

**IDENTIFICATION OF COPY NUMBER VARIANTS IN A COHORT OF WOMEN WITH
PREMATURE OVARIAN INSUFFICEINCY**

by

Natalie Joy Engel

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This thesis was presented

by

Natalie Engel

It was defended on

April 5, 2011

and approved by

Committee Chair: Aleksandar Rajkovic, M.D., Ph.D.
Associate Professor
Department of Obstetrics, Gynecology and Reproductive Sciences
Director of Reproductive Genetics
Medical Director of the Cytogenetics Lab
Co-Director of the Fetal Diagnostic Center
University of Pittsburgh, Magee Women's Hospital of UPMC

Committee Member: Elizabeth Gettig, M.S., CGC
Associate Professor and Co-Director of the Genetic Counseling Program
Department of Human Genetics
Graduate School of Public Health, University of Pittsburgh

Committee Member: John W. Wilson, Ph.D.
Assistant Professor
Department of Biostatistics
Graduate School of Public Health, University of Pittsburgh

Committee Member: M. Michael Barmada, Ph.D.
Associate Professor
Director, Center for Computational Genetics
Department of Human Genetics
Graduate School of Public Health, University of Pittsburgh

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Natalie Engel, MS

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Premature ovarian failure (POF), or premature ovarian insufficiency (POI), is characterized by the absence or early cessation of menstruation before the age of 40. Clinically this can be seen as primary amenorrhea, the complete absence of menstruation, or secondary amenorrhea, the development of amenorrhea due to cessation of ovarian function. POI affects approximately 1% of women under the age of 40 years and 0.1% of women under the age of 30. Most cases of POI are idiopathic, as only 10% have a known cause. We utilized SNP array technology to identify novel copy number variations (CNV's) in 89 women with POI. Illumina's (San Diego, CA, USA) HumanCNV370-Duo DNA Analysis BeadChip and Human660W-Quad v1 DNA Analysis BeadChip were used to identify microdeletions and microduplications on autosomal chromosomes. A total of 198 autosomal CNV's were identified ranging from 0.1 Mb to 3.4 Mb. The Database of Genomic Variants (DGV) was used as a control population. We identified seven novel microdeletions in our POI cohort, six of which contained gene-coding regions: 8q24.13, 10p15-p14, 10q23.31, 10q26.3, 15q25.2, and 18q21.32. Two of the novel microdeletions contained genes known to cause ovarian failure in knockout mice models, *SYCE1* and *CPEB1*. Seventeen novel microduplications were also detected, with the majority of CNV's detected being on autosomal chromosomes rather than the X chromosome. This pilot study demonstrates an association between specific CNV's and POI and highlights the importance for studies with larger samples sizes to confirm the findings and further support the hypothesis.

Furthermore, array technology may be a useful addition to conventional karyotyping when evaluating women with POI. Studies on the genetic factors of POI have public health significance because they search for genomic imbalances in a disease that affects 1% of the American population. Finding the genetic causes may lead to effective treatment methods and earlier recognition of those at risk, particularly before the onset of amenorrhea.

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PREFACE

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1.0 INTRODUCTION

The average woman in the American population experiences menopause naturally between 46 and 54 years of age³. Premature ovarian failure (POF), or premature ovarian insufficiency (POI), is characterized by the absence or early cessation of menstruation before the age of 40¹. Other names include "primary ovarian failure" and "hypergonadotropic hypogonadism"⁴. Clinically this can be seen as primary amenorrhea, the complete absence of menstruation, or secondary amenorrhea, the development of amenorrhea due to cessation of ovarian function. POI affects approximately 1% of women under the age of 40 years and 0.1% of women under the age of 30². POI differs from menopause in that 5-10% of women who are diagnosed with POI are able to conceive after initial onset⁴. POI has important health implications beyond infertility concerns. Women with POI have increased mortality when compared to those who experience menopause at an average age. They are known to be at an increased risk for osteoporosis, heart disease, and autoimmune diseases⁵. Hypoestrogenism for prolonged periods of time can lead to the increased risk of hypercholesterolemia, osteoporosis, as well as stroke and other forms of cardiovascular diseases². Most cases of POI are idiopathic, as only 10% have a known cause. POI is a highly heterogeneous condition and research studies have focused on finding the genetic factors that can cause POI. Several genes have been implicated in women with POI. However, the majority of cases still have an unknown cause. The aim of this pilot study was to determine whether copy number variations (CNV's) are associated with POI. CNV's have been implicated in a number

of common diseases, including Alzheimer's disease, Crohn's disease, autism, and schizophrenia⁶. Detecting pathologic CNV's in a cohort of women with POI can lead to identifying new candidate genes that affect ovarian function and provide pathways for therapy in the future.

2.0 BACKGROUND AND SIGNIFICANCE

2.1 OVERVIEW

2.1.1 Ovarian Function

During embryogenesis, the female ovaries form approximately 7×10^6 oocytes at 16-20 weeks gestation to begin the process of folliculogenesis⁷. After the seventh month of gestation, most of these primary oocytes die and the rest enter meiosis and are stored in primordial follicles arrested in the diplotene stage of prophase I until puberty⁷. At birth, a female has only 20% left of this original follicular pool and beginning at puberty 10-15 primordial follicles are recruited in the pubertal female each cycle but only 400-500 total are ovulated before menopause⁷⁻⁸. Unknown intra-ovarian factors are important for the initiation of folliculogenesis, whereas follicle-stimulating hormone (FSH) and luteinizing hormone (LH) are known to play a part in the later stages of follicle maturity⁹. FSH and LH are hormones released by the anterior pituitary gland⁷. The release of FSH and LH from the pituitary gland stimulates the growth, or maturation, of follicles in the ovary as well as estrogen release by the follicle⁷. Recruited follicles grow in volume and diameter with exposure to FSH and LH, and the amount of granulosa and theca cells surrounding the oocyte begin to grow⁷. Exposure to gonadotropins at the correct time of growth determines which follicle is chosen for ovulation⁷. The remaining recruited follicles undergo

follicular atresia. The massive oocyte wastage is important to ensure that only the highest quality oocyte is ovulated.

There are two main ways in which POI is thought to be caused: follicle dysfunction and follicle depletion¹⁰. Follicle dysfunction occurs when the follicles are present within the ovary but some mechanism is forbidding their growth, resulting in follicular arrest⁴. Follicle depletion means that the number of follicles present is different from what is expected. This may be due to an initial low number of primordial follicles, accelerated atresia at the primary level, or autoimmune or toxic destruction of the follicles⁴.

2.1.2 POI Presentation and Diagnosis

Most women who experience POI begin menstruating at a normal age and experience puberty normally⁴. POI can present in multiple ways as primary amenorrhea is thought to make up only 10% of POI cases⁴. Menses can stop abruptly, cease after the birth of a child or after one has stopped taking oral contraceptives, or cessation of menses can follow infrequent menstruation, abnormally short cycles, or uncommon uterine bleeding. Symptoms such as night sweats and hot flashes can be seen in some patients. However these are not common⁴.

POI is diagnosed by the presence of amenorrhea for at least 4-6 months in a woman less than 40 years of age with two elevated follicle-stimulating hormone (FSH) levels taken at least one month apart⁴. Before menopause, FSH levels drop after ovulation by the negative feedback of inhibin and estrogen⁷. When menopause is reached, this negative feedback is absent and therefore FSH is continually released. FSH levels greater than 40 IU/l are typically considered in the postmenopausal range. Other markers used to diagnose POI include estradiol levels, Inhibin B, anti-Mullerian hormone, and LH¹⁰.

2.1.3 Consequences and Management of POI

Infertility is the main consequence associated with POI and can be devastating to couples given this diagnosis. Reproductive assistance is available to women diagnosed with POI and family planning options can include use of donor eggs and adoption. Furthermore, approximately 5-10% of individuals diagnosed with POI will sporadically ovulate and become pregnant following their diagnosis. In such individuals oocyte reserves are diminished but not exhausted.

Other health consequences that have been associated with POI are frequently overlooked. Women with a diagnosis of POI are at an increased risk of developing heart disease, osteoporosis, autoimmune diseases such as hypothyroidism and adrenal insufficiency, and deteriorating overall well-being⁴⁻⁵. Few studies have been dedicated to long-term health consequences of women with POI. The majority of studies in the literature focus on the effects of early menopause and hormone deficiencies. However, the mean age of diagnosis of POI is markedly younger than the average age of early menopause. Hormone deficiency therefore may affect women with POI for several years more than those experiencing young menopause and this difference may cause more severe health consequences⁵. Overall, POI has been associated with a 50% higher overall mortality rate than women experiencing natural menopause at an average age¹¹.

Estrogens are important for the formation and maintenance of bone health. Therefore a deficiency of these hormones due to inactive ovaries can lead to imbalances in bone formation and breakdown¹². Women with POI have been shown to have lower bone density compared to controls⁵. Bone loss occurs naturally following menopause, and patients with earlier onset POI may have never reached their peak bone mass that on average occurs in women in their late

twenties⁵. Maintaining bone health is an important factor in the management of women with POI.

Increased mortality due to cardiovascular disease has been reported in the literature in women with POI¹². Estrogen is known to play a large role in the protection of blood vessels and inhibition of atherosclerosis formation¹³. Cardiovascular disease risk is greatest in postmenopausal women and the risk is reduced when estrogen replacement therapy is implemented¹¹⁻¹². Cardiovascular mortality has been shown to be increased in women with earlier natural menopause compared to women experiencing late natural menopause with an 80% increase risk of mortality from ischemic heart disease if menopause is experienced before the age of 40¹¹⁻¹². Low estrogen levels have also been associated with risk factors for cardiovascular disease including increased triglycerides, reduced HDL, and reduced insulin sensitivity^{11, 14-15}.

