

**Thrombosis In Pregnancy: The Role of Factor V Leiden, Prothrombin 20210 G to A, Angiotensin Converting Enzyme and Methylenetetrahydrofolate Reductase in a U.S. Population**

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**CONDENSATION**

Factor V Leiden, the prothrombin 20210 G to A variant, and a deletion polymorphism in the angiotensin converting enzyme gene increase the risk of thrombosis during pregnancy.

**ABSTRACT**

**OBJECTIVES:** Polymorphisms in the genes for factor V (Factor V Leiden), prothrombin, methylenetetrahydrofolate reductase (MTHFR), and angiotensin converting enzyme (ACE) have been associated with the occurrence of venous thrombosis. The objective of this study was to determine the relationship of these polymorphisms to thrombosis during pregnancy.

**STUDY DESIGN:** This is case-control study of 41 cases of venous thrombosis during pregnancy and 76 controls matched on hospital and race (white versus black) who had a normal pregnancy.

**RESULTS:** Among whites, mutations in the factor V gene and the prothrombin gene were associated with increased risk of venous thrombosis during pregnancy (the factor V odds ratio was 18.3 (P = 0.001) with 95% confidence interval 2.7 - 432; the prothrombin odds ratio was infinity (P = 0.01) with a 95% lower confidence limit of 1.7. No black subject had either of these two mutations. For black and white subjects, the *D/D* genotype of the ACE gene compared with the other genotypes entailed increased risk (odds ratio of 2.7 (P = 0.02) with 95% confidence interval, 1.2 - 6.3). The MTHFR polymorphism was unrelated to thrombosis during pregnancy in both blacks and whites.

**CONCLUSIONS:** Women who experience thrombotic complications in pregnancy have an increased prevalence of genetic mutations related to coagulation. The additional risk of thrombosis during pregnancy associated with such genetic mutations can be substantial.

Key Words: pregnancy; blood coagulation factors; genetics; risk factors; thrombosis

## INTRODUCTION

Pregnancy and the postpartum period are considered to be hypercoagulable states that are associated with increased concentrations of von Willebrand factor, fibrinogen, factor VIII, other vitamin K-dependent clotting factors, and decreased concentrations of protein S as well as inhibition of fibrinolytic activity.<sup>1</sup> Although these physiologic changes in the coagulation system are important to minimize blood loss during gestation and delivery, the risk of venous thromboembolism during pregnancy and after delivery is increased about five-fold compared with non-pregnant women.<sup>1</sup> As a result, thromboembolic complications are a major cause of maternal morbidity and mortality in the United States and Europe occurring in approximately 1 in 1,000 to 1 in 2,000 pregnancies.<sup>1,2</sup>

Recent research has indicated that the risk of thrombosis is increased greatly when these normal changes occurring during pregnancy are accompanied by genetic risk factors for thrombosis. A hereditary abnormality in the protein C/protein S anticoagulant system involving a mutation of Arg506 to Gln506 on the factor V gene leads to an impaired response to activated protein C, termed resistance to activated protein C (APC-R).<sup>3</sup> APC-R/Factor V Leiden has been found to be a risk factor for venous thrombosis in several studies, including some of pregnant women.<sup>4,5,6,7</sup>

Recently a described genetic variation in the prothrombin gene located in the 3'-untranslated region at position 20210 where a G to A transition occurs has been related to thrombosis.<sup>8</sup> The A allele is associated with higher plasma prothrombin levels and carriers of this allele are at increased risk of thrombotic events.<sup>8</sup> One study found the risk of cerebral-vein thrombosis was increased among women with the prothrombin variant who used oral contraceptives.<sup>9</sup> This observation suggests that this genetic trait may be a risk factor for thrombosis during pregnancy. In a recent report from an Italian research

group, a higher prevalence of this genetic mutation was observed among cases of thrombosis during pregnancy compared with controls.<sup>7</sup>

