

**Molecular genetic analysis of the
neurokinin B (*TAC3*) and neurokinin B
receptor (*TACR3*) genes as candidates
for pre-eclampsia**

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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.

Signature

Date

Summary

Hypertensive conditions of pregnancy, such as pre-eclampsia, are the principal direct cause of maternal morbidity and mortality and affect up to 10% of first pregnancies worldwide. The placenta is vital in the pathogenesis of pre-eclampsia since the condition only occurs in the presence of placental tissue and the only cure is delivery of the placenta and the fetus. It has been hypothesised that the placenta may be the source of a circulating factor(s), which transports freely in the maternal system, resulting in the multi-systemic and immunological responses that are characteristic of pre-eclampsia. Among the potential “circulating” candidates currently being investigated worldwide, is the tachykinin member, neurokinin B (NKB).

The aim of this project was to use a novel approach and investigate the role of Neurokinin B in pre-eclampsia on a genetic level. This would be achieved by bioinformatic characterisation of the neurokinin B (*TAC3*) and neurokinin B receptor (*TACR3*) genes. Samples from thirty pre-eclampsia patients (of whom 10 also had abruptio placentae) and twenty control individuals were used for mutation detection analysis involving Multiphor gel electrophoresis and automated sequencing.

Three sequence variants were identified in the *TAC3* gene and include: (i) 5' UTR variant (-25 c-t); (ii) intronic variant IVS3-53 (t-g) and (iii) 3' UTR variant exon 7 (479, t-c). Only the -25 c-t variant had been reported before (SNP database). A further two variants were identified in the *TACR3* gene: (i) exon 3 variant (nt 857, a-t) and (ii) 3' UTR variant, amplicon 5b (nt 1471, t-c), of which the latter had previously been reported in the SNP database. In the analysis of allele and genotype frequencies, only variant homozygosity for *TAC3* -25 c-t could be associated with increased risk of pre-eclampsia (RR 3.33, p=0.03). Follow-up work will include extended genotyping in further stratified and larger patient cohorts and transfection studies to assess splicing potential and functional consequences of the mutant alleles.

These data represent the first documented mutation screen of the *TAC3* and *TACR3* genes and report novel variants in patients with pre-eclampsia. This study contributes to the knowledge of neurokinin B as a circulatory molecule and confirms the heterogeneity of pre-eclampsia.

Opsomming

Die belangrikste direkte oorsaak van moedersterftes is hipertensiewe toestande in swangerskap, insluitende pre-eklampsie. Hierdie toestande kompliseer wêreldwyd 10% van alle swangerskappe. Die plasenta is kardinaal in die ontwikkeling van die siekte aangesien dit slegs voorkom terwyl die plasenta *in-situ* is en die simptome opklaar na verlossing van die plasenta. 'n Moontlike hipotese is dat die plasenta 'n sirkulerende agens afskei wat in die moederlike sisteem beland en die uiteenlopende multi-sistemiese simptome en tekens van die siekte veroorsaak, asook aktivering van die immuunsisteem. Een van die moontlike kandidate wat tans wêreldwyd ondersoek word as moontlike sirkulerende agens, is Neurokinien B (NKB), 'n lid van die Tachikinien familie.

Die unieke benadering van hierdie projek was om die rol van Neurokinien B in pre-eklampsie te ondersoek op 'n genetiese grondslag. Dit is bereik deur bio-informatiewe karakterisering van die neurokinien B (*TAC3*) en neurokinien B reseptor (*TACR3*) en deur mutasie sifting op DNA monsters van 30 pasiënte met pre-eklampsie (waarvan 10 ook abruptio placentae gehad het) en twintig kontrole individue met behulp van Multiphor gel elektroforese en ge-outomatiseerde volgorde bepaling.

Drie volgorde variasies is geïdentifiseer in die *TAC3* geen en sluit in: (i) 5' UTR variant (-25 c-t); (ii) introniese variant IVS3-53 (t-g) en (iii) 3' UTR variant in ekson 7 (479, t-c). Slegs die -25 c-t variasie is voorheen raporteer (SNP databasis). Nog twee variante is ook gevind in die *TACR3* geen: (i) ekson 3 variant (nt 857, a-t) en (ii) 3' UTR variant, amplikon 5b (nt 1471, t-c); hierdie laaste een is al in die SNP databasis raporteer. In 'n analise van genotipe en allele frekwensies is slegs homosigositeit vir variant *TAC3* -25 c-t geassosieër met 'n verhoogde risiko vir pre-eklampsie (RR 3.33, $p=0.03$). Verdere werk sal nou fokus op die genotipering van groter en gestratifiseerde pasiënt kohorte en transfeksie studies om splitsing potensiaal en funksionele gevolge van mutante allele te ondersoek.

Hierdie data is die eerste gedokumenteerde mutasie sifting van die *TAC3* en *TACR3* gene en verslag word gelewer van unieke variasies in pasiënte met pre-eklampsie.

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To my Creator, for granting me the health and strength to persevere.

List of Abbreviations

°C	Degrees Celsius
µl	Microlitre
ACE	Angiotensin converting enzyme
AGT	Angiotensinogen
APS	Ammonium persulphate
C-terminal	Carboxy-terminal
cm	centimetre
CMC	Chemical mismatch cleavage
DGGE	Denaturing gradient gel electrophoresis
dNTPs	Deoxynucleotide triphosphates
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
eNOS	endothelial nitric oxide synthase
F	Forward
fVL	factor V Leiden
g	grams
HA	Heteroduplex analysis
HELLP	Haemolysis, elevated liver enzymes, low platelets
IUGR	Intra uterine growth retardation
l	litre
mg	milligrams
MgCl ₂	Magnesium chloride
mRNA	messenger ribonucleic acid
ml	millilitre
mmHg	millimetres of mercury
mM	millimolar
MMP	Metalloproteinases
MMR	Maternal mortality ratio
MTHFR	Methylenetetrahydrofolate reductase
NKA	Neurokinin A
NKB	Neurokinin B
NO	Nitric oxide

NOS	Nitric oxide synthase
PAGE	Polyacrylamide gel
PIGF	Placental growth factor
pmol	picamolar
PCR	Polymerase chain reaction
PGI ₂	prostacyclin
PTT	Protein truncation test
Prothr	Prothrombin
R	Reverse
rpm	revolutions per minute
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel
SMS	Sequence manipulation suite
SNP	Single nucleotide polymorphism
SP	Substance P
SSCP	Single stranded conformational polymorphism
STMs	Syncytiotrophoblast microvillus membranes
<i>TAC3</i>	Tachykinin 3
<i>TACR3</i>	Tachykinin 3 receptor
TEMED	Tetramethylethylenediamine
TXA ₂	Thromboxan A ₂
U	Unit
UTR	Untranslated region
V	Volts
VEGF	Vascular endothelial growth factor
WHO	World Health Organization

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Chapter 1

Introduction

1.1 Normal Pregnancy

Human pregnancy is a remarkable experience with overwhelming emotions and immense expectations from the expectant parents and also society at large. The normal mean duration of human singleton pregnancy is 40 weeks (280 days) from the first day of the last menstrual period (Gabbe *et al.*, 2002). The period of gestation can also be divided into three units of three calendar months each, or three trimesters, since important obstetrical milestones can be designated by trimesters (Cunningham *et al.*, 2001). A fetus would be considered mature enough for birth at 34 weeks due to lung maturity. According to South African law, a fetus is considered to be viable at 28 weeks gestation. However, by First World standards, an infant of 24 weeks gestation may be expected to survive when extensive life-support and intensive care facilities are available.

1.2 Morphological Growth

Fertilisation of the human ovum by a spermatozoan occurs in the fallopian tube within hours after ovulation. The structure of the matured ovum is now referred to as the zygote. The fertilised zygote then undergoes several phases of cleavage resulting in the formation of the blastocyst. This process occurs in the first two weeks after fertilisation and is followed by the embryonic period starting at week three. At the end of this period, the heart is completely formed, the fingers and toes are present, the upper lip is complete and the external ears are being formed. At around eight weeks after fertilisation, the fetal period begins. During this developmental stage, a major portion of lung development occurs, however, this gestational period consists mainly of growth and maturation of the structures that were formed during the embryonic period. Term is reached at 40 weeks gestation when the fetus is fully developed and has a weight of approximately 3400g (Cunningham *et al.*, 2001). In South Africa, according to a study by Theron and Thompson (1995), analysing birth weight at the

Tygerberg Hospital (Western Cape Province), the mean birth weight determined for the hospital population was 2995g with a range of 760 – 5080g. The authors established a weight-for-gestational-age centile chart specific for the population served by the Tygerberg Hospital obstetric service (Appendix A).

It is with this fundamental knowledge of the miracle of pregnancy that one tries to envisage the expectations of the parents-to-be, but more specifically the mother. This sensitive development process may undergo problems at a myriad of stages, resulting in complications of pregnancy.

1.3 Global Maternal Mortality

According to the World Health Organization (WHO), an estimated 210 million women become pregnant every year. Approximately 30 million (~15%) of these women develop complications, of which 1.7% is fatal.

(<http://www.who.int/reproductive-health>).

A direct obstetric death is a death resulting from obstetric complications of pregnancy, labour, or the puerperium. Direct obstetric deaths arise from six major areas: hypertensive diseases of pregnancy, haemorrhage, infections/sepsis, thromboembolism and in developing countries, neglected obstructed labour and complications from illegal abortion. Other causes of direct obstetric deaths include ectopic pregnancy, complications of anaesthesia and amniotic fluid embolism.

Maternal mortality is estimated at >600 000 maternal deaths worldwide per year from pregnancy - related conditions. A striking analogy would be to imagine six jumbo jet crashes per day, resulting in the deaths of all 250 passengers on board; all of these passengers being women in their reproductive years of life (Gabbe *et al.*, 2002).

According to the WHO, every minute a woman dies from complications related to pregnancy and childbirth. In other words, 1600 deaths every day. What is more cause for concern is that 99% of these deaths occur in developing countries (<http://www.who.int/reproductive-health>).

Due to this high incidence of maternal deaths, an estimated one million children are left motherless each year. These children are three to ten times more likely to die within two years than children who live with both parents (<http://www.who.int/reproductive-health>).

According to WHO, pre-eclampsia accounts for about 12% of all maternal deaths. More shockingly, in the least developed countries, it can cause complications in an estimated 50 000 deliveries per year. In the more industrialised countries approximately 15% preterm deliveries are induced due to pre-eclampsia (<http://www.who.int/reproductive-health>).