Few studies have been performed to assess the overall well being of women experiencing POI. Menopause has been known to affect cognitive function as well as memory¹². Therefore women experiencing POI may be at an increased risk. In one study, one hundred women with premature ovarian failure and a normal karyotype were compared to control women with normal menstrual cycles to assess their shyness, self-esteem, and social anxiety levels¹⁶. Significant differences were seen between controls and women with POI, with cases self-reporting higher levels of anxiety and shyness and lower levels of self-esteem¹⁶. Other studies have shown increases in hostility and psychological distress in women with POI^{5, 17-18}.

Autoimmune disorders have been shown to be associated with POI, and at least 20% of women with POI will present with an autoimmune disease^{11, 19}. These autoimmune disorders include, but are not limited to, hypothyroidism, diabetes, systemic lupus erythematosus, rheumatoid arthritis, and Addison's disease¹¹. There is a 50% risk of the development of adrenal

insufficiency and Addison's disease in women with adrenal autoimmunity, which can be fatal if unrecognized^{4,11}.

Several professional organizations recommend that women with POI undergo a routine karyotype and Fragile-X testing, screening for adrenal antibodies, as well as an ultrasound of the ovaries and uterus in order to elucidate the cause of their POI²⁰⁻²². For those with a positive adrenal antibody test, annual evaluation should include a corticotropin stimulation test to monitor for adrenal autoimmunity⁴. Bone health should be addressed by a bone mineral density test and women should be educated regarding strategies to maintain bone health including 1200 mg of calcium per day and maintenance of adequate vitamin D status⁴. Women should be encouraged to exercise appropriately including walking, jogging, and resistance exercising⁴. Hormone replacement therapy can be considered to eliminate the hypoestrogenism that can lead to the increased risks for cardiac disease, osteoporosis, autoimmune adrenal insufficiency, and hypothyroidism. The American Society for Reproductive Medicine and the International Menopause Society recommend estrogen-replacement therapy for women with primary ovarian insufficiency⁴.

2.2 CAUSES OF PREMATURE OVARIAN INSUFFICIENCY

Most cases of POI are idiopathic as 90% of cases have an unknown cause. It is a highly heterogeneous disease with a wide spectrum of known causes. Known causes of POI, excluding iatrogenic causes such as hysterectomy and oophorectomy, include autoimmune disorders, metabolic disorders, and infectious disease, cancer treatments such as radiation or surgery, smoking, and genetic causes.

2.2.1 Non-Genetic Causes

Ovarian Toxins

Chemotherapy and radiation therapy are known to cause premature ovarian insufficiency in women receiving them for cancer treatment²³. There is wide variability in how women are affected with chemotherapy. Women under the age of 40 receiving cancer treatments have been seen to experience amenorrhea during treatment; menstrual function and fertility have been known to return years after treatment has ceased in a subset of women²⁴. Cancer drugs are known to affect both dividing somatic cells in the follicles and mature oocytes²³. Alkylating agents are more damaging to ovarian cells than non-alkylating agents²³. Drugs such as cyclophosphamide have been shown to cause DNA single strand breaks and change base pairs and can affect both resting oocytes and dividing follicles. Studies have shown the effects are age and dose dependent, for example, Koyama et al studied the effects of cyclophosphamide on premenopausal women²³. Women in their 40's experienced permanent amenorrhea at an average dose of 5.2 g compared to 9.3 g for women in their 30s²⁵. The argument for this discrepancy is the differing number of oocytes still present in these women, presumably with women in their 30s having more oocytes remaining²³. Radiation therapy is more damaging to ovarian function than chemotherapy drugs, and it specifically affects mature oocytes²³. Especially in younger women, radiation can cause amenorrhea that resolves after 6 to 18 months, presumably after recruitment of a new cohort of primary follicles²³⁻²⁴. Such a response also has been documented in women receiving radioactive iodine therapy for thyroid cancer, in whom approximately 30% had reversible primary hypogonadism²³.

Autoimmune disorders

Autoimmune disorders are seen in approximately 20% of women who present with POI¹⁹. Autoimmune oophoritis may occur as part of type I and type II syndromes of polyglandular autoimmune failure (PGA), which are associated with auto-antibodies to multiple endocrine and other organs⁴. The mechanism behind autoimmune oophoritis resulting in POI is unknown at this time. Other autoimmune disorders that have been described with POI include insulin dependent diabetes, thyroid autoimmunity, and acetylcholine receptor autoimmunity²⁶. In most cases, it is not able to be determined if the autoimmune disease is the cause of POI or a secondary complication.

Other causes

Different viruses have also been implicated in POI including mumps and HIV²⁴. Cigarette smoking is the best-known environmental toxin to affect ovarian function. Those who smoke reach menopause approximately two years earlier than nonsmokers²⁴.

2.2.2 Genetic Basis of POI

There are several reasons why it is thought that POI can be greatly attributed to genetics. Multiple animal and human studies have demonstrated that POI can be caused by single gene mutations, including syndromic and non-syndromic forms²⁷. Second, there is a large familial pattern seen with POI. Studies have found that 4-31% of POI cases are present in a familial form². Different patterns of inheritance can be seen in pedigrees of families with POF including autosomal dominant, autosomal recessive, and X-linked patterns. Because of the supporting factors of a genetic basis for POI, research groups have focused on finding the genetic causes. POI has devastating effects on fertility as well as the other health problems. Therefore, early

detection of individuals at risk for POI could aid in family planning as well as oocyte cryopreservation. Family studies have shown that POI is a highly heterogeneous multifactorial disorder with variable expressivity and reduced penetrance²⁷. Therefore, even though certain genes have now been linked to POI, most cases of POI are still considered idiopathic to date.

2.2.2.1 Chromosome Disorders

Chromosome conditions such as Turner syndrome, as well as translocations and inversions, are implicated in POI. Turner syndrome is one of the most common chromosomal defects seen in humans and is seen at 1/2500 live births²⁸. It is caused by the lack of an X chromosome (45,X) and its symptoms include webbed neck, short stature, lymphedema in the newborn, and streak gonads (fibrous tissue that replaces a functioning gonad)²⁸. Approximately 50% of patients with gonadal dysgenesis are found to have Turner syndrome⁹. Primary amenorrhea and absence of pubertal development is seen in most affected women, however, some have been known to develop normally. The cause for the variability is unknown, but studies between karyotypes and phenotype differences in women with X chromosome abnormalities have demonstrated the importance of certain X-linked genes to ovarian function and fertility and has led to the delineation of three X chromosome critical regions: POF1 at Xq27.2-q27.3, POF2 at Xq13.3-q22, and a third region at Xp11²⁹. 47,XXX karyotype is also known to be implicated in POI. Trisomy X is thought to affect one in one thousand female births, however only 10% are thought to be diagnosed²⁸. In most cases, there are no major health concerns associated with the extra X chromosome²⁸. Multiple case reports in the literature report POI in women with trisomy X, most of these reports involve autoimmune disorders as well²⁸.

2.2.2.2 Mendelian Disorders

Monogenic POI can be separated into two categories: syndromic and non-syndromic POI. There are several syndromes known to be associated with POI. Table one outlines the syndromes as well as the genes implicated. Typically, patients with one of these syndromes do not present first with POI. However, these syndromes should not be overlooked when working up an individual affected with POI.

Table 1. Syndromes Associated with POI²⁷

Syndrome/Symptoms	Gene	Position
Galactosemia	<i>GALT</i>	9p13
Ataxia Telangiectasia	<i>ATM</i>	11q22.3
Frasier Syndrome	<i>WT1</i>	11p13
POF + dilated cardiomyopathy	<i>LMNA</i>	1q21.2
Blepharophimosis-Ptois-Epicanthus syndrome	<i>FOXL2</i>	3q23
Nijmegen breakage syndrome	<i>NBS1</i>	8q21
Autoimmune polyendocrine syndrome	<i>AIRE</i>	21q22.3
Werner Syndrome	<i>RECQ2</i>	8p12-p11.2
Bloom Syndrome	<i>RECQ3</i>	15q26.1
Rothmund-Thomson syndrome	<i>RECQ4</i>	8q24.3
Vanishing white matter + POF(ovarioleukodystrophy)	<i>EIF2B2</i>	14q24
Congenital disorder of glycosylation type Ia	<i>EIF2B4</i>	2p23.3
	<i>EIF2B5</i>	3q27
	<i>PMM2</i>	16p13.3-p13.2
Chronic progressive external ophthalmoplegia + POF	<i>POLG</i>	15q25
Mullerian anomalies + adrenal failure + POF	<i>SF1</i>	11q13
Mayer-rokitansky-kuster-hauser syndrome	<i>WNT4</i>	1p35
Fanconi Anemia	FA genes	
Lipoid congenital adrenal hyperplasia	<i>STAR</i>	8p11.2

Several genes have been implicated in non-syndromic POI and can be seen in table two. Most of these genes were discovered through animal studies or through studies on women affected with POI. Only 1-2% of women with POI show a mutation or perturbation in one of these genes. Therefore large heterogeneity exists for POI²⁷.