Two other genetic traits which have recently been shown to increase the risk of venous thrombosis are the angiotensin I-converting enzyme gene (ACE) and the 5,10-methylenetetrahydrofolate reductase (MTHFR) gene. Though these genes do not specifically regulate coagulation, they affect the coagulation system indirectly. The ACE gene is involved in the regulation of vascular tone by converting angiotensin I to the potent vasoconstrictor angiotensin II by the inactivation of the vasodilator bradykinin.<sup>10</sup> An insertion/deletion polymorphism in the ACE gene has been identified and the *D/D* genotype has been associated with increased plasma ACE levels and has been linked to an increased risk of cardiovascular disease in several epidemiologic studies.<sup>11,12</sup> Two epidemiologic studies implicate the *D/D* genotype as a risk for venous thrombosis. In a case-control study of African Americans, the *D/D* ACE genotype was associated with a 3-fold increased risk of deep vein thrombosis among men, but no excess risk was found among women.<sup>13</sup> The *D* allele recently has been related to increased risk of venous thrombosis following hip replacement surgery.<sup>14</sup>

The MTHFR gene encodes for a key enzyme involved in the remethylation of homocysteine to methionine.<sup>15</sup> One polymorphism in the MTHFR gene occurs where a C to T substitution at nucleotide 677 converts alanine to valine.<sup>16</sup> The V allele is associated with reduced MTHFR activity and elevated levels of plasma homocysteine.<sup>16</sup> Hyperhomocysteinemia has been shown to increase the risk of venous thrombosis.<sup>17</sup> Recently, Grandone et al reported a weak association between difference in homozygosity for the MTHFR mutation and thrombosis during pregnancy.<sup>7</sup>

In this study, we investigate the relationship between the occurrence of thrombosis in

pregnancy and the presence of Factor V Leiden, the prothrombin 20210 variant, the ACE polymorphism, and the MTHFR polymorphism in a United States population.

## **METHODS**

Women experiencing a deep venous thrombosis and/or pulmonary embolism during pregnancy at two New Jersey and two Georgia hospitals were eligible as cases. These cases were identified retrospectively by computer search for the appropriate ICD-9 codes (671.30-671.54, 671.90-671.94, 673.20-673.24) at the hospitals from 1991 through 1996. A total of 158 women were identified as potential cases at the four hospitals. However, 45 were excluded either because their medical records did not include radiological confirmation of a clot during pregnancy or because the medical record indicated that the clot occurred in the presence of infection. Of the remaining 113 eligible cases, 65 could not be located or did not respond to our mailings, and 7 were contacted but refused participation. Thus, 41 cases are included in the study.

Controls were selected from among women at these hospitals who experienced a live birth during the study period. At three of the hospitals, a list of potential control subjects was generated from a computer search for the ICD-9 code for a normal vaginal or cesarean delivery (650). At the remaining hospital, controls were selected from among women attending the obstetric clinic for the six week postpartum visit. For each case, we attempted to obtain 2 controls of the same race. Women who had experienced a clot during the index pregnancy were not eligible as controls. We identified 248 potential control subjects. One hundred forty-four of these women could not be located or did not respond to our mailings, while 28 refused participation. Thus, the study includes 76 controls.

Participants were administered an in-person questionnaire by study interviewers. The instrument collected information on demographics, lifestyle habits, medical history, reproductive history, and family history of thrombosis. A venous blood sample was collected for DNA analysis of the factor

V, prothrombin, MTHFR, and ACE genes.

Human investigation approvals from the institutional review boards of the Centers For Disease Control and Prevention, Emory University, Northside Hospital, and Robert Wood Johnson Medical School were obtained prior to the initiation of the study.

### **Laboratory Methods**

The presence of factor V Leiden, zygosity for the 20210 G to A polymorphism of the prothrombin gene, zygosity for the alanine/valine polymorphism of the MTHFR gene, and zygosity for the insertion/deletion polymorphism of the ACE gene were determined from DNA extracted from a blood sample. DNA was extracted from 3 ml of whole blood using a Gentra\* DNA Extraction kit (Minneapolis, Minnesota) per the manufacturer's instructions and stored at -20 degrees centigrade. Polymerase chain reaction (PCR) was used to amplify DNA fragments in the factor V<sup>18</sup>, prothrombin<sup>8</sup>, MTHFR<sup>16</sup>, and ACE<sup>11</sup> genes. Restriction enzyme analysis for factor V Leiden and MTHFR was carried out using Mnl and Hinf1, respectively. The digested products were then run on a ethidium bromide stained 3% metaphor gel and the results were determined from the restriction enzyme digestion pattern. A subset of at least twenty samples was selected at random and confirmed by direct nucleotide sequencing. For the prothrombin gene, restriction enzyme analysis was performed using Hind III. The digested products were then run on an ethidium bromide-stained 3% metaphor gel, and the results were determined from the restriction enzyme pattern. The results were confirmed by both