1.4 Pregnancy Statistics in South Africa

South Africa has an estimated birth rate in excess of 1 million births per year. Recent findings by WHO state that the highest maternal mortality rates are in sub-Saharan Africa, where a woman has a one in sixteen chance of dying in pregnancy or childbirth. In black South Africans, hypertension in pregnancy affects 18% of all pregnancies (Chikosi *et al.*, 1999).

The maternal mortality ratio (MMR) is the number of women that die as a result of pregnancy, birth or within the first 42 days after delivery or termination of pregnancy per 100 000 livebirths during that year. This figure is estimated to be in the vicinity of 175-200/100 000 livebirths in South Africa and 38/100 000 livebirths for the Western Cape (NCCEMD, 2001). This ratio varies from province to province, with the figure for Gauteng 67.4 in 100 000 livebirths and Free State 135 in 100 000 livebirths (Cronjé and Grobler, 2003). During the triennium, 1999-2001, the National Health Department reported 507 deaths associated with hypertensive disorders of pregnancy in South Africa. This constituted 20.7% of all maternal deaths in the country. Of these, 139 deaths were reported to be due to pre-eclampsia. Thus, in this triennium, hypertensive deaths in mothers constituted the second most common cause of death (Moodley and Molefe, 2001), second only to HIV/AIDS.

Since the setting of this study is Tygerberg Hospital in the Western Cape Province, a closer look at a study conducted by Hall *et al.* (2000), over the five year period (April 1992 - March 1997), indicated that 33 832 women delivered their babies at this referral centre (approximately 6 766 births per year). In a more recent survey conducted at the Tygerberg Hospital, between 1 May 2000 and 30 April 2001, 4 735 births above

500g were recorded (Hall, personal communication). With an incidence of hypertensive conditions occurring in 10% of these tertiary referral cases, 470 cases of pre-eclampsia (more than 1 patient per day), are expected at one institution alone. A personal communication revealed that during the time period 1 May 2000 - 30 April 2001, 169 early onset pre-eclampsia patients (20-34 weeks gestation), had been admitted to Tygerberg Hospital.

1.5 Maternal Adaptation to Pregnancy

The anatomical, biochemical and physiological adaptations of the pregnant woman are profound. Many of these changes start soon after fertilisation and continue throughout gestation in response to fetal stimulation (Cunningham *et al.*, 2001). Some of the changes relevant to this study will be dealt with briefly.

During normal pregnancy the total circulating blood volume increases by approximately 45%, a process modulated by the interaction of the fetal (paternal) allograft with the maternal tissue (Zeeman and Dekker, 1992). This increase refers to both red blood cell mass and plasma volume and consequently a rise of 30-40% in cardiac output occurs. This increase is the result of an increase in both the heart rate and stroke volume. The heart rate increases on average by 15-20 beats per minute and the stroke volume by 5-10ml/min. A decrease in the arterial blood pressure arises with an average decrease in systolic pressure of 5-10mmHg and a decrease in diastolic pressure of 10-15mmHg (Cronjé and Grobler, 2003). As arterial pressure is determined by the peripheral resistance as well as the cardiac output, a decrease in peripheral vascular resistance must take place. The developing placenta is still too small in the first trimester to account for this decrease. The reduced peripheral resistance is most likely modulated by prostaglandins and nitric oxide and is also

associated with an arterial refractoriness to the constricting actions of infused angiotensin II (de Swiet, 1995). A failure of this expansion in plasma volume is associated with an increase in the incidence of hypertension and intrauterine growth restriction (Thorburn, 1994).

The kidneys enlarge during pregnancy as renal flow is increased by approximately 40% by 20 weeks gestation and with a further 10% thereafter. A decrease in plasma sodium concentration occurs due to a much greater re-absorption of water compared to sodium.

1.6 The Placenta

The placenta is a specialised organ of exchange, located at the interface between the fetal and maternal circulations. This interface (placental syncytiotrophoblast) comprises a multi-nucleated true syncytium with an extensive microvillus brush border that is in direct contact with maternal blood (Thorburn *et al.*, 1994). The maternal surface is composed of chorionic villi, arranged in cotyledons (lobules) that are separated by furrows. The maternal blood gives it a bluish-red colour and the surface is covered by a thin layer of trophoblastic cells. The fetal surface on the other hand, is smooth and shiny and the branches of the umbilical veins and arteries and the umbilical cord protrude from it (Myles, 1985).

1.7 Functions of the Placenta

The placenta provides nutrients and oxygen to the fetus and excretes waste products from the fetus. The placenta plays an essential role in the synthesis and transformation of a variety of hormones necessary for the normal maintenance of pregnancy and preparation for lactation (Thorburn *et al.*, 1994). The placenta and the fetus therefore, function as a unit and the fetus is dependent on the placenta for its quality of life *in utero* (Myles, 1985). The main functions of the placenta are thus:

- (i) Nutrition;
- (ii) Respiration;
- (iii) Excretion;

(iv) Endocrine.

During the embryonic period, the placenta develops from a highly vascularised membrane known as the chorion. The development of the placenta is largely dependent on the successful attachment of the blastocyst to the uterine endometrium (Redman *et al.*, 1993).

1.8 The Decidua

The decidua refers to the functional layer of the endometrium of a pregnant woman. This layer comprises of three sections namely:

- (i) decidua basalis: the part of the decidua that forms the maternal part of the placenta;
- (ii) decidua capsularis: the superficial part of the decidua overlying the conceptus; and
- (iii) decidua parietalis: all the remaining parts of the decidua.

In normal pregnancy the spiral arteries increase in diameter by four- to six fold compared with the non-pregnant state. The endothelium, internal elastic lamina and smooth muscle layer is replaced by trophoblast and amorphous fibrin-containing matrix.

1.9 The Mature Placenta

The mature placenta is discoid and flattened in shape and weighs approximately 500g at birth. Figure I depicts a schematic cross-section through a mature placenta, indicating the fetal and maternal circulations. It is about 20cm in diameter and 2.5cm thick at the centre. The placenta is normally positioned in the upper part (fundus) of the uterus (England, 1996).

Placental ischemia is a possible reason for widespread activation of the maternal vascular endothelium that results in the enhanced formation of endothelin, thromboxane, increased vascular sensitivity to angiotensin II and decreased formation of vasodilators such as nitric oxide and prostacyclin (Slowinski *et al.*, 2002).

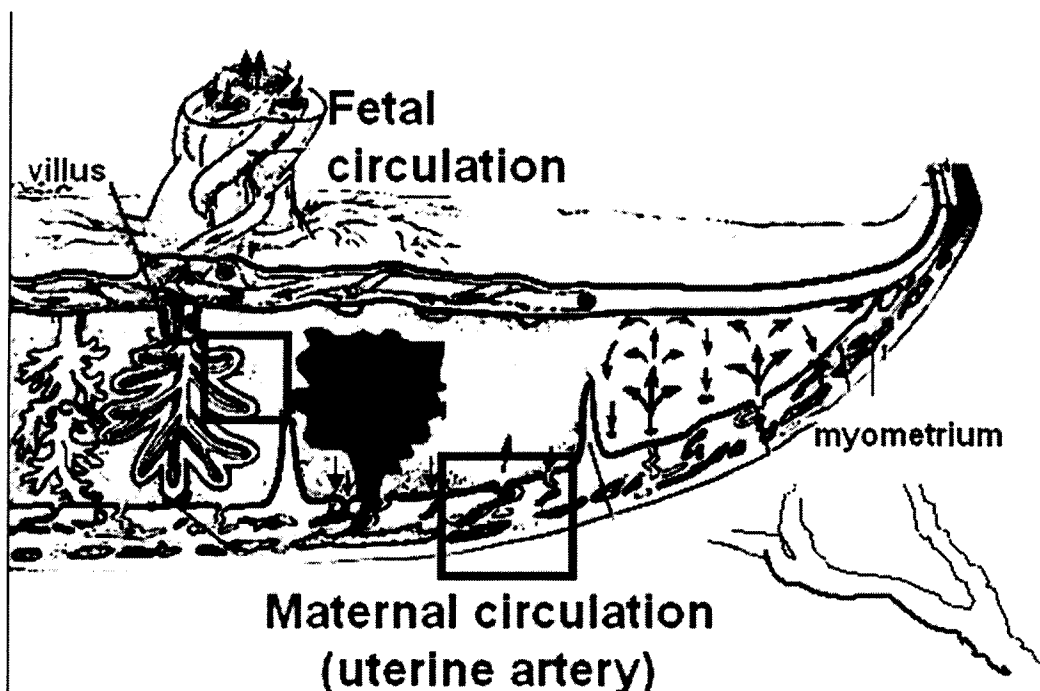


Figure I: Schematic view of a section through a mature placenta (not drawn to scale). Adapted from Cunningham *et al.*, 2001.

1.10 Conditions of the Placenta

Since two particular pathological conditions of the placenta are significant to this study, a short description of each is given. They are complete hydatidiform mole (molar pregnancy) and abruptio placentae.

1.10.1 Complete Hydatidiform mole

Hydatid means ‘drop of water’ and mole means ‘spot’ (Figure II). Hydatidiform mole is a mass of vesicles resulting in the cystic proliferation of the chorionic epithelium. The chorionic villi form these vesicles which can vary in size from a pin-head to a small grape. The embryo is absent and the karyotype is usually 46,XX but with all genetic material of paternal origin (Kajii and Ohama, 1977). This karyotype is a result of duplication of a paternal haploid set in a functionally empty ovum (Matsuda and Wake, 2003). These molar pregnancies are more common in younger (under 20 years) and older (over the age of 45) women. Interestingly, pre-eclampsia is observed in ~25% of women with complete hydatidiform mole.