Table 2. Genes Known to be Involved in Non-Syndromic POI²⁷

Gene	Position
<i>BMP15</i>	Xp11.2
<i>GDF9</i>	5q31.1
<i>FIGLA</i>	2p12
<i>NOBOX</i>	7q35
<i>POF1B</i>	Xq21
<i>DIAPH2</i>	Xq22
<i>FOXO3</i>	6q21
<i>FSHR</i>	2p21-p16
<i>LHR</i>	2p21
<i>CYP17</i>	10q24.3
<i>CYP19A1</i>	15q21.1
<i>FMR1</i>	Xq27.3
<i>FMR2</i>	Xq28

BMP15 and GDF9

Bone morphogenic protein 15 (MIM 300247) is part of the TGF β family and has been found to encode an oocyte derived growth and differentiation factor known to be involved in follicular development¹⁰. Some of its main roles include the promotion of follicle maturation, prevention of cell apoptosis, promotion of oocyte growth, and the regulation of oocyte recruitment numbers¹⁰. Most of the research on *BMP15* have been done through animal studies and have shown *BMP15* knockout female mice are subfertile and homozygous mutations in yews lead to infertility³⁰. The *BMP15* gene is located on the X chromosome in the third POF critical region and studies have shown heterozygous mutations (mostly missense) in the gene in women with both primary and secondary amenorrhea³⁰. However, the actual mechanism by which these mutations impede *BMP15* functionality is presently unknown.

GDF9 (MIM 601918) is another gene in the TGF β family known to be associated with folliculogenesis and POI. *GDF9* is located at 5q31.1 and like *BMP15*, animal and functional

studies have shown its importance in folliculogenesis²⁷. Several case control studies have shown a possible prevalence of *GDF9* heterozygous mutations in POI women of 1.4%; these variants have not been found in control samples¹⁰.

BMP15 protein forms homodimers as well as heterodimers with *GDF9*. The dominant negative hypothesis is a possible mechanism for interrupted ovarian function through heterozygous mutations in these genes by contributing to abnormal dimerization of these proteins²⁷.

FIGLA and NOBOX

FIGLA (MIM 608697) and *NOBOX* (MIM 610934) are transcription factors and were first identified as candidate genes for POI through animal studies³¹. Mouse models have demonstrated the importance of *NOBOX* in oogenesis and ovarian development based on effects of deleting part of the coding region³². This led to accelerated oocyte loss and absent transition of primordial germ cells to growing follicles³². Sequencing of the *NOBOX* gene in 96 women with POI revealed four novel variations, 2 resulting in missense mutations that affected transcription binding thus leading to ovarian failure³³. Zhao et al 2008 studied one hundred Chinese women with POF and identified three variants in four women in the *FIGLA* gene³⁴.

POF1B

POF1B (MIM 300603) maps to the POF2 critical region at Xq21. *POF1B* was first identified through studying a woman with POI due to an X:autosome balanced translocation³⁵. Studies on the effects of *POF1B* on ovarian function have been mixed. Several studies have found point mutations in *POF1B* in women with premature ovarian failure, and mouse studies have suggested that *POF1B* is critical for early ovarian development and establishing the final

number of germ cells³⁵⁻³⁶. Other studies have failed to find mutations or chromosome changes in women with POI that disrupt the *POF1B* gene³⁵.

DIAPH2

DIAPH2 (MIM 300108) is the homologue of *Drosophila diaphanous* (*dia*), which is known to be involved in establishing cell polarity, govern cytokinesis, and reorganize the actin cytoskeleton²⁷. In flies, disturbance of this gene causes sterility. In one case report, a X:autosome translocation affecting the *DIAPH2* gene was described in a woman with POI³⁷.

FOXO3

Studies on the *FOXO3* (MIM 602681) locus have shown it plays a major role in primary follicle apoptosis in the mouse³⁸. Loss of *Foxo3* function in the mouse leads to premature ovarian failure due to global follicular activation and oocyte death, resulting in sterilization by 15 weeks of age³⁸. Studies have identified variations in the *FOXO3* gene in women with POI³⁹, however it is unknown at this time whether these variations are causal or benign polymorphisms.

FSHR and LHR

FSHR (MIM 136435) and *LHR* (MIM 152790) are hormone receptor proteins and are essential for normal reproductive function in both men and women. Both FSH and LH are important sex hormones needed for activation of follicular development and maturation of oocytes as well as other processes. Defects in the *FSHR* gene can lead to decreased follicle growth due to inability to bind FSH or to activate signaling pathways²⁸. The homozygous mutation C566T has been found in many Finnish families studied by Aittomaki et al, heterozygous females showed normal fertility⁴⁰. This mutation has not been found in other ethnic populations, perhaps due to the founder effect among the Finnish. Abnormal LH levels have the potential to lead to disorders such as polycystic ovary syndrome, infertility, and

miscarriages²⁸. A common variant in the *LHR* gene, G1502A, causes an amino acid substitution that may affect LH function, however this hypothesis remains to be proven²⁸.

CYP17

CYP17 (MIM 609300) encodes steroid 17-alpha-hydroxylase, and 46,XX patients with a defect in this gene may present with primary amenorrhea. Some studies report cystic follicles in these women as well as streak gonads. The gene is important for male sex patterning, therefore the more common finding is sex reversal in 46,XY individuals with defects in this gene²⁷.

CYP19A1

CYP19A1 (MIM 107910), located at 15q21.1, codes for aromatase and is involved in the conversion of androgens to estrogens. Two studies have found compound heterozygous mutations in the aromatase gene in 2 women presenting with primary amenorrhea and cystic ovaries⁴¹⁻⁴². In one study, it was shown that the mutant protein exhibited no activity⁴¹.

FMRI

POI is associated with premutations in the *FMRI* gene. Alleles in the *FMRI* gene are separated into number of CGG repeats present including normal (6 to 55 repeats), premutation (55 to 200 repeats) and full mutation (>200)²⁸. Approximately 2% of women who present with sporadic POI and 14% with familial POI will have a *FMRI* premutation⁴. Women with premutations in the *FMRI* gene have a 20% risk of developing FXPOI (Fragile X associated POI). These women are also at risk of having a child with Fragile X syndrome. Penetrance of FXPOI seems to be highest in carriers of mid premutation alleles (80-99 repeats) (18.6%) when compared with low repeat numbers (59-79) or high repeat (>100) premutation carriers, 5.9% and 12.5%, respectively⁴³. Interestingly, smoking has been found to cause menopause to occur an

average of one year earlier in permutation carrier women compared to non-smokers⁴⁴. This is similar to what is experienced in women who do not carry a premutation in the *FMRI* gene.

FMR2

FMR 2(MIM 300806) is located distally to *FMRI*, the gene responsible for Fragile X Syndrome. *FMR2* also has a similar pattern of trinucleotide repeat (GCC): normal (<50), premutation (50-200) and full mutation variants (>200) as in the *FRAXA* site of the *FMRI* gene. Murray et al first reported small alleles (less than 11 repeats) in a cohort of women with POI, but no premutations or full mutations⁴⁵. In 1999, Murray et al also reported microdeletions in this gene in 3 women with POI⁴⁶.

2.3 GENOMIC STUDIES AND POI

2.3.1 Genome Wide Association Studies

Separate approaches have been used to find genes associated with POI, including targeted gene approaches driven by animal models, genome wide association studies (GWAS), linkage analysis in families with more than one affected individual, and translocation breakpoint mapping⁴⁷. Traditionally, use of conventional karyotyping could detect larger deletions, duplications, and rearrangements within the genome of women with POI. With the development of microarray technology, array-comparative genomic hybridization (aCGH) platforms can be used to detect smaller deletions and duplications that conventional karyotype cannot⁴⁸. This technique involves labeling a reference genome and a test genome with different fluorescent

markers, hybridizing to the microarray, and analyzing the intensity of the hybridization signal for each clone.

GWAS can scan the genome of hundreds of POI patients to search for regions that may be associated with POI when compared to control samples. Early GWAS studies on women with POI have been sparse but have shown promise. A study performed on 99 women with idiopathic POI and 235 female controls using a SNP array revealed a significant association of one SNP contained in the *ADAMSTS19* gene⁴⁹. This gene is expressed in female mice gonads during sexual differentiation⁴⁹. No other SNP's were significantly associated with POI, including those in 74 candidate genes selected based on previous literature reports⁴⁹.

Kang et al revealed 10 SNPs in the *BCKDHB* gene that significantly associated with POI⁵⁰. The authors hypothesized that alterations in this gene may decrease LH levels and increase FSH levels through a complex involvement of neurotransmitters and gonadotropin stimulation release⁵⁰. In this study, 16 patients with POI were matched with 16 controls⁵⁰. Kang et al later reported the *PTHBI* gene to associate with POI and also identified a haplotype in this gene that was associated with susceptibility to POI⁵¹. The functional role of *PTHBI* is not yet known; it is known to be expressed in several tissues including the heart, placenta, liver, kidney, and brain⁵¹. Twenty-four individuals with POI and 24 matched controls were used for this analysis on a SNP array. A two step association study was performed, with the first stage based off of linkage disequilibrium to identify genes that associate with POI and the second stage focused on analyzing additional SNP's in the *PTHBI* gene in a larger population⁵¹. Causative SNPs were not identified in the analysis⁵¹.

Another study involving 24 individuals with POI and 24 matched controls revealed SNP's that significantly associated with POI in the *PCMT1* gene⁵². This gene is highly expressed

in the human brain and has been associated with epilepsy, spina bifida, and Alzheimer's disease⁵². Some reports have found this gene to be expressed in the pituitary gland of rats and is a substrate for some pituitary hormones⁵². The authors report that *PCMT1* is expressed in the human pituitary gland and that variants of the gene may lead to an altered structure of the FSH receptor binding region⁵².

Lacombe et al utilized microarray and linkage analysis to find a region on the X chromosome that was homozygous in all daughter affected with POI in a large Lebanese family³⁶. A single point mutation in the *POF1B* gene, resulting in an amino acid change from arginine to glutamine, was reported to be the cause of the POI in the five sisters with POI³⁶. Statistical significance were reached in all of these studies for at least one SNP or genomic region, however in all of these GWA studies were low numbers of patients and lack of reproducing the same results in different populations or ethnic groups of patients.

