The R563Q mutation of the β-subunit of the epithelial sodium channel gene associated with hypertensive disease and related complications in pregnancy.

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Declaration

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

J E VIljoen

Date: December 2010

Abstract

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Viljoen JE, Theron GB, Hillerman R.

Introduction: Hypertensive disease is one of the cardinal causes of maternal morbidity and mortality in South Africa. According to the National Confidential Enquiry into Maternal Deaths (NCEMD) report for 2005-2007, the "big five" causes of maternal death have remained the same as in the previous triennium, with hypertensive disease in second place, being the causative factor in 15.7% of cases.[1] Women under 20 years of age were at greater risk of dying due to complications of hypertension. In this light, the early identification and treatment of hypertensive disease remains important priorities in improving maternal care. Various serum markers have been studied to identify women at risk of pre-eclampsia, including biological markers and genetic factors.[2] It is also well known that chronic hypertension is one of the major predisposing factors to the development pre-eclampsia.[2] A continued search for a genetic screening test to assist in early diagnosis could facilitate a reduction of maternal morbidity and mortality.

Aims: The aim of this project is to determine the prevalence of the R563Q mutation of the β-subunit of the epithelial sodium channel (β-ENaC) gene in a cohort of primigravid women with hypertensive disease in pregnancy and to compare pregnancy outcomes in this group of hypertensive patients to those not identified to be carriers of the mutation.

Methodology: A retrospectively collected study cohort of patients with early onset pre-eclampsia, obtained from pooled samples and data from the GAP study (Genetic Aspects of Pre-eclampsia, project number C99/025), was used. The planned sample size was 200, with 200 controls who were ethnic-matched, normotensive women. Exclusion criteria were gestation ≥34 weeks, multiple pregnancy, known underlying collagen vascular disease and type I Diabetes Mellitus. Outcome criteria: The pregnancy outcomes were analysed with respect to the degree of hypertensive disease and related complications (maternal, placental and neonatal).

Results: Blood samples form 104 patients and 80 control samples were analysed. Pre-eclamptic patients were significantly younger than controls ($p<0.0001$). The presence of the mutation was not significantly increased in the pre-eclamptic group ($p=0.33$). The mutation bearers did not exhibit a significant tendency towards a specific degree of pre-eclampsia ($p=0.51$). There were no significant differences in the other studied maternal or fetal outcome measures. A composite outcome (the presence of ≥ 1 adverse outcome compared to no adverse outcome) was created which did not differ between the mutation positive and negative pre-eclamptic patients. Data of the index study was combined with the data form a prior relevant study[9] and combined odds ratios were calculated. The increased mutation frequency amongst pre-eclamptics compared to healthy controls then remains significant, OR 2.57(95%CI 1.23-5.36).

Conclusion: In this study the R563Q mutation of the ß-subunit of the epithelial sodium channel gene was not linked to pre-eclampsia. No significant negative correlation could be established between the presence of the R563Q mutation and the outcomes of pre-eclampsia. Further research aimed at chronic hypertensive patients in pregnancy and unstable pre-eclampsia in larger study groups could shed more light on the relation between the mutation and the pre-eclamptic phenotype.

Opsomming

Die verband tussen die R563Q mutasie van die β-subeenheid van die epiteliële natriumkanaal geen met hipertensiewe siekte en verwante komplikasies in swangerskap.

Viljoen JE, Theron GB, Hillerman R.

Inleiding: Hipertensie-verwante siektes is een van die hoof oorsake van moederlike morbiditeit en mortaliteit in Suid-Afrika. Volgens die Nasionale Vertroulike Ondersoek insake Moederlike Sterftes (NCEMD) verslag vir 2005-2007, is die "groot vyf" oorsake van moedersterftes dieselfe as in die vorige triënnium, met hipertensie-verwante siektes in tweede plek, as die oorsaak van 15.7 % van die sterfgevalle. [1] Vroue jonger as 20 jaar het 'n groter risiko om te sterf aan die komplikasies van hipertensie-verwante siektes. In die lig hiervan is die vroeë identifikasie en behandeling van hipertensie-verwante siektes 'n priorteit in die verbetering van moedersorg. Verskeie serum merkers is al bestudeer met die hoop om vroue met verhoogde risiko vir die ontwikkelling van pre-eklampsie te identifiseer, wat biologiese merkers en genetiese faktore insluit. [2] Dit is ook welbekend dat chroniese hipertensie een van die hoof predisponerende faktore is vir die ontwilkkeling van pre-eklampsie.[2] 'n Voortgesette soektog na 'n genetiese siftingstoets wat kan bydra tot vroeë identifisering, sou moederlike morbiditeit en mortaliteit kon verminder.

Doelwittle: Die doelwit van hierdie projek is om die prevalensie van die R563Q mutasie van die β-subeenheid van die epiteliële natrium kanaal (β-ENaC) geen te bepaal in 'n kohort primigravida vroue met hipertensie-verwante siekte in swangerskap en om die swangerskapsuitkomste van hierdie groep te vergelyk met pasiente wat nie draers van die mutasie is nie.

Metodologie:

'n Retrospektief versamelde studie kohort met vroeë aankoms pre-eklampsie, verkry van die monsterbank en data van die GAP studie (Genetic Aspects of Pre-eclampsia, projek nommer C99/025) is gebruik. Die beplande steekproef grootte was 200, met 200 kontroles, wat etnies- en ouderdomvergelykbare normotensiewe vroue was. Uitsluitingskriteria was gestasie ≥34 weke, onderliggende bindweefselsiekte en tipe I

Diabetes Mellitus. Uitkomskriteria: Swangerskap uitkomste was geanaliseer met betrekking tot die graad van hipertensiewe siekte en verwante kompliksies (moederlik, plasentaal en neonataal).

Resultate: Bloed monsters van 104 pasiënte en 80 kontroles is ontleed. Pre-eklampsie pasiënte was betekenisvol jonger as kontroles ($p<0.0001$). Die teenwoordigheid van die mutasie was nie betekenisvol verhoog in die pre-eklampsie groep nie ($p=0.33$). Die mutasie-draers het nie ‘n geneigdheid tot ‘n spesifieke graad van pre-eklampsie getoon nie ($p=0.51$). Daar was geen betekenisvolle verskille tussen die ander moederlike of fetale uitkomste wat bestudeer is nie. ‘n Gesamentlike uitkoms (teenwoordigheid van ≥ 1 swak uitkoms vergeleke met geen swak uitkoms) is geskep; daar was geen verskil tussen die mutasie-positief en negatiewe pasiënte met pre-eklampsie nie. Data van die indeks studie en relevante data uit ‘n vorige studie[9] is saamgevoeg en die gesamentlike kansverhouding is bereken. Die verhoogde mutasie frekwensie onder pasiënte met pre-eklampsie vergeleke met gesonde kontroles was betekenisvol, KV 2.57(95%VI 1.23 - 5.36).

Gevolgtrekking: In hierdie projek was daar nie ‘n verband tussen die R563Q mutasie van die β-subeenheid van die epiteliële natrium kanaal (β-ENaC) geen en pre-eklampsie nie. Geen betekenisvolle negatiewe korrelasie tussen die R563Q mutasie en pre-eklampsie uitkomste kon aangetoon word nie. Verdere navorsing gerig op pasiënte met chroniese hipertensie of akute, onstabiele pre-eklampsie in groter studiegroepe kan die verband tussen die mutasie en die pre-eklampsie fenotipe moontlik beter toelig.