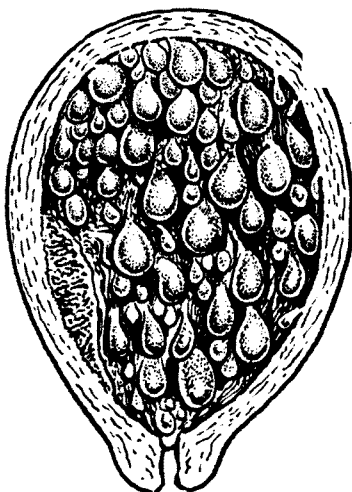
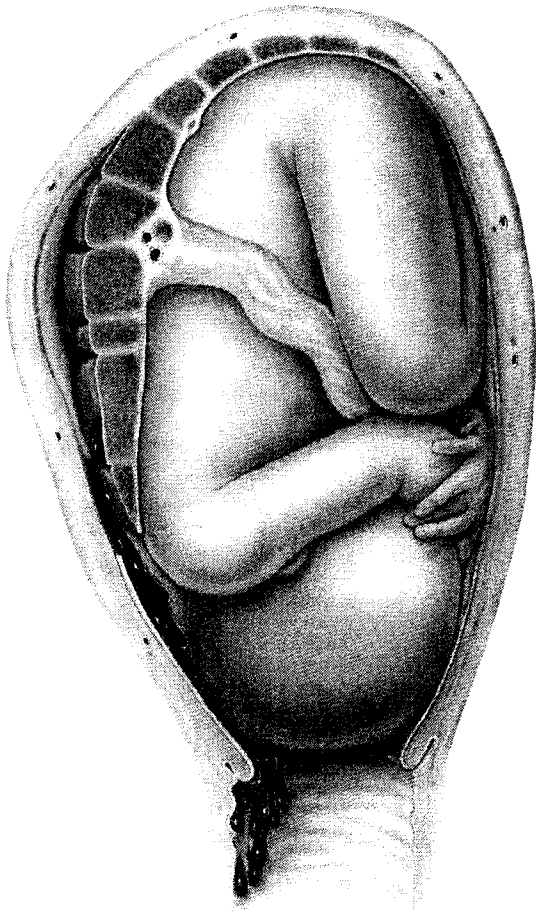


Figure II: Schematic representation of a Hydatidiform Mole (not drawn to scale). Adapted from Garrey *et al.*, 1972.

1.10.2 Abruption Placentae



**Selected Cases of
Abruption Placentae**

Figure III: Schematic representation of Abruption Placentae (not to scale).
Adapted from Young, 2002.

Abruption placentae is a major complication of hypertensive disease in pregnancy (Odendaal *et al.*, 2000). It involves the premature separation of a normally-situated placenta resulting in haemorrhage from the decidua basalis, with bleeding between the placenta and the uterine wall (Figure III). This dangerous and unpredictable condition occurs in 0.5-2% of pregnancies and in 25-30% of cases, results in fetal death. No specific cause for abruption placentae has yet been found, although it is associated with many clinical conditions (Odendaal and Gebhardt, 1999). These include: pre-eclampsia, homocysteinaemia, cigarette smoking, cocaine abuse, pre-labour rupture of

the membranes and chorio-amnionitis (Cronjé and Grobler, 2003). Other factors such as advanced maternal age, grand multiparity, polyhydromnios and previous history of placental abruption have also been reported (Ananth *et al.*, 1996).

De Jong *et al.* (1997), reported a study which investigated the role of nutrition in the development of abruptio placentae in the Western Cape population. Although no nutritional anomaly could be found in the study group, the mean birth weight in the abruptio placentae group was lower than the control group of similar gestation. It was concluded that abruptio placentae was possibly a disorder of poor placentation and that poor vitamin B₆ status could be the aggravating factor in susceptible patients.

In 2000, Hall *et al.* and Odendaal *et al.* each indicated that 20.2% of patients hospitalised for early onset, severe pre-eclampsia developed abruptio placentae. Abruptio placentae has been implicated as a major cause of intra-uterine death in South African hospitals (Odendaal, 1991; 1994). The perinatal mortality rate associated with abruptio placentae ranges from 119 per 1000 to 580 per 1000 (Odendaal *et al.*, 2000).

1.11 The Endothelium

The endothelium is a single cell layer that lines the internal surface of all blood vessels (de Swiet *et al.*, 2002). The endothelial cells have a very important metabolic and secretory function to help maintain homeostasis in pregnancy. Any alteration in the endothelial cell function results in an increased sensitivity to pressor agents, reduced plasma volume and the activation of the coagulation cascade.

Normal endothelial cells modify the contractile response of adjacent smooth muscle cells by the secretion of vasoconstrictors like (i) endothelin and (ii) angiotensin and vasodilators like (iii) nitric oxide (NO) and (iv) prostacyclin (PGI₂) (Wilkinson *et al.*, 1996).

(i) **Endothelin**

Endothelin is a 21-amino acid peptide vasoconstrictor that is derived from a 23-amino acid peptide precursor pre-proendothelin that is cleaved after translation to form pro-endothelin. A converting enzyme then cleaves proendothelin to produce the 21-amino acid endothelin. Endothelin exerts its vasoconstricting effects via endothelin A receptors on the vascular smooth muscle. The endothelin B receptors enable endothelin to release NO and PGI₂ from the endothelium, thereby inhibiting platelet activation (Granger *et al.*, 2001). The endothelin system has been shown to be activated in pre-eclamptic pregnancies since endothelin concentrations are significantly higher than in non-pre-eclamptic pregnancies (Slowinski *et al.*, 2002).

(ii) **Angiotensin II**

The renin-angiotensin system is involved in the regulation of blood pressure and electrolyte metabolism. Angiotensinogen is a α_2 -globulin produced in the liver and is a substrate for renin. Renin is an enzyme produced in the juxtaglomerular cells of the kidney that acts upon angiotensinogen to produce the decapeptide, angiotensin I. Two carboxyl terminal amino acids of angiotensin I are then removed by angiotensin converting enzyme (ACE), a glycoprotein found in lungs, endothelial cells and plasma to form angiotensin II. Angiotensin II is a potent vasoconstrictor that increases the blood pressure by its action on the arterioles (Murray *et al.*, 1996).

(iii) **Nitric Oxide (NO)**

NO is an organic free-radical that is synthesised from L-arginine by a family of enzymes known as NO synthases (NOS) (Granger *et al.*, 2001). NOS exists in both a constitutive (calcium-dependent) and inducible (calcium-independent) form (Norris *et al.*, 1999). Human endothelial NOS is widely distributed in placental tissue and a reduction in placental NOS activity has been demonstrated in pre-eclamptic placentae (Guo *et al.*, 1999). NO reduces platelet sensitivity to pro-

aggregatory agents, thus inhibiting platelet aggregation (Wilkinson *et al.*, 1996). In women developing pre-eclampsia, NO generation was shown to be reduced. Inhibition of NO synthesis in rats during pregnancy produces hypertension and proteinuria, making the endothelial NO synthase (*eNOS*) gene a primary candidate for pre-eclampsia (Arngrímsson *et al.*, 1997).

(iv) **Prostacyclin (PGI₂)**

Prostacyclin is an endothelial-derived prostanoid which is synthesized from arachidonic acid. It is a potent vasodilator, an inhibitor of platelet aggregation and a stimulator of renin secretion (Lyll and Greer, 1996 and Wilkinson *et al.*, 1996). In normal pregnancy, the placenta produces approximately equal amounts of prostacyclin and the vasoconstrictor, thromboxane. However, in pre-eclampsia, the placenta produces in excess of seven times more thromboxane than prostacyclin (Walsh, 1985). Cyclooxygenase metabolises prostaglandins, and aspirin has been found to be an inhibitor of cyclooxygenase (Merviel *et al.*, 2004). Therefore, 75-100mg aspirin per day is sometimes prescribed to patients with a history of early-onset, severe pre-eclampsia from about 8 weeks gestation of the following pregnancy. This is thought to diminish the excess thromboxane and thus re-establishes the PGI₂/TXA₂ balance. This role of aspirin in reducing the risk for severe early-onset pre-eclampsia was first examined in a randomised way in the CLASP (Collaborative Low dose Aspirin Study in Pregnancy) trial (CLASP Collaborative Group, 1994). A subsequent study by Bower *et al.* (1996), confirmed that low dose aspirin therapy was effective in significantly reducing the incidence of pre-eclampsia.

Chapter 2

Pre-eclampsia

Hypertensive conditions of pregnancy, including pre-eclampsia, are the principal direct cause of maternal morbidity and mortality, affecting 5-10% of first pregnancies worldwide (Page *et al.*, 2000). One in six stillbirths can be associated with hypertensive disorders (Wareing *et al.*, 1993 and Prins *et al.*, 1997).

Hypertension can exist prior to pregnancy (essential hypertension) or it can be induced by pregnancy (pregnancy-induced hypertension). Pregnancy-induced hypertension is defined as a diastolic pressure of ≥ 90 mmHg or an increment of ≥ 20 mmHg from the first trimester diastolic blood pressure measurement. The absolute blood pressure levels have to be observed on two occasions, ≥ 4 hours apart. This form of hypertension can be accompanied by significant proteinuria (≥ 300 mg protein per 24 hours urine collection) (Dekker *et al.*, 1995), coagulopathy and peripheral and cerebral oedema when it is then referred to as pre-eclampsia. Pre-eclampsia may swiftly become more complicated and affect the maternal liver, kidneys, lungs, blood vessels and nervous system. These clinical problems only become apparent in the second half of the pregnancy even though they are believed to start during the first trimester (Page and Lowry, 2001).

Pre-eclampsia is a condition unique to human pregnancy and can lead to iatrogenic prematurity. It had been termed the “disease of theories” by Zweifel as early as 1916 (Walker and Gant, 1997), since several models for its pathogenesis have been proposed. However, no single hypothesis has yet been shown to comprehensively define the disorder (Luttun and Carmeliet, 2003). A current theory proposes a two-stage model in the development of the condition. The first stage appears to involve the defective trophoblastic invasion of the placental bed, which results in hypoperfusion and an ischemic placenta. The second stage then appears to involve the release of an unknown factor(s) into the maternal circulation from the placenta in response to the first stage (Page and Lowry, 2001). Various factors have been named as likely candidates, including neurokinin B (NKB), vascular endothelial growth factor (VEGF) and placental growth factor (PIGF).

2.1 Pathophysiology of pre-eclampsia

Recent work has indicated that a key feature of placental pathology is an insufficient uteroplacental circulation which leads to placental hypoxia and oxidative stress. More severe cases result in infarction (Redman and Sargent, 2003).

Trophoblast cells normally grow from villi of the early placenta into the spiral arteries of the maternal decidua, thereby displacing the muscular layer of arteries. The cytotrophoblast secretes enzymes (Gelatinase A and B) to digest the extracellular matrix of the endometrium (Bischof, 2001). These enzymes are also called matrix metalloproteinases (MMP) (MMP2 and MMP9, respectively). After replacement of the muscular (and vasopressor-responsive) media with vasopressor-inert trophoblast cells, the arterioles now allow an increased flow of blood to the intervillous space. These changes extend from the intervillous space to the inner-third of the myometrium.

In pre-eclampsia, however, this process of trophoblastic invasion occurs to a lesser extent (or not at all), resulting in narrower vessels and ultimately in placental insufficiency and fetal growth impairment (Cronjé and Grobler, 2003). Furthermore, the spiral artery dilation is only ~40% of that of normal pregnancy. The myometrial component of the spiral artery is not invaded by trophoblast and is therefore unaltered from the non-pregnant state. Ultimately, almost half of the decidual portion of the spiral arteries in women with pre-eclampsia exhibit acute atherosclerosis.