2.3.2 Copy Number Variation and SNP Arrays

Recent studies have shifted their focus to copy number variations (CNV's) in women with POI. CNV's are changes in copy number in a region of the genome. This can be a gene, a small segment of DNA, or an entire chromosome. CNV's cover at least 1000 bases and can be benign or deleterious as well as inherited or de novo. CNV's have been implicated in a number of common diseases, including Alzheimer's disease, Crohn's disease, autism, and schizophrenia⁶.

The use of single nucleotide polymorphism (SNP) arrays for the detection of CNV's is one of the more recent developments in the search for causes of complex diseases. Approximately 99% of our base pairs are the same on both chromosomes. The remaining 1% represents the diversity that is among the human race, including differences in disease

susceptibility⁵³. SNP's represent a large portion of this genetic variation between individuals, and are defined by a genomic site where there are two nucleotide possibilities, or alleles, each possibility appearing in a significant proportion in the population⁵⁴. SNP's are the most common genetic variation in the human genome, with over 15 million identified⁵⁴. They are highly conserved within a population and throughout evolution, and are therefore an excellent marker for research⁶. SNPs lying in coding regions of the genome are termed either synonymous or non-synonymous, depending whether if they change the amino acid sequence⁵⁴. SNP's lying in non-coding regions also have the potential to impact phenotype through changing splicing processes or transcription factor binding⁵⁴.

SNP arrays were originally designed for fast genotyping of thousands of SNP's across the genome in an individual or population. Recent use of these arrays to detect copy number variation detection has broadened its scope of use in research laboratories. Analysis of signal intensities across the genome can be used to identify regions with multiple SNPs that are deleted or duplicated⁵⁵. These arrays utilize SNP's spaced across the genome to detect copy number variations using statistical software.

There are several advantages to using SNP arrays to detect CNV's over other applications. SNP arrays use less sample per experiment compared to array comparative genomic hybridization(CGH)⁵⁶. Its cost effectiveness allows researchers to test more samples per run. Coverage of SNP arrays allows for this increase in number of samples without sacrificing sensitivity⁵⁶. SNP arrays allow for parent of origin studies to be conducted as well as linkage studies by homozygosity mapping for recessive disorders. Mosaicism and consanguinity can be assessed by SNP arrays, as well as mitotic recombination and gene conversion events, unlike array CGH platforms⁵⁷. SNP arrays from Illumina and Affymetrix now include hundreds

of thousands of SNPs, as well as non-polymorphic probes in regions of the genome where known CNV's are not known to be located⁵⁸.

Karyotyping is used to rule out specific causes of POI such as Turner syndrome, trisomy X, large deletions and rearrangements, and translocations between autosomal chromosomes and the X chromosome. However, karyotyping suffers from low resolution as it can only detect deletions and duplications that are larger than 5 Mb. Microdeletions and microduplications therefore cannot be detected by classical cytogenetics, and may be implicated in 15% of all human diseases⁵⁹. Therefore, use of classical cytogenetics for women with POI may fail to identify a large proportion of the genetic causes of the disease. The use of methods such as oligonucleotide arrays and SNP arrays have allowed for a higher resolution and detection of microduplications and microdeletions.

2.3.2.1 SNP Array Methodology

Illumina is one of the forerunners of SNP array platforms. Their BeadArray has increased in the amount of SNP's interrogated for each sample from 100,000 to 650,000⁶. Illumina platforms call for single stranded DNA fragments to hybridize to thousands of probes along the array, each designed to bind a specific DNA sequence⁶. The signal intensity between the probe and the target DNA sequence is used to determine the SNP genotype.

Every SNP site is determined by sets of probes that are 50 nucleotides in length⁶. The probe is meant to be complementary to the DNA sequence adjacent to the SNP site. Each set contains probes associated with either the A allele or the B allele of that specific SNP. The probe that is complementary to that SNP site will bind and results in the appropriately colored signal (red for A/T, green for C/G)⁶. Illumina's computer algorithms are responsible for transforming signal colors into genotype calls AA, AB, or BB. Besides SNP's, these platforms

also interrogate non-SNP CNV regions using additional markers. These markers are found in regions where common CNV's are known to exist.

2.3.2.2 SNP Array Data Analysis for CNV Detection

Illumina's Genome Studio Genotyping Module Software was developed for use with the Illumina platforms. It can utilize several algorithms, including *cnvPartition*, designed for detecting CNV's with the GenomeStudio software⁵⁸. *cnvPartition* is capable of generating a report of CNV's in the genome of the samples using confidence values where the user can remove CNV's with low values from further analysis⁶⁰. To identify CNV's across the genome, the software utilizes two different measurements to determine the copy number at each SNP.

B Allele Frequency

The B allele frequency (BAF) is defined as the ratio of A to B. For example, at a given SNP the possibilities are A(A) or C(B). The possible genotypes for that SNP are then AA, AB, or BB. After standardization at 0.5, if the genotype is AA, the BAF is given at 0, if the genotype is AB the BAF is 0.5, and if the genotype is BB the BAF is 1. In the case where there is a deletion on one chromosome containing the SNP, the BAF could then either be A or B, thus making the BAF either 1 or 0. Duplication on one chromosome containing the SNP can result in genotypes of AAB, AAA, ABB, or BBB again altering the BAF.

Log R Ratio

The Log R Ratio is defined as the overall fluorescence intensity of the given SNP. This can be standardized at normal copy number = 0. Therefore any fluctuation from this line may be due to a CNV. Table three provides information on expected Log R ratio and BAF readings for different CNV's.

Table 3. Copy number state information from SNP array data⁶

CNV type	Possible SNP genotypes	Expected A+B signal	Expected BAF
Homozygous gain	AAAA	4	0
	AAAB	4	0.25
	AABB	4	0.5
	ABBB	4	0.75
	BBBB	4	1
Hemizygous gain	AAA	3	0
	AAB	3	0.33
	ABB	3	0.67
	BBB	3	1
Normal	AA	2	0
	AB	2	0.5
	BB	2	1
Hemizygous loss	A-	1	0
	B-	1	1
Homozygous loss	--	0	Undefined

The GenomeStudio software is capable of transforming the raw data from the image scan system and converting the signal intensities and colors into the BAF and LRR⁶¹. When the LRR and BAF are looked at in unison across a chromosome, a CNV can be distinguished from copy neutral loss of heterozygosity (LOH), as well as determining how many copies have been lost or duplicated within a region. *cnvPartition* compares LRR and BAF values of the alleles in the samples to expected values (see table three) to determine CNV regions⁶⁰. The user can then determine the CNV's to focus on for their analysis.

3.0 METHODS AND MATERIALS

3.1 SPECIFIC AIM OF STUDY

The aim of this pilot study was to determine whether CNV's are associated with premature ovarian insufficiency.

Hypothesis: A subset of women with premature ovarian insufficiency harbor pathologic microdeletions or microduplications that are not detectable with classical cytogenetics currently used for evaluation of POI.

To test this hypothesis, we utilized SNP array technology on a subset of women with POI to delineate whether copy number variants are present on autosomal chromosomes. Further investigation of the genes present in these CNV's may provide insight into other possible causes for idiopathic POI. Furthermore, the use of genome wide studies may show the need for further assessment of women with POI beyond standard karyotyping and *FMRI* status screening during their initial workup following their diagnosis.

3.2 RECRUITMENT OF SUBJECTS

89 women with POI were recruited through Baylor College of Medicine's POI research study. Women were recruited in the Houston, Texas area as well as through the POFsupport.org

website, a support group designed for women with POI. Approval was received from BCM's Institutional Review Board (IRB) as well as the University of Pittsburgh's IRB. Inclusion criteria for the study included: primary amenorrhea or secondary amenorrhea experienced for at least 6 months prior to age 40, and at least one FSH serum level greater than 40 IU/L. Women with a history of pelvic surgery, cancer, radiation exposure, smoking, and genetic syndromes associated with POI were excluded from the study. Of these 89 women, 8 presented with primary amenorrhea, 60 presented with secondary amenorrhea, and in 21 cases the type of amenorrhea is unknown. Ages of the subjects at the time of diagnosis ranged from 12 to 39 with the average age being 27. A case was considered familial if the individual had at least one family member with a known diagnosis of POI. Table four summarizes the patient population and breaks the cohort up into familial versus isolated cases, as well as age of onset of POI.

Table 4. Subjects by Type of POI and Age at Diagnosis

	Total	Percent of Total	Number of Familial Cases	Number of Idiopathic Cases	Family history unknown
PA*	8	9%	3	3	2
<25	17	19%	2	14	1
25-35	34	38%	6	28	0
>35	9	10%	0	9	0
Unknown	21	24%	0	1	20
	89	100%	11	55	23

*PA = primary amenorrhea

3.3 SAMPLE COLLECTION AND PROCESSING

Peripheral blood samples were collected from all 89 women after informed consent was received. Genomic DNA (gDNA) was extracted from 5 mL of whole blood (Qiagen Puregene protocol, Valencia, CA, USA). gDNA was precipitated by centrifugation and washed with 70%

ethanol, then rehydrated overnight in DNA Hydration Solution (Qiagen, Cat # D-5004). DNA concentrations were determined on the NanoDrop instrument.

Illumina's (San Diego, CA, USA) HumanCNV370-Duo DNA Analysis BeadChip with more than 318,000 tag SNP markers and 52,000 markers targeting additional CNV regions was used to examine CNV's among gDNA for 38 individuals on the BeadArray 500GX. Illumina's Human660W-Quad v1 DNA Analysis BeadChip with 550,000 tag SNPs and an additional 100,000 markers targeting common CNV was used for an additional 51 individuals on the iScan System. The median spacing between markers for the arrays was 5.5 kb and 2.5 kb, respectively. The array data was analyzed using Illumina's GenomeStudio Genotyping Module software. A call rate of >99% was accepted as the validity cutoff for each sample on both chips, which indicates >99% of the probes successfully identified a genotype. CNV's were identified using the B-allele frequency and Log R Ratio in GenomeStudio, congruently. CNV's were determined by visual scanning of all of the chromosomes of the 89 subjects.