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Table of Contents

List of Figures

List of Tables

List of Addendums

Chapter 1 - Literature review

1.1 Introduction

Hypertensive disease is one of the cardinal causes of maternal morbidity and mortality in South Africa. According to the National Confidential Enquiry into Maternal Deaths (NCEMD) report for 2005-2007, the "big five" causes of maternal death have remained the same as in the previous triennium, namely non-pregnancy related infections – mainly AIDS (43.7%), complications of hypertension (15.7%), obstetric haemorrhage (12.4%), pregnancy-related sepsis (9.0%) and pre-existing maternal disease (6.0%).[1] Women under 20 years of age were at greater risk of dying due to complications of hypertension; women 35 years and older were at greater risk of dying of obstetric haemorrhage, ectopic pregnancies, embolism, acute collapse and pre-existing medical disease. Thus, as the second foremost cause of maternal death and the foremost direct obstetric cause of maternal death, the early identification and treatment of hypertensive disease remains important priorities in improving maternal care. Fortunately, from the latest NCEMD report there has been a significant decrease in the institutional Maternal Mortality Rate (14%) for complications of hypertension in pregnancy. The reason for this reduction, however, is unclear.

Various blood tests have been suggested to identify women at risk of pre-eclampsia, including hCG, plasma fibronectin, increased circulating free fatty acids, hyperlipidemia, and markers of platelet activation.[2] Several genetic studies have been undertaken and will be elaborated upon in the text. It is also well known that chronic hypertension is one of the major predisposing factors to the development pre-eclampsia.[2] A continued search for a genetic screening test to assist in anticipating which women might be at higher risk for developing hypertensive disease holds the potential to impact positively on the reduction of maternal morbidity and mortality.

The aim of this project is to determine the prevalence of the R563Q mutation of the β-subunit of the epithelial sodium channel (β-ENaC) gene in a cohort of primigravid women with hypertensive disease in pregnancy and to compare pregnancy outcomes in this group of hypertensive patients with those not identified to be carriers of the mutation.

1.1.1 Implication of the epithelial sodium channel (ENaC) in hypertension

The body's sodium balance is a critical factor in blood pressure regulation. The kidney acts as a central regulator of sodium homeostasis by controlling renal salt handling by means of the renin-angiotensin system. Aldosterone is the primary hormonal effector in this system. Its principal effect is to stimulate sodium (Na+) absorption in the distal renal tubules and distal colon.[3]

The epithelial sodium channel (ENaC) is a membrane bound protein consisting of three homologous sub-units (α, β and γ). These components are situated in the apical membrane of epithelial cells of the distal nephron. ENaC forms the controlling mechanism for sodium re-absorption in epithelial cells that line the distal nephron, distal colon, ducts of several exocrine glands and lung airways. The three ENaC subunits have been demonstrated in the renal epithelial cells of the distal convoluted tubule, connecting tubule, cortical collecting tubule and collecting duct, as well as in the medullary collecting duct. The sodium-potassium-ATP-ase (Na,K-ATPase) on the basolateral side of the cells provide the driving force for transepithelial Na+ transport. Aldosterone has been shown to strongly affect the ENaC. Upon stimulation, aldosterone binds to the intracellular mineralocorticoid receptor (MR). The active hormone-receptor complex interacts with hormone responsive elements in gene promoter areas, e.g. the β2 sub-unit of the Na,K-ATPase and modulates transcription. An increase in epithelial Na+ absorption is achieved by directly or indirectly activating promoter genes via aldosterone induced proteins. Thus, highly selective aldosterone-dependent epithelial sodium channels (ENaC) mediate Na+ transport in the distal nephron.[4]

Genetic studies have made several attempts to prove that ENaC mutations result in hypertensive disease. Significant linkage was found between systolic blood pressure and micro-satellite markers on chromosome16p12. This region contains the genes that encode for the β- and γ-ENaC subunits.[5] Molecular analysis of two well described human genetic diseases, Liddle's syndrome and pseudohypoaldosteronism type 1 (PHA-1), provided further direct evidence that ENaC dysfunction influences blood pressure.[3]

Liddle's syndrome is characterised by increased sodium transport despite low aldosterone levels. Clinical presentation is hypertension with low plasma levels of

renin and aldosterone, and hypokalemia in some cases. In PHA-1 there is decreased sodium absorption despite high levels of aldosterone. In both of these conditions, mutations in the ENaC subunits were demonstrated. In Liddle's syndrome there are several activating mutations of the β- and γ sub-units, leading to constitutive re-absorption of Na+ and subsequent low levels of renin and aldosterone. In PHA-1, all three subunits (α, β and γ) are affected and hypofunction of the ENaC ensues with urinary salt wasting.

Different mutations of ENaC were linked to other forms of hypertension: the T594M polymorphism and the R563Q mutation. The T594M variant, a polymorphism that is highly prevalent in black population groups, was linked to hypertension in a British study.[6] The R563Q mutation was linked to low-renin, low-aldosterone hypertension.[7] This group also found that only a minority of the patients with R563Q allele fully express the Liddle's syndrome phenotype.

1.1.2 Pre-eclampsia and the ENaC

In renal principal epithelial cells, mineralocorticoid receptor (MR) activation leads to an enhanced activity of ENaC and Na+ /K+ -ATPase. ENaC, with its three different subunits, seems to be differentially regulated in response to mineralocorticoids and glucocorticoids in different target tissues. It is described that (MR) mutations can cause severe pregnancy–induced hypertension, as a result of aldosterone mediated receptor activation through the 100-fold increased progesterone levels in pregnancy.[8] Hypertension is caused by gain-of-function mutation in the mineralocorticoid receptor, the mediator of aldosterone-induced sodium transport in the distal nephron, has been described, with the notable finding being that pregnancy causes a severe worsening of blood pressure. This finding stimulated further interest in the possible role for renin-angiotensin-aldoserone system abnormalities in the disease process of pre-eclampsia.

In South Africa, epidemiologic information indicate that black women are prone to developing severe pre-eclampsia and suffering the consequences, with high maternal mortality and morbidity.[9] The question arose whether a gene mutation could explain this association. The T594M variant of the β-ENaC, which has been shown to be highly prevalent in black population groups, has been associated with

hypertension in a London population.[6] This was however not confirmed in a South African study.10 In 2006 however, the R5630 β-ENaC mutation was linked to pre-eclampsia in black and mixed ancestry individuals in South Africa.[9]

1.2 The molecular biology and genetics of pre-eclampsia

A brief outline

Pre-eclampsia is a multi-system pregnancy disorder characterised by hypertension and proteinuria on the grounds of diffuse endothelial dysfunction. It has a complex etiology and is not yet clearly understood. Etiological factors include maternal and fetal genetics and environmental factors.