Trophoblast invasion by MMP2 and MMP9 is mediated by several autocrine factors (such as human chorionic gonadotrophins and leptin secreted by the trophoblast) as well as in a paracrine way by uterine factors (such as tumor necrosis factor and interleukins). It is possible that disruption of these early response genes may be the earliest insult leading to defective placentation (Bischof, 2001). This defective trophoblastic invasion leads to vascular damage with increased endothelial permeability. Normal endothelial cells modulate complex actions like immune and inflammatory responses, maintenance of vascular integrity and anticoagulant effects. However, in pre-eclampsia, where these endothelial cells become damaged, they lose

their normal function and start to produce vasoconstrictors (Cronjé and Grobler, 2003).

Van Beek *et al.* (1998) have hypothesised that the formation of oxygen-free radicals caused by immune maladaptation, results in the activation of lymphoid cells and an ischemic placenta. When the production of these free radicals exceeds the neutralization capacity of the free radical scavengers, a condition of imbalance is created which favours the formation of lipid peroxides from unsaturated fatty acids. The oxygen-free radicals together with the lipid peroxides are toxic to endothelial cells and is another possible contributor to the pathophysiology of pre-eclampsia.

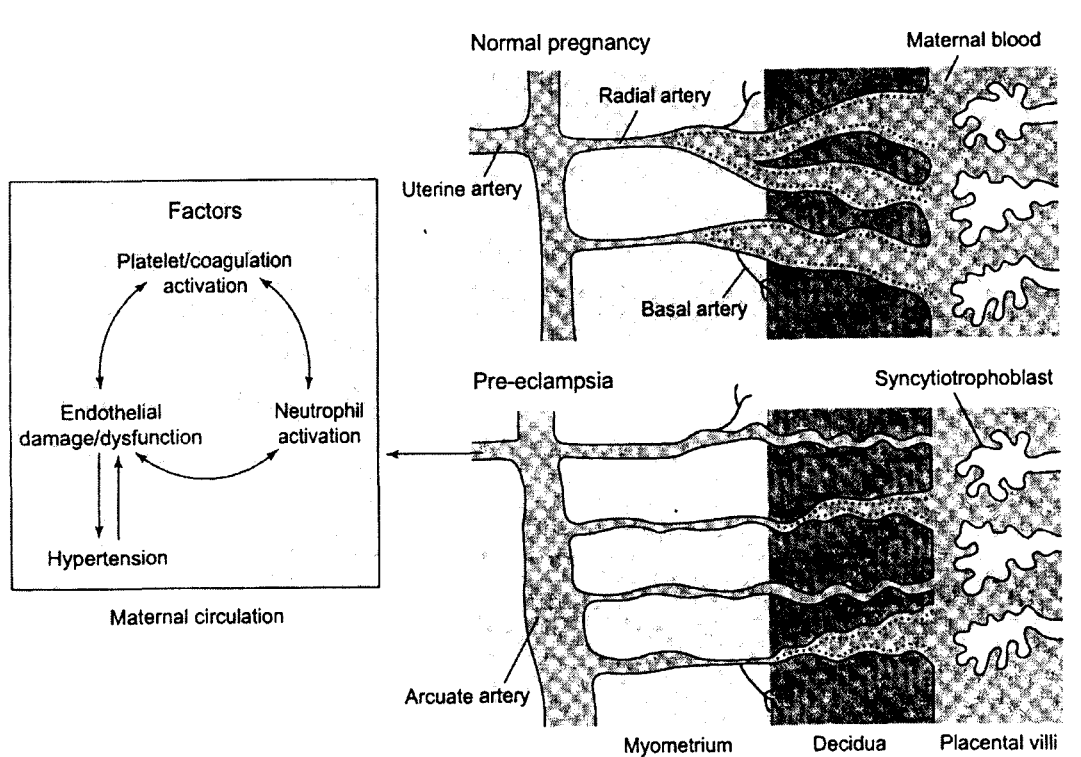


Figure IV: Schematic representation of trophoblastic invasion of spiral arteries. Dotted lines indicate trophoblast invading the vessels. Adapted from Lyall and Greer, 1996).

Pre-eclampsia has been reported in abdominal pregnancies, therefore a uterine cause has been eliminated and it has also been shown that the presence of a fetus is not essential, since pre-eclampsia is characteristic of molar pregnancies (Wilkinson *et al.*,

1996). It has been proposed that placental tissue is probably vital to the pathogenesis of pre-eclampsia as this condition only occurs if the placenta is present and the only cure is the delivery of the fetus and placenta (Wareing *et al.*, 2003).

2.2 HELLP Syndrome

Pre-eclampsia can be further complicated by accompanying HELLP (haemolysis, elevated liver enzymes, low platelet) syndrome. In normal pregnancy, the platelet count may drop to below $200 \times 10^9/L$, because of the normal maternal blood volume expansion. However, in pre-eclampsia, the platelet count can drop even further. This drop is associated with the HELLP syndrome (Walker, 2000). Liver involvement in pre-eclampsia is the cause of the epigastric pain commonly reported in severe pre-eclampsia and results in swelling of the liver and stretching of the liver capsule. The haemolysis is caused by the damaged endothelium. The incidence of HELLP syndrome at Tygerberg is about 3% of severe pre-eclampsia cases and as high as 9% in patients with abruptio placentae (Odendaal *et al.*, 2000).

2.3 The Genetics of Pre-eclampsia

Various hypotheses have been proposed to explain the mode of inheritance of pre-eclampsia, assuming that it does indeed have a genetic basis. In the 1980's, Chesley and Cooper (1986) studied the sisters, daughters, granddaughters and daughters-in-law of eclamptic women and concluded that pre-eclampsia was clearly heritable.

Recent reports have also established a familial predisposition to pre-eclampsia. Esplin *et al.*, 2001 proposed that since the fetal genotype is a combination of the maternal and paternal genotypes, it may be important to determine the contribution of the fetal and paternal components to the predisposition to pre-eclampsia. They found that men and women who were the products of pregnancies complicated by pre-eclampsia were significantly more likely to have pregnancies affected by pre-eclampsia. A primigravida woman with a family history of pre-eclampsia was three times more likely to develop pre-eclampsia during pregnancy than a primigravida woman without

such a family history. Also, a man born of a pregnancy complicated by pre-eclampsia is twice as likely to father a child from a pre-eclamptic pregnancy than a man who was born of an uncomplicated pregnancy. Furthermore, a man who had previously fathered a child from a pre-eclamptic pregnancy, who then fathers a child with a different partner, was twice as likely to have the latter pregnancy also complicated by pre-eclampsia (Lie *et al.*, 1998). Therefore, the role of the paternal and fetal genes and their contribution to pre-eclampsia warrants further investigation.

2.3.1 Linkage Studies

In order to identify areas within the genome that may harbour pre-eclampsia causative genes, a genome-wide linkage study was performed by Arngrimsson *et al.* (1999) utilising the homogenous island population of Iceland. The data indicated a chromosome 2p13 locus (lod score 4.70), however, a closer look at the study group revealed that these peaks were attributed mainly to the two larger families in the study cohort. These data provide indirect evidence for the existence of several genes which are likely to contribute to the clinical phenotype.

An independent genome scan carried out in families of Australian and New Zealand descent (Moses *et al.*, 2000), confirmed the presence of a chromosome 2 candidate locus (LOD score 2.58, located between D2S112 and D2S151) and suggested an additional locus on chromosome 11q23 (LOD 2.02, located between D11S925 and D11S4151).

2.3.2 Candidate genes (case-control association studies)

The specific gene variants that have been investigated in pre-eclampsia include factor V Leiden (G1691A) mutation in the *factor V* gene, the G20210A mutation in the *prothrombin* gene, the M235T mutation in the *angiotensinogen* gene, the Glu298Asp mutation in the *eNOS* gene and the C677T and A1298C mutations in the *MTHFR* gene, among others.

Since pre-eclamptic women exhibit haematological (bleeding) abnormalities, genes encoding factors involved in the coagulation cascade have been investigated. The

factor V gene, which is located on chromosome 1, carries the G1691A (Leiden) variant. This mutation results from the substitution of the normal guanine with an adenine nucleotide at position 1691 of the *factor V* gene (Bertina *et al.*, 1994). The prothrombin mutation results from an adenine substitution for a guanine at position 20210 in the 3' UTR region of the *prothrombin* gene (O'Shaughnessy *et al.*, 2001).

Several genes encoding "vascular" factors have been extensively investigated in pre-eclampsia. The *angiotensinogen* gene is located on chromosome 1 and contains the variant M235T that has been identified as a risk factor in some pre-eclamptic women (Pipkin 1999). A missense Glu298Asp variant was identified in exon 7 of the *eNOS* gene. This variant results from the substitution of an aspartate from the normal glutamic acid residue (Yoshimura *et al.*, 1998) and represents a risk factor for pre-eclampsia in Japanese women.

Another common polymorphism is found in the *methylenetetrahydrofolate reductase* (*MTHFR*) gene, namely the C677T variant, which is responsible for reduced MTHFR activity. This polymorphism is a cytosine to thymine substitution at nucleotide position 677, converting an alanine residue to a valine. The human *MTHFR* gene has been mapped to chromosome 1p36.3 and catalyses the conversion of 5,10-MTHF to 5-MTHF, a co-substrate for homocysteine remethylation to methionine. Altered levels of homocysteine are a recognised risk factor for cardiovascular disease and were thought to contribute to the hypertension component of pre-eclampsia. MTHFR variant C677T has been documented as a risk factor for the development of pre-eclampsia (Prasmusinto *et al.*, 2002).

It is apparent that several genes may be involved in the aetiology of pre-eclampsia, and they may be population-restricted (Table I). For example, in a very recent publication, the association of the factor V Leiden mutation with pre-eclampsia in the Caucasian population has been reported to be rare in other ethnic groups (Prasmusinto *et al.*, 2004). Rosenberg *et al.* (2002) reported that the frequency of the MTHFR C677T allele varies phenomenally in different regions of the world and across ethnic groups. The allele frequency is 0.07 in sub-Saharan Africans and 0.06 in Canadian Inuit, whereas in Caucasians, Japanese and Chinese, the allele frequencies are 0.24-0.54 (Pepe *et al.*, 1998). In keeping with this, Chikosi *et al.* (1999) reported that the

polymorphic C677T mutation was not an important factor in the pathogenesis of pre-eclampsia in black South African women.

Thus one concludes that the varying disease allele frequencies in different population groups influence the pathogenesis of pre-eclampsia and so further contribute to the complexity of the disease.

