	G/T	Leu/Met	T=0.00	HapMap-CEU
rs7196804	missense	C/T	Val/Met	T=0.00	HapMap-CEU
rs1801902	missense	G/A	Thr/Ala	NA	NA

Chromosome 16p12-p13: NMDAR2A

ref SNP ID	Function	ref SNP Allele	Protein Change	Minor Allele Frequency	Test Population
rs60429225	missense	C/G	Trp/Ser	NA	NA
rs59975221	missense	G/C	Pro/Ala	NA	NA
rs56241810	missense	C/A	Gly/Val	NA	NA
rs4781938	missense	T/G	Asn/Thr	G=0.00	HapMap-CEU

Chromosome 11q14-22: TRPC6

ref SNP ID	Function	ref SNP Allele	Protein Change	Minor Allele Frequency	Test Population
rs36111323	missense	G/A	Ala/Val	NA	NA
rs35857503	missense	T/G	Asn/Thr	G=0.02	HapMap-CEU
rs3802829	missense	G/A	Pro/Ser	NA	NA

Chromosome Xq23: TRPC5

ref SNP ID	Function	ref SNP Allele	Protein Change	Minor Allele Frequency	Test Population
rs36047478	missense	G/A	Arg/His	A=0.00	HapMap-CEU
rs28470854	missense	A/G	Leu/Pro	NA	NA
rs3027722	missense	A/T	Tyr/Phe	T=0.00	HapMap-CEU
rs3027721	missense	G/A	Ala/Thr	A=0.00	HapMap-CEU

Chromosome 6p21: MLN

ref SNP ID	Function	ref SNP Allele	Protein Change	Minor Allele Frequency	Test Population
rs2281820	missense	C/T	Val/Ala	T=0.46	HapMap-CEU

Chromosome 12q13: VDR

ref SNP ID	Function	ref SNP Allele	Protein Change	Minor Allele Frequency	Test Population
rs58915677	missense	C/T	Ala/Thr	NA	NA
rs28934607	missense	C/T	Pro/Ser	NA	NA
rs28934606	missense	C/G	Arg/Pro	NA	NA
rs28934605	missense	A/G	Gly/Glu	NA	NA
rs28934604	missense	A/G	Arg/His	NA	NA
rs13377933	missense	C/T	Ser/Asn	NA	NA
rs11574115	missense	C/T	Thr/Ile	T=0.00	HapMap-CEU
rs11574090	missense	C/G	Leu/Val	G=0.01 G=0.00	PDR90 HapMap-CEU
rs8176344	missense	G/C	Val/Leu	C=0.00 C=0.02	HapMap-CEU PDR90
rs2229103	missense	T/C	Val/Ala	NA	NA
rs2228570	missense	C/T	Met/Thr	T=0.31	PDR90

Proposal: Investigation of Genetic and Environmental Factors that Impact Variation in Enzymes and Metabolites Obtained from the Iowa Newborn Screen

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K99/R00 Pathway to Independence Award: Investigation of Genetic and Environmental Factors that Impact Variation in Enzymes and Metabolites Obtained from the Iowa Newborn Screen.

RESEARCH PLAN

Newborn screening programs are extremely important public health initiatives for detecting hereditary metabolic disorders, congenital endocrinopathies, hemoglobinopathies and cystic fibrosis. However, several of the enzymes, particularly immunoreactive trypsinogen and 17-hydroxy progesterone utilized in newborn screening programs have high variability among healthy newborns that may be attributed to environmental and genetic factors. This can lead to false positives and decreased sensitivity of the screening test which can cause adverse psychological and social problems for the infants and their families. Additionally, several of the enzymes such as thyroid stimulating hormone are sensitive to gestational age and birth-weight and can result in false negatives in premature infants. Many of these enzymes remain to be evaluated in detail with respect to gestational age and birth-weight.

The main objectives of this study are as follows:

Specific Aim 1 (K99 phase): To identify enzymes, metabolites and tandem mass spectrometry measurements from the newborn screen that correlate with gestational age, birth-weight, gender and region (designated by mother's zip code).

Specific Aim 2 (K99/R00 phase): To estimate the heritability and variance explained by shared genetic and/or environmental variables for all measurements obtained from the newborn screen by analyzing monozygotic and dizygotic twin pairs.

Specific Aim 3 (R00 phase): To identify genetic polymorphisms associated with normal variation in measurements obtained from the newborn screen. The initial focus will be on those measurements with high heritability as identified in aim 2.

1. Abstract and Proposal

We request use of the biomarker and tandem mass spectrometry measurements collected during newborn screening as well as the blood spots for use in a K99/R00 Pathway to Independence NIH grant to be entitled "Investigation of Genetic and Environmental Factors that Impact Variation in Enzymes and Metabolites Obtained from the Iowa Newborn Screen." This grant proposal will be submitted to the National Institute of Child Health and Human Development (NICHD). This grant is available for postdoctoral researchers and provides funding for training, personnel, supplies and research costs associated with the project. If awarded this grant would provide resources for two years of research at a postdoctoral level and three years of research at a faculty level.