A clue to a genetic background for pre-eclampsia is that pre-eclampsia tends to cluster in families (Table 1).[11] Data from an unpublished Utah genealogy database, showed that the incidence of pre-eclampsia was higher in families: more than 30 standard deviations separated the occurrence in the control group. The risk for pre-eclampsia in the female offspring of a pre-eclamptic mother was 20-40% and for siblings of the index case 11-37%.[11] Much lower rates are seen in non-blood relatives. African American mothers have a higher rate of pre-eclampsia, at all socio-economic levels, suggesting that ethnicity, more than socio-economic status, is the predisposing factor[12]. Twin studies shows the heritability of pre-eclampsia to be between 22-47 %.[11,13]

The pathology underlying pre-eclampsia and the complex maternal-fetal interaction resulting in placental malfunction and the multi-system maternal syndrome may involve a multitude of ligands, receptors, amplification cascades and programmed responses on both the maternal and fetal sides. Any number of genetic disorders could affect the processes in the maternal endothelium or affect trophoblast physiology.[11]

Table 1. **Noteworthy family clustering studies of pre-eclampsia.**
Adapted form Chesley's Hypertensive disorders[11]

Author	Year	Area of study

Humphries	1960	Mother-daughter pairs
Adams and Thompson	1961	Disease of pre-eclampsia, sisters of pre-eclamptics
Chesley et al.	1968	Pregnancies of daughters and grandmothers of eclamptics compared to daughters and daughters ''in-law''
Cooper and Liston	1979	''Severe'' pre-eclampsia
Sutherland et al	1981	Increased preeclampsia in mothers and daughters of preeclamptics
Agrimson et al	1990	Increased rate of preeclampsia in mothers and daughters of preeclamptics
Alexander	2007	Mother-fetus pairs, including male transmission

In the search to unravel the etiology of pre-eclampsia, a large number of genetic studies have been performed, mostly focussing on maternal genes.[14] The full list of candidate genes for pre-eclampsia is extensive. Susceptibility to the maternal and fetal features of the condition are probably conferred by different genes.[15] Genetic research over the last decade indicate that, although some common genetic variants play an epidemiologic role in pre-eclampsia, the genetic contribution is much more complex, with non-Mendelian transmission, gene interaction, numerous variants and interaction with environmental factors in concert.[11] Although there is interesting data linking pre-eclampsia with genes encoding the hereditary thrombophilias, HLA-G and the renin-angiotensin system, the studies were not all consistent for the specific candidate genes or loci.

Genetic search strategies may include use of family studies, sib-pairs or twin studies, segregation analysis, linkage analysis, population association studies or studies of affected pedigree members of rare pedigrees, where inheritance of the condition appears to follow a Mendelian pattern.[14] Maturity onset diabetes of the young (MODY), glucokinase receptor mutations[14], Liddle's syndrome and the ENaC mutation, as discussed above, serves as examples of apparently Mendelian inheritance of genetic susceptibility to complex disorders.
Besides from ENaC mutations (including R563Q and T594M), candidate gene studies pertaining to the renin-angiotensin-aldosterone (RAA) system, and its linkage to pre-eclampsia, were the following:

- RFLP in the maternal renin gene in Icelandic pedigrees with more than three affected women was not associated with pre-eclampsia.[16]

- T235 variant of the maternal angiotensinogen gene was linked to pre-eclampsia in a US[17] and Scottish/Icelandic population, but not in a similar UK study.[18]

- Maternal-fetal angiotensinogen gene allele sharing was associated in pre-eclampsia.[19]

- ACE and ATII type 1 receptor has been demonstrated in spiral arteries. The hypothesis is that genetic variants may promote abnormal remodelling and predispose to pre-eclampsia.[20]

- Decidual angiotensin expression was found to differ between alleles in a comparative study (T235 vs M235). These findings indicate the possible role of the fetal genotype in the RAA system in the etiology of pre-eclampsia.[21]

- ATII receptors type 1 and 2, mediated by AGTR1 and AGTR2 genes mediates vasoconstriction, cellular growth, remodelling and apoptosis.[23] Two AGTR1 alleles were found to be transmitted preferentially from the mother to the fetus in pre-eclampsia.[22] A specific AGTR2 haplotype was also found more frequently in women with pre-eclampsia.[24]

Other candidate gene studies in the hereditary thrombophilia area examined mutations in the MTHFR gene and factor V Leiden gene. Results were inconsistent.[14] Further genetic studies focussed on human leucocyte antigen that is expressed in the trophoblast at the placental decidual zone (HLA-G), nitric oxide synthetase gene, tumour necrosis factor-alpha and HADHA. For the last mentioned gene mutation, the result is long chain hydroxyl-acyl coenzyme-A dehydrogenase (LCHAD) deficiency. Certain susceptibility loci have been located by genome–wide scans. Results however were not conclusive and future large detailed studies are needed with maternal and fetal samples.[14] Table 2 summarises of the most commonly studied genes.[11]

Table 2. **Summary of most commonly researched genes for association with pre-eclampsia.**
Adapted from Chesley's Hypertensive disorders[11]

Chromosome location	Gene	Primary Polymorphism Studied	Number of studies	Presumed Biological Association with Pre-eclampsia	Cumulative Evidence of Association

Other recent molecular studies relevant to the etiology of pre-eclampsia include detection of cell-free fetal DNA in maternal circulation and soluble fms-like thyrosine kinase (sFlt-1). Cell free fetal DNA have been shown to be elevated in pre-eclampsia and further elevated in HELLP syndrome. The raised levels may even be present before the onset of pre-eclampsia and may possibly offer predictive value.[14] The elevation of sFlt-1 in the pre-eclamptic placenta is thought to be related to placental hypoxia. The sFlt-1 binds to vascular endothelial growth factor (VEGF) and deprives maternal endothelium of a vital growth factor, thus resulting in the maternal manifestation of dysfunctional endothelium.[24] Elevated sFlt-1 is thus a marker for the early detection of the inflammatory pre-eclamptic process resulting form placental stress, rather than a direct etiological factor.

Proteome analysis is a further research area to briefly mention. In a recent study published in Placenta, the analysis of 5 pre-eclamptic placentas (compared to 5 normal placentas) indicated that there was down-regulation of proteins with antioxidant properties in pre-eclamptic placentas.[25]

1.3 Overview of recent articles relevant to this study

1.3.1 Rayner study findings[7]

This study, conducted in 2003 looked at the relationship between β-ENaC mutation and low renin, low aldosterone hypertension. They found a new mutation of the β-ENaC, R568Q, and could strongly link it to this form of hypertension in their cohort of black and mixed ancestry South African patients.

The new R563Q mutation was found in 10 of 139 hypertensive patients but in none of the normotensive patients (p=0.0058). The clinical and laboratory results of this study is summarised in Table 3. The frequency of the mutation in the subgroup of 14 black patients with low renin low aldosterone hypertension was significanty greater (p=0.0001) than in normotensives, and was also greater (p=0.041) than in normal-high renin hypertensives. This suggests that R563Q is an activating mutation. R563Q was also found in seven out of 250 mixed ancestry patients and was significantly (p=0.017) associated with low-renin, low aldosterone hypertension. The mutation was found in only one of 100 mixed ancestry normotensives, but not in any of the 136 white hypertensives. Eleven of the 18 R563Q patients had severe hypertension, with 2 cases suffering renal failure. Only two had hypokalemia. Thus the minority of the affected patients displays the full Liddle's syndrome phenotype.

Table 3. **Results of clinical and laboratory findings of Rayner at al, 2003[7]**

	R563Q positive	R563Q negative
Age (years, mean ± SD)	52.7±14.1 (n=18)*	47.1±15.6 (n=172)
Sex	11males, 7 females	67 males, 105 females
Population group	10 black, 8 mixed ancestry	53 black, 105 mixed ancestry, 13 white
Serum K+(mmol/l, mean±SEM)	4.0±0.2 (n=14)*	4.2±0.04 (n=151)
PRA**(ngml per h, mean±SEM)	<0.1;0.2 (n=2)	2.8±0.8 (n=47)
Plasma active renin (mU/l,mean±SEM)	22.7±7.5 (n=11)*	61.7±8.9 (n=125)
Plasma aldosterone (pmol/l, mean±SEM)	172±18.2 (n=14)**	410±20 (n=172)

*not significantly different from R563Q negative patients

**Significantly different from R563Q negative patients; p<0.0001, Mann-Whitney test

PRA=plasma renin activity, normal range 1-8ng/ml per h. Plasma active renin normal range 7-77mU/l.

Plasma aldosterone normal range 190-970pmol/l.