Table I: Summary table of some candidate gene studies (up to 2002) and their association or lack of association with pre-eclampsia.

Gene	Positive association with pre-eclampsia	No association with pre-eclampsia
<i>AGT</i>	Ward <i>et al.</i> (1996) Arngrímsson <i>et al.</i> (1993) Takimoto <i>et al.</i> (1996) Kobashi <i>et al.</i> (1999) Morgan <i>et al.</i> (1999) Morgan <i>et al.</i> (1999) Kobashi <i>et al.</i> (2001) Hefler <i>et al.</i> (2001)	Morgan <i>et al.</i> (1994) Wilton <i>et al.</i> (1995) Guo <i>et al.</i> (1997) Harrison <i>et al.</i> (1997) Arngrímsson <i>et al.</i> (1999) Suzuki <i>et al.</i> (1999) Moses <i>et al.</i> (2000) Curnow <i>et al.</i> (2000) Lachmeijer <i>et al.</i> (2001) Bashford <i>et al.</i> (2001)
<i>NOS3</i>	Arngrímsson <i>et al.</i> (1997) Guo <i>et al.</i> (1999) Yoshimura <i>et al.</i> (2000) Bashford <i>et al.</i> (2001) Hefler <i>et al.</i> (2001) Savvidou <i>et al.</i> (2001) Kobashi <i>et al.</i> (2001) Tempfer <i>et al.</i> (2001)	Harrison <i>et al.</i> (1997) Lewis <i>et al.</i> (1999) Arngrímsson <i>et al.</i> (1999) Lade <i>et al.</i> (1999)
<i>MTHFR</i>	Grandone <i>et al.</i> (1997) Sohda <i>et al.</i> (1997) Kupferminc <i>et al.</i> (1999)	Powers <i>et al.</i> (1999) Chikosi <i>et al.</i> (1999) O'Shaughnessy <i>et al.</i> (1999) de Groot <i>et al.</i> (1999) Kaiser <i>et al.</i> (2000) Laivuori <i>et al.</i> (2000) Kobashi <i>et al.</i> (2000) Raijmakers <i>et al.</i> (2001) Kim <i>et al.</i> (2001) Lachmeijer <i>et al.</i> (2001) Livingston <i>et al.</i> (2001) Ozcan <i>et al.</i> (2001) Kaiser <i>et al.</i> (2001)
<i>FVL</i>	Dizon-Townson <i>et al.</i> (1996) Brenner <i>et al.</i> (1996) Nagy <i>et al.</i> (1998) Mimuro <i>et al.</i> (1998) Krauss <i>et al.</i> (1998) Nagy <i>et al.</i> (1998) Kupferminc <i>et al.</i> (1999) Rigo <i>et al.</i> (2000) Ozcan <i>et al.</i> (2001) Hillermann <i>et al.</i> (2002)	Lindqvist <i>et al.</i> (1998) Lindqvist <i>et al.</i> (1999) O'Shaughnessy <i>et al.</i> (1999) de Groot <i>et al.</i> (1999) Van Pampus <i>et al.</i> (1999) Kim <i>et al.</i> (2001) Livingston <i>et al.</i> (2001)

Adapted from Lachmeijer *et al.*, 2002.

2.3.3 Microarray Studies

Pre-eclampsia is undoubtedly a complex, multifactorial disease with several genes involved in its aetiology. Microarray analysis was designed to enable relative quantification of gene activity from a multitude of genes, simultaneously. Since this technology is relatively 'new', not many studies have been reported yet.

One such DNA microarray study of pre-eclamptic tissue was reported by Reimer *et al.* (2002). In this study nine categories of genes were differentiated, viz., adhesion molecules, obesity-related genes, transcription factors/signalling molecules, immunological factors, neuromediators, oncogenic factors, protease inhibitors, hormones and growth factor-binding proteins. Amongst these, the obesity-related genes were found to be most significantly associated with pre-eclampsia. The obese (*Ob*) gene was found to be significantly up-regulated, and biochemically, elevated leptin protein levels were measured.

Another microarray study by Pang and Xing (2003), found that 162 of 221 cytokine-associated genes were up-regulated in pre-eclamptic placentae. In the third of the four studies reported to date, Tsoi *et al.* (2003), demonstrated glycogen phosphorylase to be up-regulated in placentas from pre-eclamptic patients. In the most recent study by Pang and Xing (2004), apoptosis-related genes were investigated and 35% were found to be up-regulated in pre-eclamptic placentae.

2.4 Circulating factors

Since the delivery of the placenta results in the cessation of the symptoms associated with pre-eclampsia, it is believed that the placenta may be the source of a circulating factor(s) (Roberts *et al.*, 1989). This factor(s) circulates freely in the maternal system resulting in the multisystemic and immunological responses that are characteristic of pre-eclampsia.

Potential candidates, identified largely on the basis of their expression profiles and capacity to circulate within the maternal circulation, include neurokinin B, vascular endothelial growth factor (VEGF), oxidative stress and lipid peroxidases,

syncytiotrophoblast microvillus membranes (STMs) (Hayman, 2004) and placental growth factor (PIGF) (Luttun and Carmeliet, 2003). Neurokinin B will now be discussed in further detail in the quest to determine its role as such a circulating factor.

2.5 Neurokinin B

Neurokinin B (NKB) belongs to a family of neuropeptides called the tachykinins. The first and most well known is substance P (SP) that was discovered in 1931 (von Euler and Gaddum, 1931). In 1983, a further two members were designated neurokinin A (NKA) and NKB. They are classified in the same family since they share a common C-terminal sequence (Phe-X-Gly-Leu-Met-NH₂), where X is hydrophobic. The N-terminal region is believed to convey receptor specificity to each of the three known mammalian tachykinin receptors. SP, NKA and NKB are encoded by two distinct mRNAs derived from separate preprotachykinin (*PPT*) genes (Page *et al.*, 2001).

PPT-A encodes the *SP/NKA* gene which generates four mRNAs by alternate RNA processing: α - and δ -*PPT* mRNA, which encodes SP only, and β - and γ -*PPT* mRNAs, which encode both SP and NKA. The *PPT-B* gene generates only one mRNA that produces NKB (Page *et al.*, 2001).

2.5.1 The function of Neurokinin B

The tachykinins have been implicated in a variety of biological actions from smooth muscle contraction, vasodilation, pain transmission and inflammation, to the activation of the immune system (Longmore *et al.*, 1997; Page *et al.*, 2001).

2.5.2 The Neurokinin B gene

The human NKB / *TAC3* gene expressed in the placenta, comprises seven exons and spans a region of 5.4 kilobase (kb) pairs. Exons 1 and 7 correspond to the 5' and 3' untranslated regions of the mRNA, respectively (Page *et al.*, 2001).

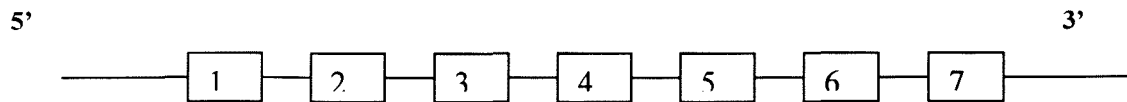


Figure V: Schematic diagram of *TAC3* gene (not drawn to scale)

2.5.3 Neurokinin B Receptors

The neurokinin B receptors are specific membrane receptors which belong to the family of G protein-coupled receptors. Three receptors have been characterised, namely, NK1, NK2, NK3. The genes encoding these are similar in structure and contain five exons (Pennefather *et al.*, 2004). Neurokinin B activates these receptors with the following order of affinity: NK1: SP>NKA>NKB; NK2: NKA>NKB>SP; NK3: NKB>NKA>SP, since SP shows preference for NK1, NKA for NK2 and NKB for NK3.

2.5.3.1 The *TACR3* Gene

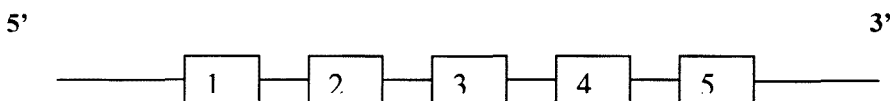


Figure VI: Schematic diagram of *TACR3* gene (not drawn to scale).

2.6 Neurokinin B in pre-eclampsia

In human pregnancy, the expression of NKB is confined to the outer syncytiotrophoblast of the placenta and significant concentrations of NKB can be detected in the plasma as early as the ninth gestational week (Page *et al.*, 2000).

These authors speculated that elevated levels of NKB in early pregnancy may be an indicator of hypertension and pre-eclampsia.

However, Sakamoto *et al.*, (2003) has shown that the concentration of NKB in early pregnancy was not significantly different from that in the non-pregnant state, but that the concentration of NKB in the blood increased with an increase in gestational age.

This trend has been previously reported by Page *et al.*, (2000) as well as D'Anna *et al.*, (2002). Schlembach *et al.*, (2003), on the other hand, has shown that NKB serum levels are higher in normotensive pregnant women than in pre-eclamptic pregnant women.

Recent studies by Laliberte *et al.*, (2004) have shown that contrary to the hypothesis that neurokinin B constricts placental resistance vessels, it does in fact, cause relaxation of isolated human placental resistance vessels.

Since biochemical determination of neurokinin B appears to be influenced by factors such as gestational age, hormones and other vaso-active substances (Schlembach *et al.*, 2003), its role in normal pregnancy and in conditions like pre-eclampsia will remain hampered. However, on the basis of localisation, expression and functions (such as vasoconstriction, immune system activation, etc.), it remains a good candidate gene for pre-eclampsia. An alternative form of measuring its candidacy is therefore warranted.

Genetic genotypes are unvarying and independent of gestational age, health status, etc., and can be correlated with both biochemical and clinical data.

Chapter 3

Identification of Mutations

3.1 Mutation Screening Techniques

The underlying principle of a mutation detection assay is that the nucleotide sequence of the gene in the affected individuals will differ from the sequence content of the same gene in individuals with a normal phenotype (Pasternak, 1999). Several mutation detection techniques have been developed, namely, single strand conformational polymorphism (SSCP), denaturing gradient gel electrophoresis (DGGE), heteroduplex analysis (HA), chemical mismatch cleavage (CMC), protein truncation test (PTT) and direct DNA sequencing, among others.

3.1.1 Single Strand Conformational Polymorphism

Single strand conformational polymorphism is one of the most commonly used methods of detecting mutations. Some, or all, of the exons of a gene are amplified individually by PCR of the affected and unaffected individuals. These PCR products are then denatured, rapidly cooled on ice, and resolved by gel electrophoresis. Each denatured single stranded DNA molecule assumes a different three-dimensional conformation and therefore migrates at a different rate on a gel during electrophoresis. Therefore the difference between affected and unaffected individuals is easily detected by direct comparison. The nature of the mutation can then be characterised by direct sequencing. This method has limitations in that it can only detect about 90% of the single base pair mutations in PCR products that are 200bp or less (Orita *et al.*, 1989).