3.3.1 Determination of Novel CNV's

All CNV's were screened against polymorphisms noted in the Database of Genomic Variants (DGV; <http://projects.tcag.ca/variation>). The DGV is a compilation of common CNV's detected in more than 10,000 healthy individuals, and it has been used as a control population in several studies⁶²⁻⁶⁴. The DGV currently reports over 100,000 CNV's that are found in the control samples. In order for a CNV to be considered present in the control population of the DGV and therefore called non-pathogenic, it was present in its entirety in the DGV in the control population. Pearson's chi squared test was used if needed to determine statistical significance.

Microdeletions or microduplications were considered novel and likely pathogenic if not present in the DGV.

3.3.2 Corroboration of Novel Microdeletions

Quantitative real-time PCR (qPCR) was utilized to corroborate SNP array findings⁶⁵. Emphasis was placed on unique microdeletions as opposed to microduplications. Where possible, a relevant protein-coding gene within each deleted region was chosen as a target, and oligonucleotides were designed for qPCR analysis (Table 5). Primers were custom-designed through Primer3Plus (<http://frodo.wi.mit.edu/primer3>). Table five below lists the primers used for qPCR corroboration of the autosomal microdeletions deemed novel in the analysis.

Table 5. Oligonucleotides designed for qPCR Corroboration of Novel Autosomal Microdeletions Within Coding Regions⁶⁶

Microdeletion locus	Patient	Targeted Gene	Forward primer (5' -> 3')	Reverse primer (5' -> 3')
8q24.13	POF-65	<i>FER1L6</i>	CGA ACC ACA GTG CAG AAG AA	AGG GCT ACG TCA TTA ATG CT
10p15-p14	POF-105	<i>AKR1C1</i>	TGT GGA TGG TGA CAC AGA GG	TCT TTC CTT TCT GGC CAA TG
10q23.31	POF-58	<i>STAMBPL1</i>	TAA GCC CAG AAG AGC GAG TC	CGC CAT CCT CTC CAT CTC TA
10q26.3	POF-108	<i>SYCE1</i>	CCA GTG ACA TGG TGG AGT TG	GGT GTC TTC ATT GCC ACT CA
15q25.2	POF-87	<i>CPEB1</i>	CCT GGG TAT TAG CCG ACA GT	AAT CCC GGC ATA CAC CAC T
18q21.32	POF-119	<i>PMAIP1</i>	CAC CGT GTG TAG TTG GCA TC	CCT TCT TCC CAG GCA TCT

Quantitative real-time PCR was conducted using 100 ng of gDNA, 0.4 μM forward and reverse primers, 1X iQTM SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) and nuclease free water, with a total volume of 20 μL per well. Five replicates were run for each POI individual for the targeted gene within each respective deleted region. A pooled human gDNA sample from five unaffected women was used as a control (Promega, Cat # G1521). Threshold

cycle (Ct) values were calculated and averaged for each set of five replicates; these values were then used to calculate the standard error of the mean. The $2^{-\Delta \Delta C_t}$ method of quantification was used to analyze fold changes in copy number between control (2n) and deleted regions (1n or absent).

4.0 RESULTS

Of the 89 women whose gDNA was run on the SNP arrays, 88 were used in the analysis. One individual did not pass quality control on the array (call rate of >99%), therefore the subject was excluded. A total of 50 microduplications and 148 microdeletions on autosomal chromosomes, ranging in size from 0.1 Mb to 3.4 Mb, were detected. These CNV's were found on 72 of the 88 women. In order to distinguish novel CNV's from seemingly benign, non-pathogenic imbalances, we compared our data to the Database of Genomic Variants. 33/50 microduplications and 141/148 microdeletions were present in the control population of the DGV. Therefore, 24 autosomal CNV's in a total of nineteen women were considered novel. There were seven novel autosomal microdeletions (Table 6), one in each of seven different women, and seventeen novel autosomal microduplications (Table 7), one in each of eleven women and two in each of three women.

Table 6. Novel Hemizygous Microdeletions in Cohort of Women with POI⁶⁶

Microdeletion Locus	Length(Mb)	Genes Within Deleted Region	Patient	Age at POI onset	Family history of POI?
chr 8: 124,964,990-125,101,913	0.14	<i>FER1L6</i>	POF-65	36	No
chr 10: 4,867,522-5,196,096	0.33	<i>AKR1E2, AKR1C1, AKR1C2, AKR1C3, AKR1CL1</i>	POF-105	35	No
chr 10: 90,517,549-90,648,588 ^a	0.13	<i>LIPN, LIPM, ANKRD22, STAMBPL1</i>	POF-58	36 ^b	No
chr 10: 135,092,227-135,256,027 ^c	0.16	<i>CYP2E1, SYCE1</i>	POF-108	21	Unknown
chr 14: 40,060,168-40,305,161	0.24	None	POF-66	31	No
chr 15: 80,972,574-82,640,853	1.67	<i>CPEB1, AP3B2, FSD2, HOMER2, WHAMM, FAM103A1, C15orf40, BTBD1, TM6SF1, HDGFRP3, BNC1, SH3GL3, ADAMTSL3</i>	POF-87	PA	Sister, aunt
chr 18: 55,460,039-55,783,281	0.32	<i>CCBE1, PMAIP1</i>	POF-119	PA	Unknown
chr X: 48,104,013-52,083,957	3.98	<i>SSX4.....BMP15....AK09637^d</i>	POF-37	34	No

^a Unable to corroborate via qPCR

^b Age at onset of POI unknown; age at time of POI diagnosis indicated

^c Pearson's chi squared test was used to show statistical significance:

3/2,802 control individuals showed a variation of this deletion in the DGV at the indicated locus⁶⁷⁻⁶⁸ compared to 1.1% in our cohort; $P < 0.0001$

^d A total of 65 genes deleted; the first and last genes in the deleted interval are listed, as well as the *BMP15* gene

Table 7. Novel Microduplications in Cohort of Women with POI⁶⁶

Microduplication locus	Length(Mb)	Genes within duplicated region	Patient	Age at POI onset	Family history of POI?
chr 1: 119,652,659-119,966,935	0.31	<i>HAO2, HSD3B2, HSD3B1</i>	POF-106	Unknown	Unknown
chr 1: 119,719,096-119,953,605	0.23	<i>HAO2, HSD3B2, HSD3B1</i>	POF-21	33	No
chr 1: 237,422,778-237,602,136	0.18	None	POF-56	33	No
chr 2: 51,536,418-51,865,782	0.33	None	POF-87	PA	Sister, aunt
chr 3: 7,979,828-8,127,315	0.15	None	POF-87	PA	Sister, aunt
chr 4: 4,705,258-4,939,891	0.23	<i>MSX1</i>	POF-124	Unknown	Unknown
chr 4: 6,003,730-6,247,313	0.24	<i>CRMP1, C4orf50, JAKMIP1</i>	POF-124	Unknown	Unknown
chr 6: 57,377,166-57,503,129	0.13	<i>PRIM2</i>	POF-34	25	Unknown
chr 7: 70,257,034-70,592,167	0.34	<i>WBSCR17, CALN1</i>	POF-37	34	Unknown
chr 7: 75,984,206-79,394,983	3.41	<i>PMS2L11, CCDC146, FL2, PION, PTPN12, RSBNIL, TMEM60, PHTF2, MAGI2, RPL13AP17</i>	POF-83	29	No
chr 7: 97,252,046-97,393,215	0.14	<i>ASNS</i>	POF-10	PA	No
chr 8: 509,919-835,807	0.33	<i>ERICH1</i>	POF-16	17	No
chr 8: 2,442,385-3,126,569	0.68	<i>CSMD1</i>	POF-45	20	No
chr 8: 47,626,500-48,043,110	0.42	<i>BEYLA</i>	POF-13	38	No
chr 9: 71,619,570-72,483,319	0.86	<i>C9orf135, MAMDC2, SMC5, KLF9, TRPM3</i>	POF-119	PA	Unknown
chr 11: 48,556,067-51,447,829	2.89	<i>FOLH1, OR4C13, OR4C12, OR4A5, OR4C46</i>	POF-10	PA	No
chr 16: 14,983,979-16,367,263	1.38	<i>PCXDCl, NTANI, RRN3, MPV17L, C16orf45, NDE1, MIR484, MYH11, C16orf63, ABCC1, ABCC6, NOMO3</i>	POF-11	27	No

We specifically focused on microdeletions, as genomic losses are more likely to result in a clinical phenotype than genomic gains⁶⁹. Eight women with POI exhibited novel hemizygous microdeletions greater than 0.1 Mb in size. These were present on chromosomes 8, 10, 14, 15, 18, and X. Although our study focused on autosomes, we detected one novel interstitial microdeletion on the X chromosome in a woman with secondary amenorrhea, which mapped to Xp11.23-p11.22 where the *BMP15* gene is located (Table 6).

qPCR was utilized to verify the novel autosomal microdeletions. A $2^{-\Delta \Delta C_t}$ (fold change) quantification analysis was performed with the standard error of the mean Ct difference for each group of replicates given as the error bars. Using this method, the initial amount of DNA can be determined by when the sample reaches a standardized concentration threshold. The fold change was plotted against the mean intensity shift (log R ratio), as measured on the array and shown in the GenomeStudio software (Figure 1). All plotted microdeletions showed considerable fold reductions, as indicated by the top bars that lie below the shaded region ($2n = \text{diploid}$). A cutoff value of 0.8 was used to determine diploid status. Six of the novel autosomal microdeletions contained coding regions, and five of the deletions were verified by qPCR analysis: 8q24.13, 10p15-p14, 10q26.3, 15q25.2, and 18q21.32 (Table 6).