1.3.2 Dhanjal study findings[9]

The frequency of the R563Q mutation in black and mixed ancestry (MA) patients with pre-eclampsia was compared to that in normotensive, ethnic matched controls. Data from 412 women were obtained, of which 192 normotensive women and 230 women with pre-eclampsia. The significant differences in demographics included age, birth weight and gestation at delivery. Pre-eclamptic women were also older than controls, with smaller babies born at younger gestations. A total of 23 women were identified as mutation carriers. The frequency of pre-eclampsia (7.8%) was significantly higher amongst the mutation bearers than in the control group 2.6% (p=0.014). With subgroup analysis according to ethnicity, the frequency of the mutation was significantly higher in black women. In the MA group the mutation frequency was increased but not significantly so. The mean renin level was significantly lower in pre-eclamptic mutation positive subjects while aldosterone and potassium levels were not different. These levels were however low in all groups compared to levels in white population groups. The comparative prevalence of the mutation in the study is summarised in Table 4.

Table 4. **Prevalence of R563Q mutation in pre-eclamptics compared to controls[9]**

		Normotensive pregnant women	Women with pre-eclampsia	p-value
All subjects	n	192	230	
	age	25.7±6.0	27.6±6.8	0.0027*
	mutation+ (%)	5 (2.6)	18(7.8)	0 014**
	mutation-	187	212	
Black	mutation+ (%)	3(2.3)	12(8.1)	0.031**
	mutation-	124	136	
MA	mutation+(%)	2 (3.1)	6(7.3)	0.22**
	mutation-	63	76	

* Student's unpaired t-test (two tailed)

**Fisher's exact test (one sided)

1.4 Aim of index study

Following to the linkage of the R563Q allele to hypertension and pre-eclampsia in black and mixed ancestry patients in a South African cohort, the index study further explores the relationship between this specific genetic defect and pre-eclampsia. As little is known regarding the effect of the mutation on pre-eclampsia outcomes, a retrospective study cohort in an existing database of blood samples from pre-eclamptic women were analyzed and correlated to the course and complications of their pregnancies. The group consisted of a cohort of primigravid patients with early onset pre-eclampsia. The allele frequency in this group was compared to that in a healthy control group. The pregnancy outcomes in subjects with and without the affected R563Q allele were compared.

Chapter 2 - Methods

A retrospectively collected study cohort was used. The selected study population was primigravid patients with early onset pre-eclampsia. The control group consisted of age matched women who had uncomplicated pregnancies. Pooled blood samples and data from the GAP study (Genetic Aspects of Pre-eclampsia, project number C99/025) were used for both the study group and the control group. Patients originated from the eastern Metropole of Cape Town and surrounding areas, including two regional referral hospitals (Paarl and Worcester) and rural areas of the Western Cape.

The GAP study was conducted in Tygerberg Academic Hospital (a tertiary referral center) and focused on genetic associations (MTHFR, Factor V Leiden, Prothrombin and LDL) with pre-eclampsia. The initial phase of the GAP study was from 1998 to 2001. The study recruited patients with early onset pre-eclampsia (primigravidae and multigravidae), pre-eclampsia beyond 34 weeks, intra-uterine growth restriction and abruptio placentae. A sample bank of maternal and fetal specimens was established that was subsequently used for further genetic studies. Recruitment resumed periodically for this purpose and concluded in September 2008. Patients were enlisted consecutively during recruitment periods and had to be able to give consent for participation in genetic research. (Addendum A) Data for each patient was captured and stored in a database by means of completion of a data collection sheet (Addendum B).

The planned sample size was 400 - 200 study patients and 200 control patients. Sample size was determined assuming a polymorphism frequency of 5% in hypertensives and 0.1% in the normotensive controls. (α = 0.05, β = 0.20). Controls were ethnic-matched women who were normotensive during pregnancy and delivery.

Included samples were specimens from primigravidas with early onset pre-eclampsia (33 weeks and under). Exclusion criteria were gestation 34 weeks and over, multiple pregnancy, patients known with underlying collagen vascular disease or type I Diabetes Mellitus.

Pre-eclampsia was defined as hypertension with onset after 20 weeks gestation (mild/moderate >140/90 mmHg, <160/110 mmHg, severe ≥160/110 mmHg) and proteinuria (2+ protein on side room urinalysis on 2 occasions, or 24h urine protein ≥0.3g). Outcome criteria were the severity of the hypertensive disorder, (severe pre-eclampsia, mild-moderate pre-eclampsia or eclampsia) placental function (according to Doppler studies of the resistance index in the umbilical artery), the presence of abruptio placentae, delivery (gestational age, route of delivery, reason for delivery), maternal complications (HELLP syndrome, pulmonary oedema, renal compromise or other) and neonatal outcome (intrauterine death, termination before viability, low birth weight and small for gestational age, Apgar score, admission to neonatal intensive care, hospital stay and neonatal complications). Estimation of weight for age for was performed using a centile charts for birth weight for the local urban population of the Western Cape.[26,27]

In the laboratory, DNA extraction and purification was performed by the overnight salt extraction method, followed by PCR amplification with designated primers. Overnight digestion of the resulting product was allowed. The restriction enzyme was *Sfc1*. Analysis of the product was achieved by gel electrophoresis (4% agarose gel). Results were stained with ethidium bromide and visualised under UV light. An example of the electrophoresis strips is displayed in Figure 1.

On the marker strip as illustrated in Figure 1, The *GG* type is the normal form of the genotype. The *GA* type is the mutant heterozygous form and the *AA* type the mutant homozygous form. In some of the DNA samples, analysis was unsuccessful (Fig. 2).

Statistical processing of categorical data was done using the Chi^2 test with Yates correction. For categories with expected values of less than 5, the two-tailed Fisher's exact test was used. Means of normally distributed data were compared with the Student's t-test. Medians of data with an abnormal distribution were compared with the Mann-Whitney U-test. Comparable data from the previous study by Dhanjal et al[9] was combined with that of the index study and combined odds ratios and 95% confidence intervals were calculated using Revman 4.2 software.

The laboratory specimens and data sheets did not contain information of the patient's name and file number to allow identification of individuals. Permission for the project was obtained from the Tygerberg Ethics Committee (Project number N07/07/163).