3.1.2 Denaturing Gradient Gel Electrophoresis

Denaturing gradient gel electrophoresis involves electrophoresis of PCR products in a polyacrylamide gel containing a gradient of two denaturants, viz. urea and formamide. DNA fragments are separated according to their melting pattern. This melting behaviour is highly sequence-dependent, therefore when a DNA molecule is

partially melted it undergoes a conformational change and experiences a decrease in electrophoretic mobility. Double-stranded DNA molecules that differ by even a single base substitution show different melting behaviour, thus melting at a different position along the gel. A critical step in this mutation detection technique is the introduction of a GC-clamped primer that prevents the fragment from melting completely. This GC-clamp also alters the melting characteristics of the fragment thereby allowing the detection of mutations in the melted part of the fragment (Wu *et al.*, 1998). This method can detect more than 95% of the single base differences present in PCR products that are 600bp or less, however it does demand meticulous technical skills (Pasternak 1999), as it needs optimised experimental conditions for each DNA fragment (Wu *et al.*, 1998).

3.1.3 Heteroduplex Analysis

Heteroduplex analysis involves the amplification of DNA from two different sources that differ by only a single nucleotide pair, with the same primers. The PCR products are combined, denatured, and renatured. Homoduplex and heteroduplex DNA molecules are formed and these molecules migrate at different rates through a gel. This method can detect more than 95% of the single nucleotide mismatches in DNA fragments of 300bp or less (Keen *et al.*, 1991).

3.1.4 Chemical Mismatch Cleavage

Chemical mismatch cleavage is a variant of heteroduplex analysis. DNA samples are amplified and one of these PCR products is radiolabeled during amplification. The PCR products are combined, heat-denatured, and then left at room temperature to renature. These samples are then split into two aliquots. One aliquot is treated with hydroxylamine hydrochloride and the other with osmium tetroxide. Each aliquot is then treated with piperidine alkaloid, which cleaves the DNA strand at a modified nucleotide. These samples are then resolved on a denaturing polyacrylamide gel and viewed by autoradiography (Gunther, 1997). The presence of a heteroduplex, rather than homoduplexes, indicates the presence of a sequence variant. This method detects more than 95% of the single nucleotide mismatches in DNA fragments up to 1700bp in length (Cotton *et al.*, 1988).

3.1.5 Protein Truncation Test

Protein truncation test is based on reverse transcriptase-PCR and targets mutations that generate shortened proteins, mainly premature translation termination. Briefly, RNA is reverse transcribed to generate a cDNA copy. This cDNA is then amplified using PCR in combination with a specifically tailed F primer facilitating *in vitro* transcription by T7-RNA polymerase. The products are then analysed on an agarose gel to verify amplification, determine yield and also check the size. Abnormally migrating products point to mutations (deletions, duplications, etc.). Lastly, *in vitro* transcription/translation is used to generate peptide fragments. These are then analysed on a SDS-PAGE gel in order to detect translation terminating mutations.

The advantages of this technique are that it pinpoints the site of a mutation, has good sensitivity, has a low false-positive rate and it highlights any possible disease-causing mutations. The technical problem is that it largely uses an RNA target and in order for it to be used for high throughput, major improvement has to be made (Den Dunnen and Ommen, 1999).

3.1.6 Direct DNA Sequencing

Direct DNA sequencing is employed to identify both known and unknown sequence specific nucleotide variants. It has been termed the “gold standard” of mutation detection techniques. Various automated systems are available, like the ABI 3100 automated DNA analyser (Applied Biosystems, USA). Each nucleotide base in a DNA strand is differentially labelled by a fluorescein. When combined in a single lane on a gel matrix, each fluorescein is excited at a particular wavelength when the product passes through a laser beam, thus producing a distinct “peak” pattern, which is subsequently read as “sequence”.

Ultimately, the choice of method for mutation detection depends on the characteristics of the gene(s) being screened and the availability of resources (eg, funding and staff). The identification of a mutation would warrant further functional analysis and this could facilitate the molecular and biochemical characterisation of the genetic defect. Finally, this would result in more specifically targeted treatment/management of the specific disorder/disease.

Aim

The aim of this study is to establish (by molecular genetic techniques) whether variants exist in the *TAC3* and *TACR3* genes, and if so, whether they are associated with pre-eclampsia.

This can be achieved by mutation analysis of the novel *TAC3* and *TACR3* genes in a clinically-defined pre-eclampsia cohort and a control panel.

Chapter 4

Materials and Methods

4.1 Materials

4.1.1 Patient Selection

Institutional and ethical approval (C99/025) was granted for the project entitled “Genetic Aspects of Pre-eclampsia” (GAP). Written consent was obtained from each participant and a questionnaire was completed before blood was collected in EDTA tubes. A copy of the consent form and questionnaire are included as Appendix B and C, respectively .

The patients involved in this study were identified and recruited in the labour ward at Tygerberg Hospital since 1999 and recruitment is ongoing. Participants were restricted to a single ethnic group (South African Coloured) who represent the majority of patients at this institution.

Since pre-eclampsia is a complex clinical condition, various criteria were applied in the selection of patients for this study. The patients were assigned to various groups, (A-H), based on distinct clinical criteria. See Appendix D for details of GAP participants.

For the purposes of investigating the role of Neurokinin B in pre-eclampsia, a pilot study was initiated which involved mutation analysis of the *TAC3* and *TACR3* genes in a subset of GAP patients.

- (i) Group 1: 20 controls (multigravidae) who had pregnancies uncomplicated by hypertensive disease in the index and all other pregnancies;
- (ii) Group 2: 20 primigravidae with onset of severe pre-eclampsia before 34 weeks gestation;
- (iii) Group 3: 10 patients with pregnancy complicated abruptio placentae (with or without the HELLP syndrome).

Although this pilot study was restricted to 50 patients, any significant findings that may be demonstrated could result in an extension of the project, to include the other GAP patients.

4.1.2 Blood sampling

Following recruitment, a peripheral venous EDTA-preserved sample of maternal blood was collected by a registered research nurse or the clinician on duty. Cord blood was subsequently obtained during delivery. The fathers of the babies were encouraged to participate as well, but unfortunately, due to varying circumstances, they were largely absent.

4.2 Methods

4.2.1 DNA Extraction

DNA was extracted from the whole blood using the GENTRA™ PureGene® genomic DNA purification kit (Minneapolis, USA). Briefly, the 0.3ml blood was first added to an aliquot of red blood cell lysis solution, which lysed the red blood cells. Thereafter, a second buffer solution was added to the sample to lyse the nucleated white blood cells. This was followed by the addition of the protein precipitation solution that resulted in the separation of aqueous DNA from proteins, which formed a pellet that was discarded. The DNA was then precipitated from the remaining solution in isopropanol. The DNA pellet was then washed with 70% ethanol before being rehydrated with the DNA hydration solution and stored at 4°C until required.

4.2.2 Bioinformatics

Several databases were trawled and sequences relating to NKB and its receptor documented. Intron/exon boundaries and regulatory domains were designated and the novel *TAC3* and *TACR3* genes fully annotated using Locuslink at the NCBI (National Center for Biotechnology Information), locus link (<http://www.ncbi.nlm.nih.gov/>)

(Appendices E and F). Primers were then designed for each exon and flanking intronic regions using Primer3 at http://frado.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi (Rozen and Skaletsky, 2000). This facilitated the design of different sets of primers with common parameters such as melting temperature, GC content and length. Primer specificity was verified by BLAST (Basic Local Alignment Search Tool) : (<http://www.ncbi.nlm.nih.gov/BLAST/>) (Altschul *et al.*, 1990).

4.2.3 PCR Amplification

The sequences, exon size, expected product size, predicted annealing temperature and melting temperature used for the PCR amplification for all amplicons are summarised in Table III. Each reaction was carried out in a total volume of 50µl and consisted of: 5µl 10x reaction buffer, 15pmol of each primer (Inqaba Biotec, RSA), 200µM dNTPs (Invitrogen, USA), 3µl MgCl₂ (25mM), 0.1µl Taq polymerase (5U/µl) (Bioline Biotaq, Celtic Diagnostics) and ~100ng genomic DNA as template.

The PCR thermal cycling (GeneAmp[®]PCR System 9700) for the *TAC3* gene was initiated at 95°C for 2 minutes, followed by 40 cycles of denaturation at 94°C for 30 seconds, annealing at 52°C (exon 1), 55°C (exons 2, 4, 5, 6, 7), 57°C (exon 3), for 15 seconds and extension at 72°C for 1 minute. A final extension step at 72°C for 5 minutes was carried out. The amplification was verified by resolving PCR products on a 1.5% agarose gel containing ethidium bromide, visualised by UV transillumination.

The *TACR3* gene was amplified as described for the *TAC3* gene above. However, the annealing temperatures were as follows: 53°C (exon 1c), 55°C (exons 1a, 3, 5a, 5b); 59°C (exons 1b, 4) and 61°C (exon 2).

4.2.4 Mutation Detection

Following confirmation of a PCR product by agarose gel electrophoresis, an aliquot of each sample was taken for Multiphor Gel Electrophoresis (Pharmacia LKP 2117 Multiphor Electrophoresis Unit, Pharmacia).

4.2.4.1 Multiphor Electrophoresis System

The Multiphor Electrophoresis System combines single strand conformation polymorphism and heteroduplex analysis. This method thus increases the detection rate, allows for greater and very fast sample throughput and extends the range of amplification analysis to 500-600bp fragments. The reaction conditions are the same for all DNA fragments thereby recognising all classes of point mutations viz. base substitutions, deletions and insertions. Therefore this assay provides rapid and efficient screening without the use of dangerous compounds such as ^{32}P . It also allows for the ability to detect up to 97.5% of all point mutations in the coding region of a disease gene (Liechti-Gallati *et al.*, 1999).