Newborn screening programs are extremely important public health initiatives for detecting hereditary metabolic disorders, congenital endocrinopathies, hemoglobinopathies and cystic fibrosis. However,

several of the enzymes, particularly immunoreactive trypsinogen and 17-hydroxy progesterone utilized in newborn screening programs have high variability among healthy newborns that may be attributed to environmental and genetic factors (1). This can lead to false positives and decreased sensitivity of the screening test which can cause adverse psychological and social problems for the infants and their families. Additionally, several of the enzymes such as thyroid stimulating hormone are sensitive to gestational age and birth-weight and can result in false negatives in premature infants (2). However, many of these enzymes remain to be evaluated in detail with respect to gestational age and birth-weight.

The focus of this project will be examining genetic and environmental factors for associations with common enzymes and metabolites evaluated in the newborn screen. Of particular focus will be the associations of immunoreactive trypsinogen, 17-hydroxy progesterone, thyroid stimulating hormone, gal-1-phosphate uridyl transferase, biotinidase, hemoglobin and phenylalanine with gestational age, birth-weight, mother's zip code (in order to estimate regional effects) and common genetic single nucleotide polymorphisms. Determining differences in these measurements by relevant genetic and environmental variables will be useful in improving the sensitivity and accuracy of newborn screening tests. Additionally, identifying factors that contribute to the variability of these measurements may be relevant to human diseases.

The main objectives of this study are as follows:

- 1) To identify enzymes, metabolites and tandem mass spectrometry measurements from the newborn screen that correlate with gestational age, birth-weight, gender and region (designated by mother's zip code).
- 2) To estimate the heritability and variance explained by shared genetic and/or environmental variables for all measurements obtained from the newborn screen by analyzing monozygotic and dizygotic twin pairs.
- 3) To identify genetic polymorphisms associated with normal variation in measurements obtained from the newborn screen that have high heritability as defined by objective 2.

2. Neonatal Blood Spots (NBS)

2.1 Selection

Approximately 1000 NBS, 500 with gestational age \leq 36 weeks and 500 with gestational age \geq 37 weeks, will be selected with respect to a sampling collection time between the 2nd and 4th day of life, no transfusions and no abnormal readings or positive results for any of the biomarkers. NBS will be selected randomly with respect to gender, race or ethnicity, geographic location, birth-weight and other demographic variables. These criteria will ensure collection of a random sample that represents normal variation of particular enzymes in relatively healthy newborns. Additionally, we request NBS from a portion of same-gender twins to genotype a subset of markers in order to determine zygosity. The zygosity information will be used in heritability studies of each measurement from the newborn screen to determine the proportion of variance that can be explained by shared genetic and/or environmental factors.

2.2 Collection Procedures

We request one full spot in order to obtain enough DNA for initial genetic analysis as well as genome wide association studies (GWAS). NBS should be cut from the collection card using the appropriate technique and protocols. Each NBS should be sealed in an individual bag and labeled with a de-identified code that can be linked to information collected at screening such as first or second screen information, gender, gestational age, multiple birth and birth order, transfusion information, birth-weight, age at collection, mother's zip code, feeding method and race/ethnicity. However, as mentioned above

no identifiable information will be attached to these samples. DNA will be extracted using standard procedures and aliquots will be prepared and stored in the laboratory for genotyping or sent with anonymized id numbers only to an outside facility for genotyping.

2.3 Storage and Confidentiality

NBS spots will be received and stored in the Murray laboratory and either genotyped on-site or sent to a genotyping core off-site. However, any unused sample will be sent back to the Murray laboratory for storage. NBS will be stored intact at -80F until needed. As stated above, data will be stored on a secure server and accessed only by the principal investigator and staff. Also, as mentioned above, no identifiable information will be provided with the NBS spot.

Variables	Parameters	Notes
Gestational Age	Weeks documented on card	
Birth-weight	Wt in grams	
Multiple Birth	Singletons and Multiples	Would require linking
Zip Code	As listed on card	If have IRB waiver
Gender	Male and Female	
First or Second Screen Information	First Screen Only	
Transfusion	No Transfusion	
Age at Screen	Hours of age	< 96 hours of age at collect
Feeding Method	As listed on card	
Race/Ethnicity	If noted on card	May require IRB waiver
Screen Measurements:		
17-Hydroxy Progesterone	Result level	
Thyroid Stimulating Hormone	Result level	
Gal-1-Phosphate Uridyl Transferase	Result level	
Biotinidase	Result level	
Hemoglobin Phenotype	Result level	
Phenylalanine	Result level	
Expanded Screening Panel - MS measurements	Result level	Would like breakout of metabolite and level

Timeline of project

Objective	0-6 months	Year 1-2	Year 2-3	Year 4-5
Data Generation Based on Parameters	X			
Analysis of Biomarker Data with Environmental Variables	X	X		
Heritability analysis on biomarkers with twins		X	X	
Collection of NBS cards for DNA extraction		X		
DNA extraction and Genotyping		X	X	
Association analysis of genotype with each biomarker			X	X
Expansion of genotyping and analysis using all MS measurements				X
Genome Wide Association Study				X