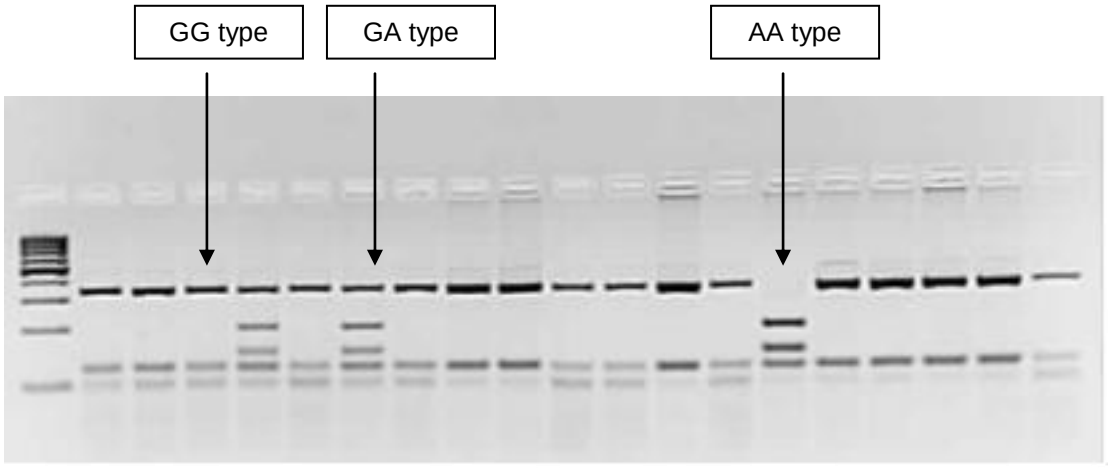


Fig.1 **Electrophoresis specimen: genotyping**

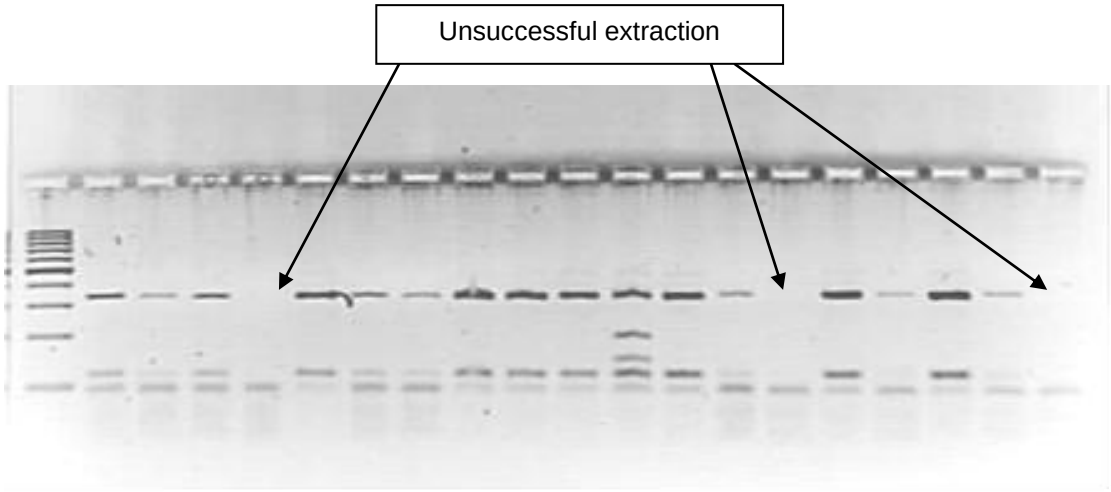


Fig. 2 **Electrophoresis specimen: unsuccessful DNA extraction**

Genotyping was performed in the laboratory of Dr Renate Hillermann, Department of Genetics, University of Stellenbosch. Costs for this project (Primers and restriction enzyme, PCR amplification and gel electrophoresis) were covered by existing funds.

Chapter 3 - Results

Of the 200 planned patients, 104 samples were analysed. Of the 200 planned controls, 80 samples were analysed. The mean age was 21.7(±4.6) in the study group compared to 27.7(±7.1) in the control group as in Table 5.

Table 5. **The age differences between the study and control groups**

		Normotensive pregnant women	Women with pre-eclampsia	p-value
All subjects	n	80	104	p < 0.0001*
	age (SD)	27.7 (±7.1)	21.7 (±4.6)	
*Mann-Whitney U-test				

The mutation was identified in heterozygous form in 12 (11.5%) of the 104 study patients. Of the 80 normal control patients, 5 (6.3%) had the mutation, of which 4 in the heterozygous form and one in the homozygous form of the gene mutation. None of the pre-eclamptic study patients had the mutation in homozygous form (p = 0.33) as illustrated in Table 6.

Table 6. **Prevalence of the mutation (homozygous + heterozygous)**

OUTCOME		Mutation +	Mutation -	p-value
Genotype				
n= 104	Patients	12 (11.5%)	92	OR = 1.96 (95%CI 0.66 - 5.80) p = 0.33*
n = 80	Controls	5 (6.3%)	75	
* Chi squared test, Yates corrected p-value				

In the study group, the racial distribution in the mutation negative group was 35 blacks, 53 of mixed ancestry (MA) and 4 whites. The mutation positive group was comprised of 5 blacks and 7 MA patients. In the control group, the mutation negative group was comprised of 9 blacks and 66 MA patients, while the mutation positive group was comprised of 0 blacks and 5 MA patients. There were no whites in the control group. No difference was demonstrated in mutation frequency in the black subgroup (p = 0.57) or the MA subgroup (p = 0.56) as shown in Table 7.

Table 7. **Racial differentiation**

PREVALENCE		Mutation +	Mutation -	p-value
Racial				
Black	Study population	5	35	p = 0.57*
	Controls	0	9	
MA	Study population	7	53	OR = 1.74 (95% CI 0.46-6.78)** p = 0.54
	Controls	5	66	
White	Study population	0	4	
	Controls	0	0	
* Fisher's exact test (two tailed) ** Chi squared test with Yates correction				

For statistical analysis, the groups grading the severity of the hypertensive disorder were combined. The patients with severe pre-eclampsia and eclampsia were combined in one group (severe) and patients with mild-moderate disease in the other group (Table 8). For hypertensive disease as an outcome the variables were as follows: 8 of the 12 mutation bearers had severe pre-eclampsia disease, 2 had mild/moderate disease and 2 had eclampsia. Among the mutation negative group (n=92), 26 (28.3%) had mild-moderate pre-eclampsia, 62 (67,0%) severe pre-eclampsia and 4 had eclampsia (p=0.018).

Table 8. **Severity of the hypertensive disorder**

OUTCOME		Mutation +	Mutation -	p-value
Severity of pre-eclampsia				
n = 76	Severe	10	66	p=0.51
n = 28	Mild-moderate	2	26	
* Fisher's exact test (two tailed)				

Doppler studies of the umbilical artery resistance index were normal in 60 of the mutation negative group and 6 of the mutation positive group. Abnormal Dopplers (above the 95th percentile (>P95), absent end diastolic flow (AEDF) and reversed end diastolic flow (REDF)) were grouped together for the statistical analysis. In the mutation negative group, 18 patients had abnormal Dopplers (>P95 8, AEDF 8, REDF 2) and in the mutation positive group 2 patients had abnormal Dopplers (>P95 1, REDF 1). Doppler studies were not done in 13 mutation negative patients and 4 mutation positive patients (Table 9).

Table 9. **Placental function according to Doppler**

OUTCOME		Mutation +	Mutation -	p-value
Placental function according to Doppler				
n = 66	Normal	6	60	p = 1.00*
n = 21	Abnormal	2	19	
* Fisher's exact test (two tailed)				

Abruptio placentae (Table 10) occurred in 6 mutation negative patients and in none of the mutation positive group (p=1.00).

Table 10. **Prevalence of abruptio placentae**

OUTCOME		Mutation + (n=12)	Mutation – (n=92)	p-value
Abruptio placentae				
n = 6	Present	0	6	p = 1.00*
n = 98	Absent	12	86	
* Fisher's exact test (two tailed)				

Gestational age at delivery for the mutation negative group was divided in births <28weeks (17 patients), between 28-33weeks (57 patients) and 34 weeks and beyond (18 patients). For the mutation positive group, 3 patients delivered before 28 weeks, 8 patients between 28 and 33 weeks and 1 patient at 34 weeks, (p=0.690). Gestational age at delivery data was then combined in the group below 34 weeks and 34 weeks and beyond for statistical analysis (Table 11).

Table 11. **Gestational age at delivery**

OUTCOME		Mutation +	Mutation -	p-value
Gestation at delivery				
n = 85	< 34 w	11	74	p = 0.69*
n = 19	≥ 34w	1	18	
* Fisher's exact test (two tailed)				

The route of delivery was known in 91 of the mutation negative patients, of whom 24 (26,3%) had normal vaginal delivery and all others were operative deliveries (Table 12). Indication for the operative deliveries were fetal distress in 43 (64,2%) of patients, failed induction of labour (6) elective (4) for fetal indication (1) hysterotomy (2) and unknown indication (11). Route of delivery was known in all of the mutation bearers. Out of the 12 patients, 4 delivered normally and 8 had caesarean sections

of which 4 for fetal distress, 3 for failed induction and 1 for unknown indication (p=0.74). There was insufficient information on the reason for induction of labour or delivery by caesarean section in both groups of patients.