4.2.4.1.1 Multiphor Methodology

The back plate (118mm x 220mm x 3mm) and the well plate (118mm x 220mm x 3mm) were wiped with ethanol three times each. The back plate was then vigorously wiped with 87 μl plate glue, until some resistance was felt. Immediately thereafter, the back plate was again wiped six to eight times with ethanol. The well plate was then wiped 3 times with acetone, remembering to be gentle over the wells. The spacers were laid down on the well plate and the plates were then sandwiched together with clamps. The gel mix contained 5.3ml 6% PAGE mix, 8.5ml Tris formate buffer, 3ml 41% glycerol, 200 μl 10%APS and 20 μl TEMED. The gel was cast and allowed to set for approximately an hour. When the gel was set, the top plate was removed, leaving the gel on the back plate. This was placed on the Multiphor platform (Pharmacia LKP 2117 Multiphor Electrophoresis Unit), using a little distilled water in order to create hydrostatic tension. The top and bottom areas of the gel were then covered with strips of chromatography paper that had been wet with Tris Borate buffer. The electrode

plate was then placed on the gel, ensuring that the electrodes were in direct contact with the chromatography paper.

One microlitre of MultiPhor loading dye (Appendix F) was added to ~ 5µl PCR product. Each sample was then denatured at 95°C for 3 minutes and then placed on ice. The samples were then loaded onto the gel and resolved at 355V for 90 minutes at 9°C. The gel was subsequently silver stained (Appendix G).

4.2.4.2 Direct Automated Sequencing

4.2.4.2.1 DNA Purification

A selection of samples was also analysed by direct sequencing. PCR products were purified using the Wizard[®] PCR Preps DNA Purification System (Promega, USA). Briefly, the 30µl PCR product was aliquoted into an eppendorf tube and 100µl Direct PCR Purification buffer was added to the tube. The tube was vortexed briefly. A minicolumn was prepared for each sample with a Syringe Barrel attached and inserted into the Vacuum Manifold. A 1ml resin aliquot was added, followed by the Resin/DNA mix. A vacuum was applied to release the liquid. Thereafter, 2ml of 80% isopropanol was added and the vacuum was again applied. The minicolumns were then transferred to a 1.5ml Eppendorf tube and centrifuged at 10,000rpm for 2 minutes. The minicolumn was then transferred to a clean 1.5ml Eppendorf tube and 30µl Nuclease-free water (preheated at 65°C) was added. After 1 minute the tube was spun for 20 seconds, to elute the DNA.

An alternative method that was used for purifying DNA for sequencing is described below.

Following successful amplification of the individual *TACR3* exons, amplicons were purified using the QIAquick[®] Gel Extraction Kit (Qiagen, USA). Briefly, the 20µl of each amplicon was resolved on a 1% agarose gel. Each band was then cut from the gel and placed in a 1.5ml Eppendorf tube. The labelled tubes had been weighed

previously and were weighed again, following the addition of the gel slice. For each 1 volume of gel, 3 volumes of buffer QG was added. The tubes were then placed on a heating block at 50°C for 10 min. This process enabled the gel slice to dissolve and free the DNA. After this incubation period, 100µl of isopropanol was added to each tube and this was allowed to stand for 5 minutes. The solution was then transferred to a prepared column that was placed in a collection tube and this was centrifuged for 1 minute at 13000rpm. The contents of the collection tube was discarded and another 500µl of the QG buffer was added to each tube. This was allowed to stand for 2 minutes and again centrifuged for 1 minute. Thereafter, 750µl PE buffer was added to each sample and incubated for 5 minutes and then centrifuged for 1 minute. The contents of the collection tubes were discarded and the columns were again centrifuged for 1 minute. Finally, 30µl SABAX distilled water was added to each column which was then placed in a fresh eppendorf tube, allowed to stand for 2 minutes and then spun for 1 minute to collect the DNA.

4.2.4.2.2 Sequencing Reaction

Following the DNA purification, the samples were resolved on an agarose gel to confirm the presence of the pure product. Successful purifications were then subjected to the sequencing reaction using the Big Dye® Terminator v.3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA).

Each reaction was carried out in a total volume of 20µl. This reaction mix was made up of: ~3ng DNA, 4pmol either forward or reverse primer (Inqaba Biotec, RSA), 2µl Terminator buffer, 1.0µl Big Dye® Terminator. These samples were subjected to thermal cycling (Touchdown Hybaid Automated Thermal Cycler) at 96°C for 5 minutes, followed by 25 cycles of denaturation at 96°C for 30 seconds and annealing at 50°C for 15 seconds, followed by an extension at 60°C for 4 minutes.

The centrifep Sephadex columns were then prepared. These columns were made with 6.25% Sephadex. Following preparation of the columns, 900µl Sephadex was added to each column and this was allowed to 'set' for about an hour. Thereafter, the columns were spun to allow any excess water to drain. A 20µl sequencing reaction

was then transferred to each column. Special precaution was taken not to disturb the column, which may lead to undesirable sequencing results.

These centrifuge columns trap the terminator and thus allowed for the desired sequence to be eluted. The eluted products were then run on a ABI 3100 Automated DNA Sequencer (Applied Biosystems, Foster City, CA).

The sequence data were analysed using BioEdit Sequence Alignment Editor. Bioedit was also used to perform multiple alignments of sequenced products and to examine sequence variants for restriction enzyme sites.

4.2.4.3 Restriction enzyme analysis

The *MspI* restriction digest was prepared in a total volume of 20.0µl comprising 10.0µl PCR product, 2.0µl 10x Buffer B and 0.5µl of 10U *MspI* (Promega, USA) enzyme. These digestion samples were then incubated overnight in a waterbath at 37°C.

Following overnight incubation, these samples were mixed with loading dye and resolved on a polyacrylamide gel (Appendix G), for 1 hour at 120Volts.

4.2.4.4 Statistical Analysis

All data were entered into an Access Microsoft database and analysed using Statistica® or EPI info® for Windows. Discrete data were compared by calculation of the relative risk and 95% confidence intervals or with the Fischer exact test where the expected values of any cell in a two by two table was less than five. Allele frequencies were determined by allele counting. Continuous data were compared with Student's t-test for data with a normal distribution and the Mann-Whitney-U test where there was not a normal distribution. A *p*-value of <0.05 was regarded as statistically significant where appropriate.

Chapter 5

Results

5.1 Patient Resource

Demographic and clinical data collected from participants are summarised in Table II.

	Control (n=20) <i>a</i>	Abruptio placentae (n=10) <i>b</i>	p-value <i>a vs b</i>	Pre- eclampsia (n=20) <i>c</i>	p-value <i>a vs c</i>
Homocysteine ($\mu\text{mol/l}$)	6.57 (2-13.45)	12.69 (4.28-37.3)	0.02	8.25 (5.5-19)	< 0.05
Age (y)	31.90 (20-44)	24.4 (18-32)	< 0.05	22 (17-29)	< 0.05
Gravidity (n)	4.0 (2-5)	2.0 (1-4)	< 0.05	1	< 0.05
Parity (n)	3.0 (1-4)	1.0 (0-3)	< 0.05	0	< 0.05
Diastolic bp(mmHg)	75.56 (60-89)	90.0 (60-120)	< 0.05	103.6	< 0.05
Gestational age (weeks)	40.0 (37-41)	34.0 (28-37)	< 0.05	27.5	< 0.05
Weight of baby (g)	3567 (2769- 4202)	1903 (916-2668)	< 0.05	803	< 0.05

Ranges are shown in parenthesis.

P values of < 0.05 are regarded as statistically significant.

Table II: Demographic characteristics of study patients

Table II demonstrated that the median age and gravidity was higher in the control group, as is expected in a control group chosen specifically for multigravidity without any previous hypertensive conditions. Homocysteine levels were significantly raised in the pre-eclampsia patients and in those pregnancies further complicated by abruptio placentae. The diastolic blood pressure was significantly raised in both patient groups, specifically more in the pre-eclamptic patients. In the control group the babies were born at term (40 weeks gestation), whereas the babies of pregnancies complicated by abruptio placentae and pre-eclampsia patients had a mean gestational age of 34 weeks

and 27.5 weeks, respectively. The control group had a median birth weight of 3567g, compared to 1903g and 803g in the patients with pregnancies complicated by abruptio placentae and pre-eclampsia patients, respectively.

5.2 Candidate gene screening

Numerous gene variants had previously been typed in the patient cohort as part of the GAP project (Appendix H). It is important to consider these gene typings in all analyses since there is likely to be a cumulative or interactive relationship between gene variants underlying pre-eclampsia.

The results of this initial gene screening are outside the immediate scope of this thesis but will be considered together with the *TAC3* and *TACR3* data as part of the GAP study.

5.3 Genetic Analysis of Neurokinin B

5.3.1 Gene Annotation of the *TAC3* and *TACR3* genes

The *TAC3* and *TACR3* genes had apparently not been examined in relation to any disease-state before. In order to ascertain the genomic distribution of the genes, full sequence annotations were carried out using data available at the NCBI locus link (<http://www.ncbi.nlm.nih.gov/>). Edited versions of the *TAC3* And *TACR3* genes annotations are given in Appendices E and F, respectively. Full annotations are available (GeneID: 6866 and 6870, respectively).

In order to fully examine the *TAC3* and *TACR3* genes by PCR, a complete set of primers had to be designed. Exons larger than 350bps were covered by two or more overlapping fragments. All primers were placed at least ~60bps into the introns, so that splice variants would not be overlooked in the screening. To minimise the level of variation in PCR conditions, the parameters in the Primer3 program (http://frado.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) were set to standardise

the following variables: primer length 18-21bps, GC content 50% \pm 2%, and melting temperature of 55°C \pm 3°C. This yielded a set of PCR reactions that were all optimised within 5°C of each other (Table III).

Name	Exon Size (bp)	Prod Size (bp)	T _m (°C)	T _a (°C)	Primer Sequence (5' – 3')
TAC3-1	117	315	58 56	52	F - TGGGATTGGTGA CTCTCAG R - GAATAAAGCAGATGGCAGC
TAC3-2	119	297	60 60	55	F - AAGCCAAGCTGCTGGTAATG R - GACAGCTGTAGTGAGGAAAC
TAC3-3	94	341	62 66	57	F - AGCACCTACTTCTCGTCCAG R - CCTTCAGATGGGAGAGAGATG
TAC3-4	30	282	60 60	55	F - TCTGAAGATAAGAGGCCTGG R - CAAACAATATGCCAGCTCCC
TAC3-5	54	290	60 60	55	F - CTGTGAGGAGTCATGCTTTG R - GAGGGAAAGACAGGACCTTT
TAC3-6	75	265	60 60	55	F - TTGAACACTGCCCGTCATAG R - CCTCCCATGCTACAGGTATT
TAC3-7	287	420	64 60	55	F - AGGATATAAGATGTGATTTTCAGTG R - CTCCTCCAGCTACATGGTAA
TACR3-1a	691	354	60 60	55	F - ATTCTTTCTGCCTGCCAGAG R - AGCCACCCAGTCTCAACTG
TACR3-1b	691	354	68 64	59	F - TGAACCTGACCGCCTCGCTA R - ACGTGTTGAAGGCGGCCATG
TACR3-1c	691	351	60 58	53	F - TACTTCCTTGTGAACCTGGC R - CACTCGAGGGCTACAAATG
TACR3-2	190	342	66 66	61	F - CCTTTGAAATACCTTTGAAACTCC R - GTTGCTCCTAATCTGTAGTTTCC
TACR3-3	153	307	60 64	55	F - AAGCTGAGCCAATTCAGTCC R - TTAACATGCCATGACTAGATTGC
TACR3-4	199	345	64 64	59	F - ATTGGCAGAAAAGCATGATTTGC R - ATTGTATGTTTCCAGTGAAGGTG
TACR3-5a	529	335	62 60	55	F - GGTAGAATTTTCTGTGGCAGC R - GCAGATTTGGAATTCCTGCG
TACR3-5b	529	384	60 60	55	F - ACCCAAGTTTCAATGGCTGC R - CGAGTTTACAAGTATTTTCTGAC

Table III: Summary of primer details and product sizes.