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direct nucleotide sequencing and fragment analysis using GENESCAN software following electrophoresis on the 310 automated ABI DNA sequencer (Applied Biosystems, Foster City, CA) on a subset of at least twenty samples selected at random. The insertion/deletion polymorphisms were determined by sizing the amplified product in a ethidium bromide stained 1.5 percent agarose gel. In order to ensure stringency for the D/D genotype, dimethyl sulfoxide was included in the polymerase chain reaction mixture. Furthermore, all D/D results were confirmed with the use of a third primer set that discriminated between the I/D and D/D. These results also were confirmed by both direct nucleotide sequencing and fragment analysis using GENESCAN software following electrophoresis on the 310 automated ABI DNA sequencer on a subset of at least twenty samples selected at random. Quality control for the DNA analyses was maintained by the use of both positive and negative controls in each set of analyzed samples and results were confirmed independently by two different laboratory workers.

### **Statistical Methods**

Most genetic analyses were performed separately for blacks and whites because the prevalence of the factor V, prothrombin and MTHFR polymorphisms varies considerably by race.<sup>19,20,21</sup> Since only one person was homozygous for factor V Leiden and none was homozygous for the prothrombin mutation, these genetic mutations are classified as either present (heterozygous or homozygous) or absent. The ACE and MTHFR polymorphisms were analyzed according to zygosity (*I/I*, *I/D* and *D/D* genotypes for ACE and *A/A*, *V/A* and *V/V* genotypes for MTHFR). Additionally, for the ACE gene, a recessive model is considered in which the *D/D* genotype is compared to the *I/I* and the *I/D* genotypes combined.

The odds ratio was used as a measure of association between genotypes and thrombosis during pregnancy and estimates the relative risk of thrombosis during pregnancy for women with a genetic defect compared with women without the defect. Confidence intervals for the odds ratios and tests of statistical significance were obtained using conditional likelihood procedures and exact mid P value methods.<sup>22</sup> The odds ratios were obtained by stratifying by the matching factors, hospital and race. All reported P values are two-tailed. We also used multiple logistic regression to evaluate trends in the odds ratios and to test for statistical interaction between genes and the risk of venous thrombosis during pregnancy.<sup>23</sup>

## RESULTS

Thirty-one of the cases had a deep vein thrombosis in the leg, hip or pelvic area, 6 had an ovarian vein thrombosis, 2 had a retinal thrombosis, and 2 cases had a pulmonary embolism. For 28 of the cases, the clot occurred during pregnancy with an average gestational age of occurrence of 22 weeks (range 4-40). Eleven women experienced their clot during the post-partum period and two women had clots after a pregnancy loss. Of these 13 women with post-partum/post-pregnancy loss clots, one had a deep vein thrombosis in the groin, four had an ovarian vein thrombosis and eight had a clot in a deep vein of the leg. Three of the women with post-partum clots had undergone caesarian section as a mode of delivery. Diagnosis of the thrombotic event was made with ultrasound for 27 of the 28 women whose clots occurred during pregnancy. One woman was given a ventilation-perfusion scan at eight weeks of gestation. Women who experienced post-partum or post-pregnancy loss clots were diagnosed by ultrasound (n=8), magnetic resonance imaging (n=4) or, in the case of pulmonary embolism, ventilation-perfusion scan (n=1). Of the forty-one cases, one case had a history of thrombosis. Descriptive data for cases and controls is presented in Table 1. Thirty-five percent of the study subjects are black and the remainder are white. The mean age at the time of the index pregnancy for cases and controls was 28 and 30 years, respectively. Both cases and controls had a mean gravidity of about three and mean parity of about two at the time of the index pregnancy. Cases and controls were similar with respect to cigarette habit and lifetime alcohol consumption. Cases were more likely to have a sibling with a history of blood clots than were controls ( $p = 0.02$ ), but no such difference was found for parents' history ( $P > 0.20$ ). The case/control distribution of the variables in Table 1 is similar for black women and white women.