Table 12. **Route of delivery**

OUTCOME		Mutation+	Mutation -	p-value
Route of delivery				
n = 75	Operative delivery	8	67	p = 0.73*
n = 28	Normal birth	4	24	
* Fisher's exact test (two tailed)				

Maternal complications were recorded in the database. In the mutation negative group, 6 patients developed HELLP syndrome and 9 had ascites. In the mutation positive group, 1 patient had HELLP syndrome and 1 had ascites.

The pregnancy outcomes for the mutation negative group were divided into live births, 68 (73.9%), early neonatal losses, 8 (8.7%), stillbirths, 4 (4,3%), and termination before viability, 12 (13.0%). The mutation positive group had 9 live births, no early neonatal losses, 1 stillbirth and 2 terminations before viability. Combining the outcomes into live babies versus pregnancy losses (Table 13), the mutation negative group had 68 (73.9%) live births and 24 (26.1%) had losses. The mutation positive group had 9 live births and 3 losses (p=1.00).

Table 13**.** **Pregnancy outcomes**

OUTCOME		Mutation +	Mutation -	p-value
Pregnancy outcome				
n = 27	Perinatal loss	3	24 (26.1%)	p = 1.00*
n = 77	Alive	9	68 (73.9%)	
* Fisher's exact test (two tailed)				

The birth weight distribution (≥24weeks, n=91) was determined according to local centile graphs.[26,27] In the mutation negative group: under the 10th centile (<P10) 35 (38.5%), between the 10th and 50th centile 35 (38.5%), more than or equal to the 50th centile (≥P50) 21 (23.0%). In the mutation positive group (≥24weeks, n=10) there were 3 babies <P10, 5 babies between the 10th and 50th centile and 2 babies ≥P50. The groups were then combined to form 2 categories, <10th centile and above or equal to the 10th centile (≥P10) (Table 14). The mutation negative group had 35

(38.5%) babies <10th centile and 56 (61.5%) ≥P10 centile. The mutation positive group had 3 babies below the 10th centile and 7 babies ≥P10 (p=0.74).

Table. 14. **Weight for gestational age**

OUTCOME		Mutation +	Mutation–	p-value
Weight for gestational age				
n = 38	< P10	3	35 (38.5%)	p = 0.74*
n = 63	≥ P10	7	56 (61.5%)	
* Fisher's exact test (two tailed)				

In the mutation negative group the birth weight distribution was as follows: 26 babies <1000g, 45 babies between 1000 -1800g, 37 babies ≥1800g. The mutation positive patients had 4 babies <1000g, 7 babies 1000-1800g and 1 baby ≥1800g. The weight categories were then combined (Table 15): In the group <1500g, mutation negative patients had 55 (59.8%) babies and mutation positive patients had 10 babies. In the group ≥1500g the mutation negative group had 37 (40.2%) babies and the mutation positive group had 2 babies (p=0.20).

Table15. **Birth weight distribution**

OUTCOME	Mutation + (n=12)	Mutation– (n=92)	p-value
Birth weight in grams			
< 1500g	10	55 (59.8%)	p = 0.20*
≥ 1500g	2	37 (40.2%)	
* Fisher's exact test (two tailed)			

In the mutation negative group, 76 babies were born alive. The available Apgar scores in the mutation negative group (n = 74): at 5 minutes 18 babies had an Apgar score <8 (24.3%), of which only 2 were under 4; 56 babies had an Apgar score ≥8 (75.7%), 3 scores were unknown and 15 not applicable because of stillbirth or non-viable termination (Table 16). At 10 minutes (n = 76) only 3 were <8 (of which none were <4) and 73 were ≥8. Apgar score was unknown in 10 patients and not applicable in 6 cases. In the mutation positive group, at 5 minutes, 2 were <8 (of which none were <4) and 7 were ≥8 (p=1.00). In three cases Apgar scores were not applicable. At 10 minutes, 9 were ≥8 and 3 were not applicable (Table 17). Babies with 5 minute Apgar scores less than 8 were compared with those with Apgar scores

of 8 or more (Table 16.) Similarly, the 10 minute Apgar scores were compared (Table 17).

Table 16. **Apgar at 5 minutes**

OUTCOME	Mutation + (n=9)	Mutation – (n=74)	p-value
Apgar at 5 min			
Apgar < 8	2	18	p = 1.00*
Apgar ≥ 8	7	56	
* Fisher's exact test (two tailed)			

Table 17. **Apgar at 10 minutes**

OUTCOME	Mutation + (n=9)	Mutation – (n=76)	p-value
Apgar at 10min			
Apgar < 8	0	3	p = 1.00*
Apgar ≥ 8	9	73	
* Fisher's exact test (two tailed)			

Of the mutation negative group (Table 18), 13 babies were admitted to the neonatal intensive care (NICU). Sixty babies did not require admission. For 18 births, NICU was not relevant (intrauterine death, non-viable termination or pre-NICU demise). In one case information was lacking. In the mutation positive group one baby was admitted to NICU. Eight babies did not require NICU and in 3 cases NICU was not relevant (intrauterine death, non-viable termination or pre-NICU demise). There was not enough information in the database about the length of stay in the NICU.

Table 18. **NICU admission**

OUTCOME	Mutation + (n=9)	Mutation – (n=73)	p-value
NICU admission			
Yes	1	13	p = 1.00*
No	8	60	
* Fisher's exact test (two tailed)			

Neonatal complications in the mutation negative group (Table 19) included prematurity 45, respiratory distress syndrome 11, necrotising enterocolitis 2, sepsis 6, early neonatal demise 8, and unknown complications 3; some babies suffered more than one complication. The number of babies in the mutation negative group

with complications was 46, with no complication in 27 babies. In the mutation positive group, the complication in 6 babies was prematurity, including respiratory distress; 3 babies had no complications.

Table 19. **Neonatal complications**

OUTCOME	Mutation + (n = 9)	Mutation – (n = 73)	p-value
Complications			
Premarurity and related complications)	6	46	p = 1.00*
No complications	3	27	
* Fisher's exact test (two tailed)			

A composite outcome was created, combining all adverse outcomes of pre-eclampsia (maternal and fetal) and a comparison was made between the mutation positive and negative groups (Table 20). The composite outcome (≥1 adverse event) was present in all the mutation bearers and in 89 of the mutation negative group. A further breakdown of the composite outcome is given in table 21.

Table 20. **Composite outcome comparison**

OUTCOME	Mutation + (n=12)	Mutation – (n=92)	p-value
Composite adverse outcome			
Present (≥1 adverse outcome)	12	89	p = 1.00*
No adverse outcome	0	3	
* Fisher's exact test (two tailed)			

Table 21. **Composite outcome**

OUTCOME	Mutation + (n=12)	Mutation – (n=92)
Composite adverse outcome		
None	0	3
Any one	1	2
Any 2	1	11
Any 3	1	14
Any 4	2	9
Any 5	6	26
>5	1	27

Chapter 4 - Discussion

The study was conducted in an attempt to gain insight into the effect of the R563Q mutation on the outcomes of pre-eclampsia. The study findings do not point to a

major relationship of pre-eclampsia, specifically of early onset with pre-eclampsia in primigravid patients, with the R563Q mutation, on the studied outcome measures. When extrapolating from the finding that mineralocorticoid receptor mutation phenotypes present with severe pregnancy induced hypertension[9], the expected association was not demonstrated (Table 8). The small sample size achieved in this study may have resulted in a Type II (β) error (failing to reject the null hypothesis when the null hypothesis is false). The relation between the prevalence of the mutation amongst pre-eclamptics that was indeed previously statistically linked to hypertension and pre-eclampsia (discussed in the studies outlined in the literature review[7,9]) was not significant. No association was found between the mutation and the degree of pre-eclampsia in this study population (Table 8). A statistically significant finding was the significantly younger age of the pre-eclampsia patients compared to the control group patients (Table 5). It is most likely a reflection of the known tendency for pre-eclampsia to occur more frequently in primigravidas, while the control group was ethnic matched women who had uncomplicated pregnancies and were likely multigravidas, who were chosen on the background of uncomplicated obstetric histories.