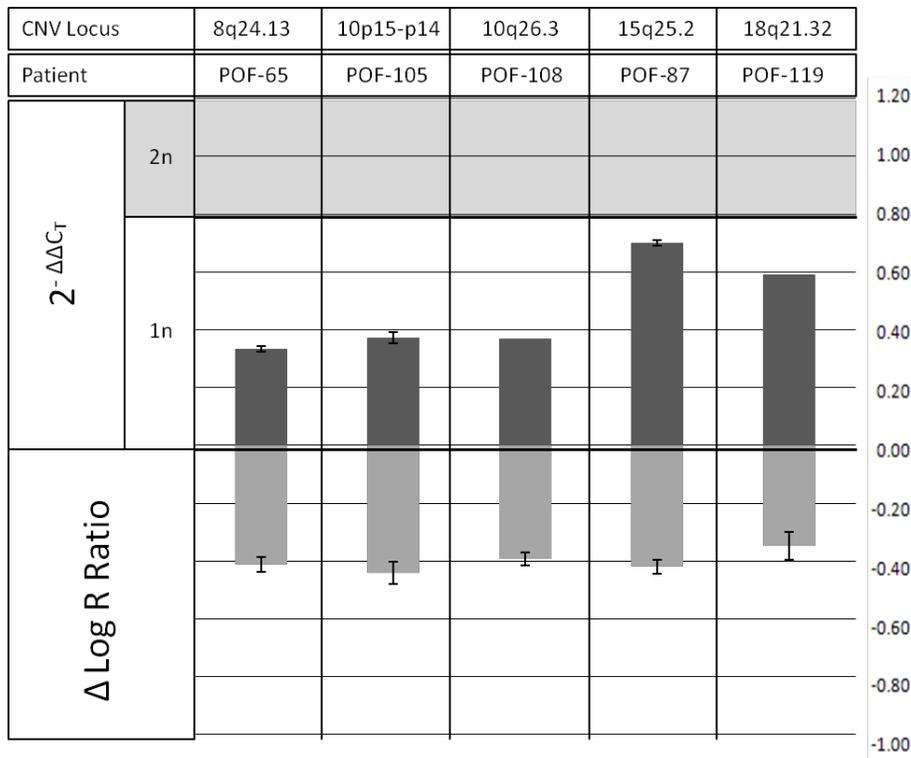


Figure 1. Data from SNP arrays and qPCR plotted as log R ratio and $2^{-\Delta \Delta C_t}$ for five autosomal microdeletions in women with POI⁶⁶

Microdeletions were found in a higher frequency in women with primary amenorrhea compared to women with secondary amenorrhea. Two of the seven genotyped women with primary amenorrhea (29%) had novel microdeletions as compared to 6/81 women with secondary amenorrhea or unknown presentation (7%). Of the nineteen total women with novel microdeletions or microduplications detected, one individual had a family history of POI in her sister and aunt (POF-87). This patient had one novel microdeletion present at 15q25.2, and two novel microduplications present, one at 2p16.3 and another at 3p26.1 (see Tables 6 and 7).

5.0 DISCUSSION

5.1 NOVEL CNV'S DETECTED BY SNP ARRAY

Genomic studies in the search for causes of complex diseases have led to the discovery that CNV's play a role in several common diseases. To date, only a few research groups have published data on copy number variants in women and families with POI.

Aboura et al studied 99 Caucasian women, using a BAC array to identify CNV's. Eight statistically significant CNVs were identified, 7 on autosomes and 1 on the X chromosome⁶² but none was considered novel. Five genes involved in reproduction were present in the CNV's and the authors concluded they may be potential candidate genes for POI⁶². These included *DNAH5*, *NAIP*, *DUSP22*, *NUPRI*, and *AKT1*⁶².

Another study focused on findings CNV's on the X chromosome⁶³. Forty-two women with idiopathic POI were studied using an X/Y tiling path BAC array to identify CNV's that may be involved in POI⁶³. Fifteen CNV's were discovered on both Xp and Xq that associated with POI⁶³. Genes located in these CNV regions included genes already thought to be associated with POI such as *POF1B*, as well as many others that may represent new candidate genes⁶³. This study also utilized a 1 Mb autosomal array; no CNV's were discovered on autosomes, likely due to the lower resolution of the array⁶³.

Another study utilized array CGH on 74 patients with POI or ovarian dysgenesis and found 15 microdeletions and 29 microduplications with only 4% present on the X chromosome²⁹. The authors focused their discussion on deletions and duplications containing genes that may be involved in fertility²⁹. The authors concluded that a majority of the genes present in the rearrangements were genes involved in meiosis, DNA repair, or ovarian folliculogenesis²⁹.

Knauff and colleagues utilized a SNP array to find CNV's on the X chromosome in 108 Caucasian women with POI compared to 235 healthy women as controls⁷⁰. After validation of CNV's by another custom designed X chromosome 60K array, there were no pathogenic CNV's detected in the POI cohort⁷⁰. The authors suggested that although surprising, it may be possible that only large deletions/duplications on the X chromosome cause POI⁷⁰. All four of the studies reported in the literature have small sample sizes and report numerous CNV's that have not yet been replicated in other women with POI. Furthermore, the studies failed to identify CNV's that could account for a majority of the POI experienced in their patients.

Our study specifically focused on autosomal microdeletions due to the overwhelming data from mice studies suggesting that autosomal genes are critical for ovarian development⁹. Mouse models include mice with prenatal ovarian failure and postnatal ovarian failure and make it possible to begin to understand the relationship between certain genes, folliculogenesis, and ovarian function⁹. More than thirty mouse models are used in research laboratories to study ovarian function, with the majority of the genes that are knocked out found on autosomal chromosomes rather than the X chromosome⁹. Furthermore, genomic losses are more likely to result in a clinical phenotype than duplications and past POI research has focused on X chromosome aberrations rather than autosomal ones⁸. We have identified a total of eight novel microdeletions among 88 women with POI. Five of six autosomal microdeletions that contained

known gene-coding regions were verified by qPCR. The last two novel microdeletions were not verified by qPCR; the deletion found on chromosome 14 of POF-66 did not contain any known genes, miRNA, or other non-coding RNAs, while the deletion found in POF-37 was present on the X chromosome.

There were a total of 92 genes involved in the novel microdeletions found in our POI cohort. Of these, two have been found to be linked to abnormal reproductive phenotypes in knock-out mice models, *SYCE1* and *CPEB1*. Synaptonemal complex central element protein (*SYCE1*) is located at 10q26.3 and has been shown to be an important structural component of the synaptonemal complex in males and females⁷¹. *SYCE1* knockout mice are infertile, with knockout ovaries being minute and knockout testes being only 20-30% the size of wild-type testes⁷¹. Histologically, knockout ovaries contained no follicles and the knockout testes contained mostly primary spermatocytes, indicating an arrest in prophase I⁷¹. Therefore, secondary amenorrhea at age 21 and POI in patient POF-108 may be due to heterozygous loss of the *SYCE1* gene and loss of functional oocytes.

Cytoplasmic Polyadenylation Element-Binding Protein 1 (*CPEB1*) is located at 15q25.2 and is known to be expressed in oocytes⁷². Studies have shown that adult female knockout mice have no oocytes present in their ovaries, and midgestational embryos had oocytes present that were arrested in pachytene⁷². The authors concluded that *CPEB1* was important in forming the synaptonemal complex⁷². As with *SYCE1*, haploinsufficiency of *CPEB1* may have played a role in the primary amenorrhea experienced by patient POF-87. This patient also had a family history of POI in her sister and aunt. Details of their age of onset or other medical history are unknown. Knowing the genetic status of other family members, including affected and

unaffected individuals, could help delineate if haploinsufficiency of this region and specifically the *CPEBI* gene is the cause for the familial POI.

Although we focused on autosomal microdeletions, one deletion was present on the X chromosome of one of the subjects (POF-37). This deletion spanned almost 4 Mb and contained 65 genes, including *BMP15*. *BMP15* is known to play a role in folliculogenesis, with studies identifying mutations in *BMP15* in women with primary and secondary amenorrhea³⁰. The presence of this gene within the large deletion on the X chromosome of this subject may provide a reason for this individual's secondary amenorrhea experienced at age 34.

The remaining microdeletions identified in our study harbor genes that are not known to play a role in reproductive biology. We used microarray data generated from wild-type newborn mouse ovaries, as well as NCBI EST databases (Unigene and Gene Expression Omnibus), to determine whether any of the genes involved in the microdeletions detected are expressed in mammalian ovaries⁷³. POF-119 harbored a deletion that resulted in deletion of one copy each of *CCBE1* and *PMAIP1*. Based on these data sets, both *CCBE1* and *PMAIP1* are expressed in mouse ovaries. Additionally, *CTNNA3* (POF-85), *ANKRD22* and *STAMBPL1* (POF-58) transcripts are present in the murine ovarian transcriptome. It is possible that such genes, as well as the others seen in our microdeletions and microduplications, play a role in ovarian development that has yet to be discovered.

Seventeen novel microduplications were identified by the SNP arrays and were not present in the DGV. Consequences of microduplications are harder to interpret than those of microdeletions. Therefore, our focus lay on analyzing and corroborating the novel deletions detected by the array. Of the microduplications found in our cohort, two individuals harbored almost identical duplications on chromosome one, POF-21 and POF-106. These

microduplications included genes *HSD3B2* and *HAO2*, both of which are expressed in ovaries of mice according to the Unigene and BioGPS databases with *HSD3B2* also being expressed in the adrenal glands of mice. The functional role of *HAO2* in the ovary has not yet been determined.