The factor V mutation is a strong risk factor for thrombosis during pregnancy among whites (Table 2). Eight cases and one control with the mutation yields an odds ratio of about 18 ( $P = 0.001$ ). The lower limit of the confidence interval indicates that women with factor V Leiden are at least 3-times as likely to have a thrombotic event during pregnancy than are women without this polymorphism. No black subject had factor V Leiden.

Four white cases had the prothrombin 20210 polymorphism, whereas no white controls had this polymorphism ( $P = 0.01$ ). The lower limit of the confidence interval indicates that women with this polymorphism have at least a 2-fold increased risk of thrombosis during pregnancy compared with women without this allele. As was also true for factor V Leiden, no blacks had the prothrombin mutation.

Among whites, the prevalence of the *D* allele in the ACE gene was 63 and 49 percent in cases and controls, respectively ( $P = 0.10$ ). Among blacks, the prevalence of the *D* allele was 79 and 63 percent in cases and controls, respectively ( $P = 0.17$ ). However, when combining whites and blacks and adjusting for race the difference between the prevalence of the *D* allele between cases and controls was statistically significant ( $P = 0.03$ ). A three-fold increase in the risk of venous thrombosis was evident in both white and black women with the *D/D* genotype compared with those of the *I/I* genotype but statistical significance was not reached. Increased risk among women with the *I/D* genotype compared to those with the *I/I* genotype was not evident. If the *D* allele is considered as a recessive trait (*D/D* genotype versus the *I/D* or *I/I* genotype), about a 3-fold increase in risk for the *D/D* genotype ( $P = 0.02$ ) is observed.

There was no association between the MTHFR polymorphism and thrombosis during

pregnancy in either whites or blacks. The overall prevalence of the *V* allele was 44 percent and 43 percent in white cases and white controls ( $P > 0.20$ ), respectively. Among blacks, 14 percent and 11 percent of cases and controls ( $P > 0.20$ ), respectively, had the *V* allele.

In Table 3 the distribution of cases and controls by the total number of defective alleles in the factor *V*, prothrombin, and ACE genes is displayed. The risk of thrombosis during pregnancy is increased by about 160% with the addition of each mutant allele (trend test  $P = 0.001$ ).

## DISCUSSION

In this case-control study, we found a higher prevalence of mutations in the factor V, prothrombin, and ACE genes among white women who experienced thrombosis during pregnancy compared with those who had not. In black women experiencing thrombosis during pregnancy, a higher prevalence of the mutation in the ACE gene was observed compared to those who had not. Mutations in the factor V and prothrombin genes were not present in any of the black study subjects. Because the population prevalence of these mutations is very low in blacks<sup>19,21</sup>, our sample size was inadequate to assess the risk of thrombosis during pregnancy for the rare black woman who possesses mutations.

The factor V mutation was found in 30 percent of white cases and only 2 percent of white controls; a control prevalence similar to that reported for other white control populations.<sup>24</sup> This finding is similar to that of a recent Italian case-control study of 42 women with deep vein thrombosis during pregnancy or in the postpartum period and 212 controls, where the prevalence of factor V Leiden was 24% and 2% among cases and controls, respectively.<sup>7</sup> Our study, and that of Grandone, suggest that factor V Leiden increases the risk of a thrombosis during pregnancy by about 20-fold. The two studies together provide 95 percent confidence that the risk of thrombosis during pregnancy is increased by at least 6-fold for factor V Leiden as evidenced by the lower limits of the confidence intervals. A causal interpretation of this finding is supported by the observations that factor V Leiden has been related to increased risk of general obstetrical complications during pregnancy.<sup>25</sup> Thus, since chance is a very unlikely explanation of this finding and since the hypothesis that factor V Leiden is associated with thrombosis during pregnancy is biologically plausible, we favor a causal interpretation of the factor V

Leiden result. Of interest is the fact that of the eight cases with factor V Leiden, one was also heterozygous for the MTHFR mutation, one was homozygous for factor V Leiden and homozygous for the ACE mutation, and six also had the MTHFR and ACE mutations in either the heterozygous or homozygous state. No subject had factor V Leiden alone. Thus, we were unable to quantify the effect of Factor V Leiden alone on the risk of thrombosis during pregnancy.