The small numbers in the index study was due to technical problems with the sample bank, i.e. samples that were not successfully analysed (Fig. 2) as DNA could not be retrieved, along with a shortage of samples in the data bank. The sample size according to the initial power calculation of the study was not achieved.

The data from the index study (Table 21) was combined with comparable data from the study by Dhanjal et al. that looked at the mutation frequency in pre-eclamptic populations (Table 22). Combined odds ratios (OR) were calculated (Fig. 3 to 5). A subgroup comparison was also performed (Tables 24 and 25), looking at the mutation frequency in the individual black and mixed ancestry (MA) subgroups. The prior study did not include any white patients and this subgroup could not be compared. When combining all study patients, the association of pre-eclampsia with the R563Q mutation remains significant [OR 2.57 (95%CI 1.23-5.36)], as was found in the original trial. This association was also significant in the black subgroup [OR 3.51 (95%CI 1.07-11.51)], but not in the MA subgroup [OR 1.99 (95%CI 0.76-5.23)].

Table 22. **Prevalence of R563Q mutation in pre-eclamptics compared to controls** – Index study data

		Normotensive pregnant women	Women with pre-eclampsia	P value
All subjects	n	80	104	
	age	27.7 (±7.1)	21.7 (±4.6)	p < 0.0001*
	mutation +	5	12	p = 0.33**
	mutation -	75	92	
Black	mutation +	0	5	p = 0.57**
	mutation -	9	35	
MA	mutation +	5	7	p = 0.56***
	mutation -	66	53	

* Mann-Whitney U-test

** Fisher's exact test (two tailed)

*** Chi squared test with Yates correction

Table 23. **Combined data of Dhanjal et al. and Index study**

Subjects		DHANJAL Controls*	INDEX Controls*	Combined Controls	DHANJAL Study patients**	INDEX Study patients**	Combined study patients
All	n	192	80	272	230	104	334
	age	25.7	27.7		27.6	21.7	
	mut+ ^	5	5	10	18	12	30
	mut -^^	187	75	262	212	92	304
Black	mut +	3	0	3	12	5	17
	mut -	124	9	133	136	35	171
MA	mut+	2	5	7	6	7	13
	mu -	63	66	129	76	53	129
White	mut +	0	0		0	0	
	mut-	0	0		0	4	

^ Mutation positive

^^ Mutation negative

* Normotensive pregnant women

** Women with pre-eclampsia

MA – Mixed Ancestry

Fig 3. **Combined Odds Ratio: All patients**

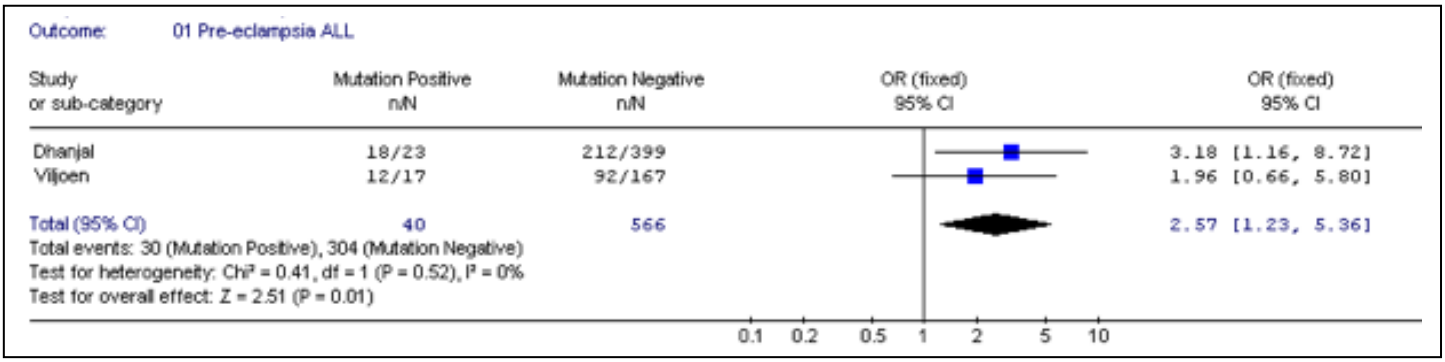


Fig 4. **Combined Odds Ratio: Black patients**

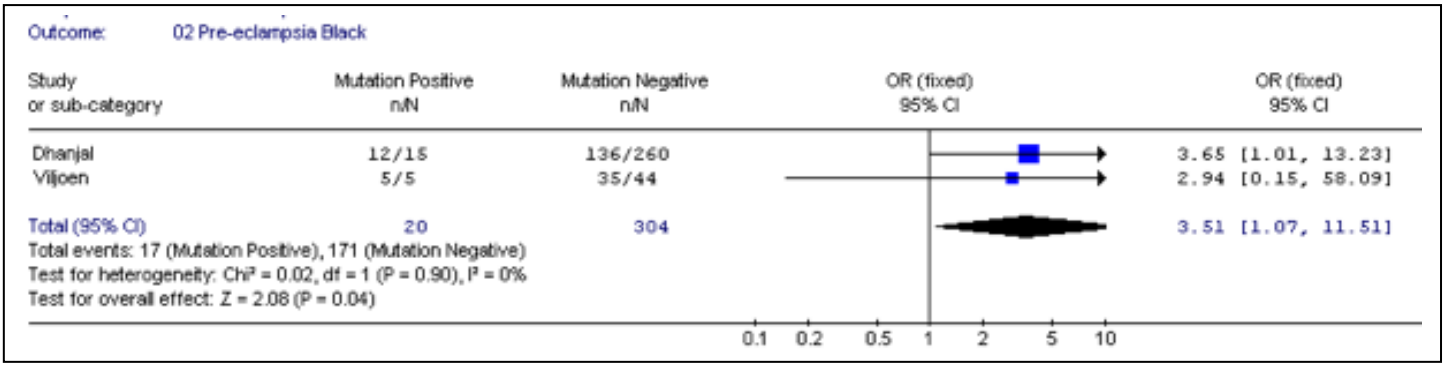
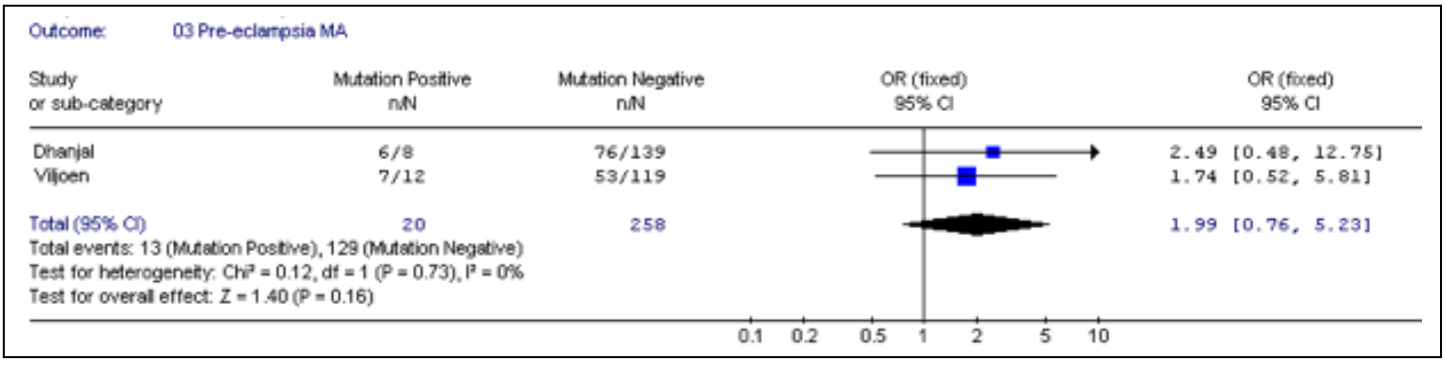


Fig 5. **Combined Odds Ratio: Mixed Ancestry (MA) patients**



When reviewing the outcome findings of the index study an interesting question arises: Would the findings change significantly should another study population be chosen?