Hydroxy-delta-5-steroid dehydrogenase (*HSD3B2*, MIM 201810) is known to be involved in steroid hormone production. Mutations in this gene have been found in individuals with congenital adrenal hyperplasia with variable presentation. Virilization can occur in genetically female patients and under-virilization can occur in genetically male patients⁷⁴. In the severe form, salt wasting and salt loss can be a common cause of death in individuals. Fertility can sometimes be an issue for individuals affected with this form of adrenal hyperplasia due to the inability of the body to produce a normal amount of sex hormones such as testosterone, androgen, and estrogen⁷⁴. Studies have reported increased expression of *HSD3B2* in the theca cells of women with polycystic ovary syndrome, a frequent cause of infertility in women⁷⁵. A possible explanation of POI in our patient with a duplication of this gene is an imbalance of androgen and estrogen levels leading to the disruption of folliculogenesis. However, further studies are warranted to test this hypothesis.

5.2 LIMITATIONS OF STUDY AND FUTURE DIRECTIONS

There are multiple limitations to the current study design. Our cohort of 89 subjects is small, and a larger cohort of women is warranted. Larger sample sizes can aid in confirmation of the novel microdeletions detected in this pilot study, as well as identify more significant CNV's that may be present in a larger proportion of women with POI. Furthermore, animal studies modeling the

novel microdeletions and microduplications seen in patients with POI may help delineate the importance of CNV's seen in these women.

SNP arrays are not capable of covering the entire genome to date. Additional CNV's may be present in our cohort of women with POI, as high resolution arrays may cover only 50% of the CNV's in the genome⁷⁶.

The amount of information available on the subjects was limited in this pilot study. In some cases, it was not known at what age amenorrhea was first experienced, which is helpful in determining whether the individual had primary or secondary amenorrhea. Family history was limited in this study as well. POI is thought to be familial in 4-31% of cases and a detailed family history with particular attention paid to ages of menstruation and cessation, as well as any immune disorders, mental retardation, or environmental exposures can help determine if there is evidence of familial POI.

Limited family data also can affect the analysis of a given CNV. In women where a novel microdeletion is discovered, information as to whether the same CNV is present in affected and unaffected family members could aid in determining if the CNV is pathogenic or non-pathogenic. However, reduced penetrance and variable expressivity have been reported in families with POI. Therefore, identification of the same variant in an unaffected family member may not rule out pathogenicity of the CNV.

The control population used in our pilot study may be limiting. The DGV has been utilized in several studies. However its makeup of men and women may not make it the best control population for studies on ovarian function. The DGV is composed of women who are considered healthy. However their complete menstrual history is not known and may not be appropriate for a study on POI.

Further studies are needed to assess the involvement of CNV's in premature ovarian insufficiency. The University of Pittsburgh is currently recruiting women with POI in the Pittsburgh area to continue identifying pathogenic CNV's. Recruitment of women with primary amenorrhea, as well as families with multiple individuals affected, may make finding genomic reasons for POI more likely. A better control population will also aid in determining CNV affects. Women who are known to have gone through natural menopause after the age of 45 and are known to be fertile, e.g. conceived children naturally, would aid in the future studies of CNV's associating with POI.

5.3 PUBLIC HEALTH SIGNIFICANCE

Premature ovarian insufficiency is a devastating diagnosis for families and affects 1% of women under the age of 40 years and 0.1% of women under the age of 30. With most cases of POI having an unknown cause, studies focusing on identifying genomic imbalances that may cause POI can lead to the discovery of more causes of this syndrome. There are no known proven methods to assist women with POI to become pregnant naturally. Discovering the genomic causes of the disease may lead to the development of safe treatment methods for these women. Furthermore, earlier detection of the disease, including before onset of amenorrhea, may aid in family planning for couples.

It is known that women with POI are at an increased risk for several common diseases, including osteoporosis and cardiovascular diseases and suffer from an increased overall mortality of 50%¹¹. Discovery of genes that are involved in POI may be determined to be risk factors for

overall morbidity and mortality and cardiovascular disease, thus aiding in the future management of patients known to have perturbations in these genes.

Further delineation of genomic imbalances in women with POI may indicate further genetic studies are warranted in the initial work up. Fragile X studies and routine karyotype are the standard of care and studies on women with POI are finding genomic changes smaller than what can be detected by conventional karyotype. In the future, array technology may be an important addition to the work up of women with POI.

5.4 CONCLUSIONS

We utilized SNP arrays technology to identify copy number variants in a cohort of 89 women with POI. The Database of Genomic Variants (DGV) was used as a control population, aiding in the identification of seven novel autosomal microdeletions in our POI cohort, six of which contained gene-coding regions: 8q24.13, 10p15-p14, 10q23.31, 10q26.3, 15q25.2, and 18q21.32. Two of the novel microdeletions contained genes known to cause ovarian failure in knock-out mice models, *SYCE1* and *CPEB1*. One individual harbored a 4Mb deletion on one of her X chromosomes resulting in haploinsufficiency of 65 genes, including *BMP15*, which is a possible explanation for her secondary amenorrhea. Seventeen novel microduplications were also detected, with the majority of CNV's detected being on autosomal chromosomes rather than the X chromosome. Two of the seven genotyped women with primary amenorrhea (29%) had novel microdeletions as compared to six of eighty women with secondary amenorrhea or unknown presentation (7%). This pilot study demonstrates the role CNV's play in POI and highlights the importance for studies with larger samples sizes to confirm the findings and further support the

hypothesis. Identification of known genetic causes could aid in development of effective treatments for women with POI, as well as earlier diagnosis which may allow for family planning before the onset of amenorrhea. Furthermore, array technology may be a useful addition to conventional karyotyping when evaluating women with POI.

APPENDIX A

IRB APPROVAL FOR POI STUDY, BAYLOR COLLEGE OF MEDICINE

January 14, 2010



ERTUG KOVANCI
BAYLOR COLLEGE OF MEDICINE
OB-GYN: REPROD. ENDOCRIN-INFIRT

Baylor College of Medicine
Office of Research
One Baylor Plaza, 600D
Houston, Texas 77030
Phone: (713) 798-6970
Fax: (713) 798-6990
Email: irb@bcm.tmc.edu

H-10662 - GENETIC BASIS OF FEMALE FERTILITY (OOGENESIS) AND PREMATURE OVARIAN FAILURE

APPROVAL VALID FROM 11/16/2009 TO 11/15/2010

Dear Dr. KOVANCI

The Institutional Review Board for Human Subject Research for Baylor College of Medicine and Affiliated Hospitals (BCM IRB) is pleased to inform you that the research protocol and consent form(s) named above were approved.

The study may not continue after the approval period without additional IRB review and approval for continuation. You will receive an email renewal reminder notice prior to study expiration; however, it is your responsibility to assure that this study is not conducted beyond the expiration date.

Please be aware that only IRB-approved informed consent forms may be used when written informed consent is required.

Any changes in study or informed consent procedure must receive review and approval prior to implementation unless the change is necessary for the safety of subjects. In addition, you must inform the IRB of adverse events encountered during the study or of any new and significant information that may impact a research participants' safety or willingness to continue in your study.

The BCM IRB is organized, operates, and is registered with the United States Office for Human Research Protections according to the regulations codified in the United States Code of Federal Regulations at 45 CFR 46 and 21 CFR 56. The BCM IRB operates under the BCM Federal Wide Assurance No. 00000286, as well as those of hospitals and institutions affiliated with the College.

Sincerely yours,



VERNON R SUTTON, M.D., B.S.

Institutional Review Board for Baylor College of Medicine and Affiliated Hospitals

APPENDIX B

INFORMED CONSENT FORM FOR POI STUDY

Institutional Review Board for Baylor College of Medicine and Affiliated Hospitals

POF Participant

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Background

Please read this information carefully. If you have any questions please ask the study doctor or staff.

Infertility or not being able to get pregnant after at least one year of trying affects 2.5-3.0 million couples in the U.S. yearly. In 50% of all cases the reason is because of the female. Each year nearly \$2 billion is spent on infertility diagnosis and treatment. However, many female patients seen for infertility evaluation or premature ovarian failure (when a woman’s ovaries stop working before she is 40) are found to have no explanations for the problem.

Study doctors know that in women with premature ovarian failure something happens to stop the normal functioning of the ovaries; but in most cases, the exact cause is not clear. This results in an inability to diagnose specific problems, inability to offer accurate prognosis, and lack of effective treatment.

Study doctors think that changes in genetic information may play an important role in infertility and premature ovarian failure (POF). Therefore they want to study genes to see if there have been any changes. In addition, study doctors are interested in also studying family members to find out whether genetic changes are related to infertility or POF.

This research study is sponsored by Baylor College of Medicine.

Purpose

The purpose of this study is to determine if there is a genetic cause for infertility or POF. This will be done by studying the genetic information in your blood or tissue sample.

Procedures

A total of 200 subjects at 1 institutions will be asked to participate in this study. You will be one of approximately 200 subjects to be asked to participate at this location.

We are providing you with the information so you know that you are not the only one that is being asked to participate in the study.

The research will be conducted at the following location(s): Baylor College of Medicine, TCH: Texas Children's Hospital, University of Pittsburgh Medical Center - Pennsylvania.

If you decide to participate, you will provide the study doctor with your medical and family history, information about premature ovarian failures or other gynecologic problems. You will also provide the study doctor with one blood sample or a tissue sample, a questionnaire, and a pedigree of your family. We will send you the materials needed to get a blood sample. We may also ask you for another sample of your blood if we are unable to obtain an adequate DNA sample from your blood or if we find new genetic information that may be important to the study.