Our finding with respect to the prothrombin G20210A mutation is striking. Although an apparent limitation of our study is its small size and the low prevalence of this mutation in the general white population (about 2%), our finding of 4 white cases with the defect versus no controls is statistically significant. In Grandone's study, the prothrombin mutation was associated with about a 10-fold increased risk of thrombosis during pregnancy<sup>7</sup> and in Kupferminc's study about a 4-fold, statistically significant, increased risk of general obstetrical complications during pregnancy<sup>25</sup>. Grandone's study and our study together suggest about 10-fold increased risk of thrombosis during pregnancy due to the prothrombin mutation with a lower 95% confidence limit for the relative risk of about 5. Thus, as with factor V Leiden, the hypothesis that the prothrombin mutation is a cause of thrombosis during pregnancy is biologically plausible and chance is an unlikely explanation. Since we cannot identify any bias that would explain the prothrombin finding, we favor a causal explanation.

We found that a recessive genetic model for the ACE *D* allele (*I/I+I/D* compared to *D/D*) was related to a statistically significant increased risk of thrombosis during pregnancy. The association was apparent both in white and black subjects. To our knowledge, the finding that the *D/D* genotype of the ACE gene increases risk of venous thrombosis during pregnancy is new. We recently reported that the ACE *D* allele was related to the occurrence of thrombosis following hip surgery.<sup>14</sup> ACE has

vasoconstrictive effects, can stimulate plasminogen activator-I inhibitor, and can activate platelets.<sup>10</sup> It follows that physiological changes in the coagulation system which occur in situations such as surgery or in pregnancy may interact with the effects of ACE to increase thrombotic risk.

We did not find an increased risk of thrombosis during pregnancy for subjects homozygous for the *V* allele of the MTHFR gene, but Grandone did. In both studies, the prevalence of the *V/V* genotype among controls was 16 percent. However, our cases had a prevalence similar to that of controls (18%), whereas in Grandone's study the prevalence of the *V/V* genotype among cases was 29%. The findings of our and of Grandone's study are consistent and together provide a summary relative risk of about 1.8 (0.9, 3.4;  $P = 0.09$ ) for the *V/V* genotype compared with the other two genotypes. Kupferminc did find a statistically significant, 3-fold increased risk of general obstetrical complications during pregnancy for the homozygous *V/V* genotype. On the other hand, the MTHFR gene polymorphism has not been related to increased risk of venous thrombosis in more general epidemiologic studies.<sup>13</sup> Thus, whether or not the MTHFR gene variant is associated with increased risk of thrombosis during pregnancy is unclear. We note however, that the absence of an association between the MTHFR genotypes and thrombosis during pregnancy may be the result of widespread folate supplementation during pregnancy. Folate supplementation would tend to reduce the elevated homocysteine levels associated with the *V/V* genotype.

It has been suggested that screening for factor V Leiden be included in routine prenatal laboratory examination.<sup>6</sup> Assuming that the prevalence of factor V Leiden is 5 percent (heterozygous genotype and homozygous genotype for the mutation) among whites and that it is associated with a 20-fold increased risk of thrombosis during pregnancy, the population attributable risk percent for factor V



Leiden is estimated as about 50 percent, i.e., about one-half of thrombosis during pregnancy is attributable to this mutation. If the overall population risk of thrombosis during pregnancy is 1 per thousand, it can be shown that if 10,000 pregnant women are screened for factor V Leiden, 500 will have the defect and that 5 (1 percent) of them would be expected to develop a clot during that pregnancy (about 5 women of the 9,500 without factor V Leiden also would develop a clot during pregnancy). Thus, in a screening program for factor V Leiden, there would be an opportunity to prevent 5 thrombotic events during pregnancy per 10,000 women screened. Assuming that the prevalence of the prothrombin mutation (heterozygous genotype and homozygous genotype) is 0.02 and that this mutation is associated with a 10-fold increased risk, the population attributable risk percent for this prothrombin polymorphism is estimated as about 15%. If screening for this gene were added to a screening program for factor V Leiden, an additional 2 cases of thrombosis during pregnancy might be prevented. We did not consider the ACE *D/D* genotype since it is so common and is associated with a comparatively small excess risk.