- Since it has been found that this ENaC mutation predisposes to hypertension independent of pregnancy, an additional population for future research could include patients with chronic hypertension.[7] When examining chronic hypertensives in pregnancy, the development of superimposed pre-eclapmpsia and outcomes could then be correlated to the presence of the mutation.

- Acutely ill patients, who could not give consent, and post-partum patients were excluded form the databank, thus effectively including only stable pre-eclamptics who qualified for expectant management. As MR defects (which activate ENaC) in pregnancy lead to severe pregnancy induced hypertension, this most likely warrants a further search into patients who present with complicated or unstable pre-eclampsia or who are emergently admitted to critical care units and thus not suitable for expectant management (i.e. pulmonary oedema, anuria with renal insufficiency, pre-hospital eclampsia, abruptio placentae).

- From the combined data, the tendency towards pre-eclampsia in black individuals with the mutation seems significantly higher than in individuals of mixed ancestry. The index study population included a majority of mixed ancestry patients, which could serve to suggest that selecting an only black cohort of patients might be of more value in examining the R563Q gene defect in pre-eclampsia. The Dhanjal study group comprised of a majority of black patients, a selection that likely was more on target to retrieve significant study results.

A further unexpected finding was the one control patient who exhibited the homozygous (AA) mutation genotype. It would be interesting to learn whether this patient developed hypertension in later life, however, the use of a retrospective, anonymous sample bank unfortunately limits elaboration on this point.

Although the association of the R563Q mutation with pre-eclampsia remains positive when looking at greater numbers, it is still not very strong, and it could not be significantly linked to specific adverse pregnancy outcomes. Other factors are likely to play a more significant role in the elusive etiology and phenotypic behaviour of pre-eclampsia.

Conclusion

In this study the R563Q mutation of the ß-subunit of the epithelial sodium channel gene was not linked to pre-eclampsia. No significant negative correlation could be established between the presence of the R563Q mutation and the outcomes of pre-eclampsia.

When combining the mutation prevalence with the data from the prior study that did prove a statistically significant link, the association with the defect and pre-eclampsia remained significant. Further suggested research includes examining the R563Q mutation in chronic hypertensives in pregnancy and unstable pre-eclampsia and examining pre-eclampsia outcomes in larger study groups.

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Addendum A

PATIENT INFORMATION AND CONSENT FORM

Genetic factors in pre-eclampsia

REFERENCE NUMBER:

DECLARATION BY PATIENT

I, the undersigned, _____ from _____(address)

Confirm that:

1. I was asked to participate in the above-mentioned research project by the department of Obstetrics and Gynaecology, division Human Genetics at the University of Stellenbosch.

2. The following was explained to me:

2.1 Aim: To see if there are genetic reasons why some pregnant patients develop high blood pressure in pregnancy.

2.2 Procedure: I will be asked to give information on my current and previous pregnancies. All information is confidential and will be kept anonymous. At birth, two extra tubes of chord blood will be taken in addition to the routine chord blood taken for blood group determination. This extra blood will be for genetic testing. A further two tubes of blood will be taken from me the next morning before breakfast. The rest of the blood samples taken from me will be the routine blood needed for the management of my pregnancy and any possible complications.

3. That all information gathered will be kept confidential, but the information could be used for presentations at a congress or for publications in medical journals.

4. That I may decline to participate in this project. If I do, this will not influence my current or future management at this or any other institution.

5. This was explained to me by _____ in English and I am fluent in this language.

I was given ample time to ask any questions and all questions were answered to my full satisfaction.

6. I was not pressurised to participate in this study.

7. There will be no additional costs involved.

Signed in _____ on _____ 19_____

_____ _____

Signature or right thumb print witness

DECLARATION BY OR FOR RESEARCHER

I, _____ declare that

1. I explained the information in this document to _____;

2. She was given ample time to ask any questions;

3. This conversation was in English and no translator was used.

Signed in _____ on _____ 19____

_____ _____

Researcher/ representative of researcher witness

Addendum B

GENETIC ASPECTS OF PREECLAMPSIA:

1. AGE: |_____|
2. RACE: |_____|
3. GRAVIDITY:PARITY: MISCARRIAGES:ECTOPIC: |____|____|____|____|
4. VDRL: NEG =0, POS TREATED =1, NOT TREATED =2 |_____|
5. BLOOD GROUP: |_____|
6. CERVICAL CYTOLOGY: NORMAL =1 ABNORMAL =2 |_____|
7. CERVICAL CULTURES: |_____|
   NOT DONE =1, NEG =2, GONO =3, CHLAMYDIA =4, GBS =5, OTHER =6
8. URINE MCS: NEG =0, ASYMP.BACTURIA TREATED =1, NOT TREATED =2,
   NOT DONE =3, UTI =4 |_____|
9. SF GROWTH: <10 th =1, NORMAL =2, >90th =3 |_____|
10. PROTEINURIA: (GESTATION) |_____| (_____)
    |_____| (_____)
    |_____| (_____)
11. BP:(ADMISSION) |_____|_____|
12. AMNIOCENTESIS FOR KARYOTYPING: YES =1 NO =2(GESTATION) |_____| (_____)
13. FETAL MOVEMENTS: NORMAL =1, DECREASED =2 |_____|
14. ULTRASOUND: YES =1, NO =2 |_____|
15. DOPPLER: N =1, >95TH =2, AEDV =3, REDF =4 |_____|
16. G.A. WHEN COMPLICATIONS DEVELOPED: |_____|
17. COMPLICATIONS: _____
18. SMOKE: YES =1, NO =2 |_____|
19. ALCOHOL: YES =1, NO =2 |_____|
20. MEDICATION AT ANY TIME DURING PREGNANCY: |____|____|____|____|____|
    FOLATE =1,Fe =2, ASPIRIN =3, ANTIHYPERTENSIVES =4, PYRIDOXIN =5 , OTHER =6

Addendum B

AT DELIVERY:

21. GESTATION: |_____|

22. BIRTH WEIGHT: |_____|

23. MALE =1, FEMALE =2 |_____|

24. DELIVERY TYPE: _____

25. APGAR SCORE: |_____|_____|_____|

26. OUTCOME: (LIST) |_____|

27. MORBIDITY:(LIST) |_____|_____|_____|_____|_____|

28. NICU: YES =1, NO =2 |_____|

30 DAYS: |_____|

31 REASON: _____

32. SPECIAL INVESTIGATIONS:

UREUM:	|_____|	LDH:	|_____|	PL:	|_____|
KREAT:	|_____|	WCC:	|_____|		
AST:	|_____|	HB:	|_____|		
ALT:	|_____|	HKT:	|_____|		