We would like to get approximately 20cc or 4 teaspoons of blood from you to possibly find out if the cause of ovarian failure or infertility is due to genetic changes. You can come into our office and have a nurse draw blood from your vein in your arm. You can also have your primary care physician or their

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nurse draw the blood and mail the sample to our office. If you would like to do this then we will be able to provide you with a laboratory order to have your primary doctor's office obtain the blood sample. Also, we will provide you with a blood collection kit , instruction sheet that tells you what to do with the sample once it is obtained and a FEDEX envelope with a pre-paid return mailer to send the sample to our office. This blood will be used only to get genetic material (DNA). The blood sample is for research purposes only. We will only be looking at genes thought to be related to infertility and POF. If tissue other than blood is already available or will become available, it may be analyzed in place of your blood

The tissue sample can be a sample that has already been removed by a past surgery or biopsy. We would like to use a small amount of tissue that is not needed by the pathologist (the doctor that studied your tissue). We will only be examining tissue that would have otherwise been destroyed. Only if excess tissue from any surgical specimen is available will it be used for this study. No surgery will be performed solely for the purpose of obtaining surgical specimens. Tissue samples used for the study will only be those obtained at an earlier time for previous testing. If you would like to provide us with a sample of your tissue then you will need to give us the name of your doctor that performed the surgery or biopsy or the doctor who ordered the procedure. We will also need you to provide us with a medical release form. The medical release form is a form that you sign that grants us permission to allow us to obtain the tissue from the doctors office.

Once we receive your blood and/or tissue specimen it will be sent to the OB/GYN laboratory at Baylor College of Medicine for analysis. Your specimen will be relabeled Unprocessed specimens will be frozen at -70C and stored in a locked freezer located in the principal investigator's laboratory. The remaining portion of the specimen(s) will be used to isolated DNA for genetic analysis. The sample will be banked at 4C and -20C, respectively, in locked refrigerator/freezers located in the principal investigator's laboratory. The results of your DNA analysis will be entered into a computerized database and reviewed by the pricipal investigator and his staff. Only the study doctor and their research assistant will have access to the original research data. Data will be completely confidential, and will not be revealed to family members, insurance companies, employers, or other individuals or organizations.

Your study records and medical records will be kept completely separate and no study data or information about your participation in the study will be entered into medical records. Your name will never appear on any reports of this study's findings. All specimens may be kept indefinitely. Your specimens will be discarded if the study is completed and no further experimentation is expected. You may withdraw from the study at any time. Your specimen will be destroyed upon withdrawal from the study. Your specimen will be available for other testing only following your authorization to the principal investigator. Your specimen(s) will not be transferred or sold in whole or part to any third party without obtaining written permission directly from you. The study doctor may contact you if he needs another blood sample from you.

The results of you DNA analysis will be entered into a computerized database and reviewed by the principal investigator and his staff. Your DNA may be analyzed in the future as new test become available that are related to premature ovarian failure. The DNA will be analyzed only for genes (functional unit of hereditary material) related to premature ovarian failure. No additional research (i.e.,

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research not related to POF) will be performed on your DNA without your written permission. You may revoke your authorization for DNA research analyses at any time. In addition, if any DNA remains, you can request that it be destroyed and discarded at any time. All aspects of this study will be kept strictly confidential.

These tests are being done for research purposes only; you and your doctor will not be informed of the results.

PEDIGREE CHART: You will complete a pedigree chart. A pedigree is like a family tree chart. It will identify family members that may have had problems with infertility or POF. We will give you a pedigree worksheet for you to complete. This form must be completed and returned to the study doctor or their coordinator.

QUESTIONNAIRE: You will complete a brief questionnaire. The questionnaire will ask you questions about your menstrual history, how you were diagnosed with POF, medical and family history. Please answer the questions as completely as possible.

FAMILY MEMBERS

We are interested in studying your family regardless of whether they can or cannot have children to help us find out if genetic changes are related to infertility or POF. We will be asking you to provide information about this study to your family members in order to test their DNA. We will provide you with a separate form for you to provide to them so they can read and possibly participate in the study. We may also ask to obtain additional samples of your family members blood if we are unable to obtain an adequate DNA sample from them or if we find new genetic information that may be important to the study.

The duration of your participation is limited to the time necessary to obtain blood or other tissue, to verify diagnosis and perform lab studies. This is expected to be 3 years or less. Blood or tissue will be stored and tested for newly detected genes that may be identified.

Your research doctor may be able to provide you with part of your information while the study is in progress. Test results will be revealed to you only at the discretion of the study doctor.

Potential Risks and Discomforts

Unforeseeable and/or unknown risks/discomforts may occur.

Blood draws: Having a blood sample taken by a medically trained person is a safe procedure, although occasionally there may be minor discomfort, and a bruise may develop around the needle puncture site. Some people may also occasionally feel faint or dizzy. Healthcare workers drawing the blood are aware of this, and they will watch you carefully before letting you get up from the chair in which you will be sitting when your blood is drawn.

Questionnaire: If you are unable to give information about your family's medical history, it may cause anxiety. It is okay if you are able to provide us with limited information you or your family knows.

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Pedigree- Although the risk is minimal it may cause anxiety.

There is some risk associated with genetic testing for uncovering and conveying unwanted information regarding specific risk of disease or not providing any new information to you about POF.

Study staff will update you in a timely way on any new information that may affect your decision to stay in the study.

Potential Benefits

You will receive no direct benefit from your participation in this study. However, your participation may help the investigators better understand genetic causes of infertility or POF. It is possible that future patients may profit from the result of this research.

Alternatives

You may choose to not participate in this study.

Investigator Withdrawal of Subject from a Study

The investigator or sponsor may decide to stop you from taking part in this study at any time. You could be removed from the study for reasons related only to you or because the entire study is stopped. The sponsor, investigator, Food and Drug Administration, or Institutional Review Board may stop the study at any time.

You may withdraw from the study at any time and you may request that your sample be permanently removed if it has not already been used.

Subject Costs and Payments

You will be reimbursed for the costs associated with the blood draw and mailing only if you have someone other than the study staff draw the blood. You will receive up to \$15 for blood draw charges. We will provide you with pre-paid shipping forms for blood that is mailed in to our office.

You will not be paid for taking part in this study.

Research personnel will try to reduce, control, and treat any complications from this research. If you are injured because of this study, you will receive medical care that you or your insurance will have to pay for just like any other medical care.

Subject's Rights

Your signature on this consent form means that you have received the information about this study and that you agree to be a part of the study.

You will be given a copy of this signed form to keep. You are not giving up any of your rights by signing this form. Even after you have signed this form, you may change your mind at any time. Please contact

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the study staff if you decide to stop taking part in this study.

The investigator or sponsor may decide to stop you from taking part in this study at any time. You could be removed from the study for reasons related only to you (for example, if you move to another city, if you do not take your study medication, or if you have a serious reaction to your study medication) or because the entire study is stopped. The sponsor may stop the study at any time.

There may be unknown risks/discomforts involved. Study staff will update you in a timely way on any new information that may affect your health, welfare, or decision to stay in this study.

If you are injured because of this study, you will receive medical care that you or your insurance will have to pay for just like any other medical care. You will not be paid for the injury.

Your Health Information

We may be collecting health information that could be linked to you (protected health information). This protected health information might have your name, address, social security number or something else that identifies you attached to it. Federal law wants us to get your permission to use your protected health information for this study. Your signature on this form means that you give us permission to use your protected health information for this research study.

If you decide to take part in the study, your protected health information will not be given out except as allowed by law or as described in this form. Everyone working with your protected health information will work to keep this information private. The results of the data from the study may be published. However, you will not be identified by name.

People who give medical care and ensure quality from the institutions where the research is being done, the sponsor(s) listed in the sections above, representatives of the sponsor, and regulatory agencies such as the U.S. Department of Health and Human Services will be allowed to look at sections of your medical and research records related to this study. Because of the need for the investigator and study staff to release information to these parties, complete privacy cannot be guaranteed.

The people listed above will be able to access your information for as long as they need to, even after the study is completed.

If you decide to stop taking part in the study or if you are removed from the study, you may decide that you no longer allow protected health information that identifies you to be used in this research study. Contact the study staff to tell them of this decision, and they will give you an address so that you can inform the investigator in writing. The investigator will honor your decision unless not being able to use your identifiable health information would affect the safety or quality of the research study.

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The investigator, ERTUG KOVANCI, and/or someone he/she appoints in his/her place will try to answer all of your questions. If you have questions or concerns at any time, or if you need to report an injury related to the research, you may speak with a member of the study staff: Aleksandar Rajkovic at 713-798-7500 during the day and/or Lisa Marsh (713) 798-7549 during the day or after hours.

Members of the Institutional Review Board for Baylor College of Medicine and Affiliated Hospitals (IRB) can also answer your questions and concerns about your rights as a research subject. The IRB office number is (713) 798-6970.

If your child is the one invited to take part in this study you are signing to give your permission. Each child may agree to take part in a study at his or her own level of understanding. When you sign this you also note that you child understands and agrees to take part in this study according to his or her understanding.

Please print your child's name here _____

CONSENT FORM

HIPAA Compliant

Institutional Review Board for Baylor College of Medicine and Affiliated Hospitals

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OVARIAN FAILURE

Signing this consent form indicates that you have read this consent form (or have had it read to you), that your questions have been answered to your satisfaction, and that you voluntarily agree to participate in this research study. You will receive a copy of this signed consent form.

Subject Date

Legally Authorized Representative Date
Parent or Guardian

Investigator or Designee Obtaining Consent Date

Witness (if applicable) Date

Translator (if applicable) Date

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