In summary, the present study showed an increased risk of thrombosis during pregnancy for white women who have factor V Leiden, the prothrombin 20210 mutation, or the *D/D* genotype of the ACE gene. The *D/D* genotype of ACE was also found to be a risk factor for thrombosis during pregnancy for black women. Although the study was too small to assess possible interactions between these mutations on the risk of thrombosis during pregnancy, it is apparent that multiple mutant alleles in coagulation genes are associated with an appreciable increased risk. Pregnant women represent a young, generally healthy population and are distinguished by the fact that the health of the mother and the health of the fetus are intertwined. Thrombosis in pregnancy, though rare, is a major cause of

maternal morbidity and mortality in the United States and Europe and its avoidance during pregnancy is of primary concern. A screening program for the factor V Leiden mutation and the prothrombin 20210 G to A polymorphism could prevent as much as half of venous thrombotic events which occur during pregnancy, though the actual number of prevented cases is very small compared to the size of the screened population. Furthermore, the potential benefit of preventing these cases must be considered in light of the costs of such a screening program and the risks involved with the medical interventions employed to lower the risk of thrombosis during pregnancy among those who screen positive. This small yield of preventable cases provides an argument against a screening program based solely on the factor V and the prothrombin mutations. On the other hand, screening for these genes may be worthwhile in women with a strong family history of venous thrombosis or a previous venous thrombosis in pregnancy or the postpartum period. A formal cost-benefit analysis is warranted prior to any initiation of a screening program.

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**Table 1: Distribution of cases and controls according to demographic and lifestyle characteristics and family history of thrombosis**

Characteristic	Controls (n=76)		Cases (n=41)	
	Mean		Mean	
Age at Index Pregnancy	30		28	
Gravidity	3.1		2.8	
Parity	2.4		2.1	
	No.	%	No.	%
Race				
Black	27	35.5	14	34.1
White	49	64.5	27	65.9
Currently Smoke				
Yes	12	15.8	5	12.2
No	64	84.2	36	87.8
Alcohol consumption (average lifetime)				
>1 drink/day	14	18.4	4	9.8
<1 drink/day or never	62	81.6	37	90.2
Parent with history of blood clot				
Yes	23	30.3	11	26.8
No	53	69.7	30	73.2
Sibling with history of blood clot <sup>†</sup>				
Yes	1	1.3	5	12.2
No	75	98.7	36	87.8

<sup>†</sup> p < 0.05

**Table 2. Distribution of cases and controls by race and the odds ratios according to Factor V Leiden, the Prothrombin 20210 polymorphism, the MTHFR polymorphism, and the ACE polymorphism**

Genetic Trait	Whites			Blacks		
	Cases n= 27	Controls n=49	Odds Ratio (95% CI)	Cases n=14	Controls n=27	Odds Ratio (95% CI)
Factor V Leiden						
Absent	19	48	18.3	13	27	
Present	8	1	(2.7-432)	0	0	---
Prothrombin 20210						
Absent	22	49	inf	14	27	---
Present	4	0	(1.7 - inf)	0	0	
ACE*						
Absent (I/I)	4	10	Referent	1	4	Referent
Heterozygous (I/D)	12	29	1.1 (0.3-4.7)	4	11	1.3 (0.1-43)
Homozygous (D/D)	11	9	2.6 (0.6-12.6)	9	11	3.2 (0.3-98)
MTHFR						
Absent (A/A)	8	15	Referent	10	21	Referent
Heterozygous (V/A)	14	26	1.0 (0.3-3.1)	4	6	1.4 (0.3-6.4)
Homozygous (V/V)	5	8	1.3 (0.3-5.6)	0	0	---

\*Rescessive model for ACE gene (D/D vs I/I and I/D) including whites and blacks

**Odds Ratio (95% CI): 2.7 (1.2-6.3)**

One black case missing Factor V gene result

One white case missing prothrombin gene result

One white control and one black control missing ACE gene result

**Table 3: Distribution of Cases and Controls and Odds Ratio According to the Total Number of Allelic Mutations in the Factor V, Prothrombin, and ACE Genes**

<b>Number of Allelic Mutations Among the Three Genes</b>	<b>Cases</b>	<b>Controls</b>	<b>Odds Ratio (95% CI)</b>
0	3	14	1.0
1	14	40	1.6 (0.4 - 7.7)
2	16	19	3.6 (0.9 - 18.6)
3 <sup>+</sup>	6	1	17 (1.7 - 491)
<b>Total</b>	<b>39</b>	<b>74</b>	

**Odds Ratio increases by a factor of 2.6 (1.5, 4.5)<sub>95%</sub> for each addition of a mutant allele**