5.3.2 PCR Amplification

The PCR amplification of each amplicon was optimised before the patients' DNA samples were used. Figure VII represents the optimised PCR amplifications of the individual amplicons 1-7 of the *TAC3* gene.

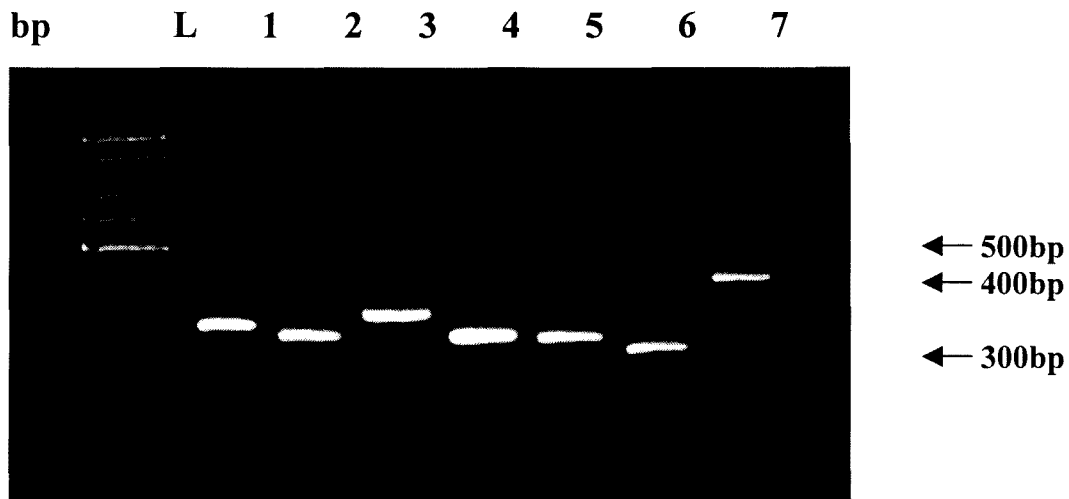


Figure VII: Photograph of *TAC3* gene amplicons (exons 1-7) and Generuler™ 100bp DNA Ladder (L) resolved on a 1.5% agarose gel. Lane 1 = 100bp DNA ladder; lane 2 = amplicon 1 (315 bp); lane 3 = amplicon 2 (297 bp); lane 4 = amplicon 3 (341 bp); lane 5 = amplicon 4 (282 bp); lane 6 = amplicon 5 (290 bp); lane 7 = amplicon 6 (265 bp); lane 8 = amplicon 7 (420 bp).

Figure VIII demonstrates the amplification products obtained for amplicons 1a-5b of the *TACR3* gene. Exons 1 and 5 were each split into three and two amplicons, respectively, to facilitate SSCP/heteroduplex analysis.

L 1a 1b 1c 2 3 4 5a 5b

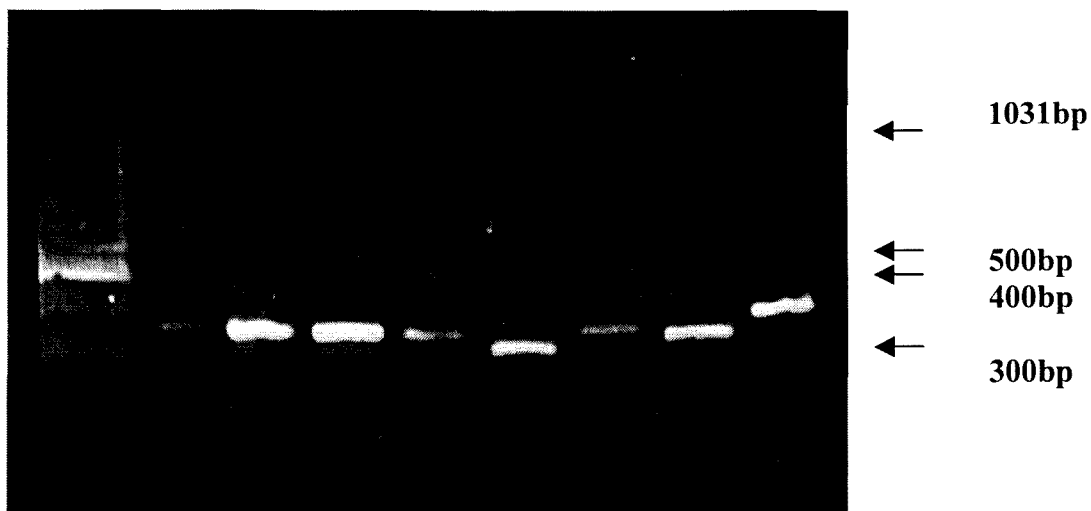


Figure VIII: Photograph of TACR3 gene amplicons and Generuler™ 100bp DNA Ladder (L) resolved on a 1.5% agarose gel. Lane 1 = 100bp DNA ladder; Lane 2 = amplicon 1a (354 bp); Lane 3 = amplicon 1b (354 bp); Lane 4 = amplicon 1c (351 bp); Lane 5 = amplicon 2 (342 bp); Lane 6 = amplicon 3 (307 bp); Lane 7 = amplicon 4 (345 bp); Lane 8 = amplicon 5a (335 bp); Lane 9 = amplicon 5b (384 bp).

5.3.3 Mutation screening

5.3.3.1 Multiphor SSCP/HA

Multiphor SSCP/heteroduplex mutation screening was performed on aliquots of the successfully amplified PCR products. Each PCR product had been diluted 1:5 in loading dye. The sample was then heat denatured and a volume of ~3ul loaded onto the horizontal gel.

The gels were run at a temperature of 9°C on a standard matrix. No conformational variants were detected in the SSCP or heteroduplex components of this system (Figure IX).

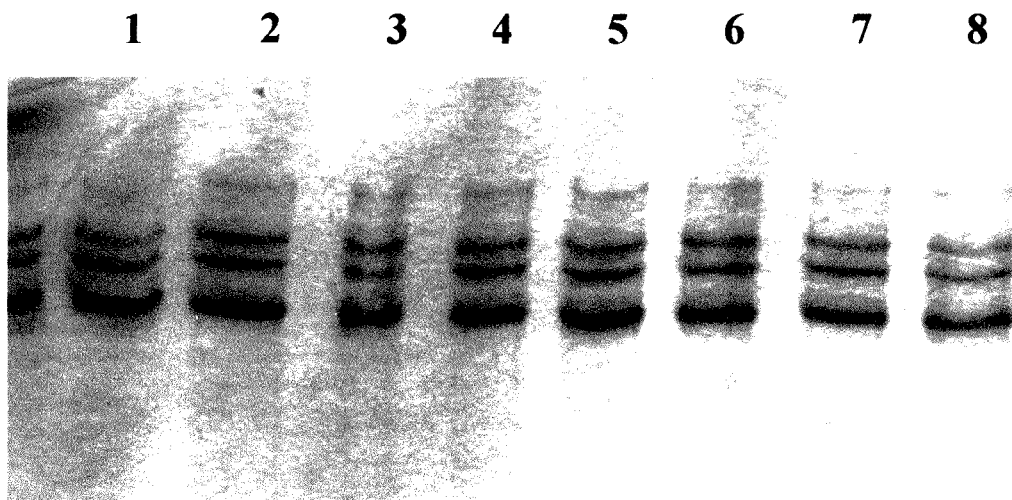


Figure IX: Photograph of a standard Multiphor/SSCP gel of *TACR3*, amplicon 5a. No conformational variants were detected.

Since no variants were discerned with the standard conditions, a sub-set of samples was selected for automated sequencing, as a direct mutation detection method. Concurrent with this, alterations were introduced to the Multiphor protocol. These included the addition of Piperazine to the gel matrix and a temperature adjustment to 12°C. Subsequently, two conformational profiles could be discerned in products representing *TACR3* exon 5b. Lanes 1, 2, 5 and 7 and lanes 3, 4, 6 and 8 in Figure X represent the two “forms”, respectively.

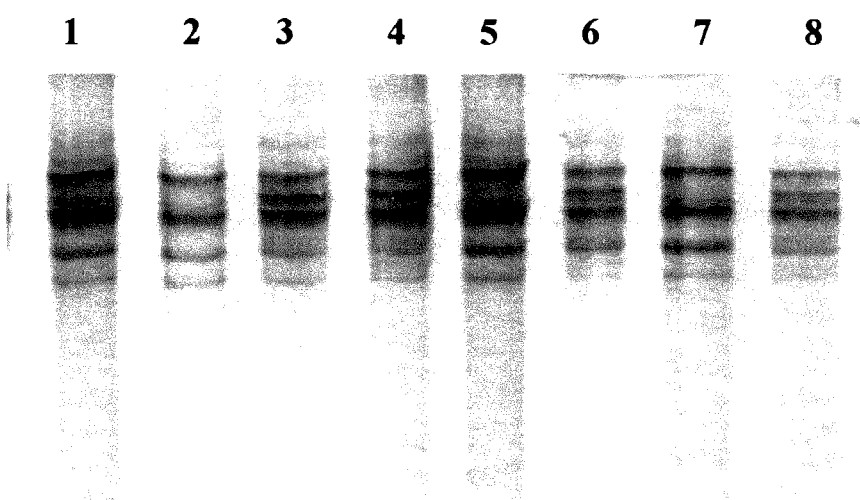


Figure X: Photograph of Piperazine Multiphor/SSCP gel run at 12°C .

5.3.3.2 Direct Automated Sequencing

Due to the initial lack of any variants being identified using the standard Multiphor/SSCP analysis, it was decided to use automated sequencing as a confirmatory technique. Because of the relative expense in using this technique, only a subset of samples was selected.

A subset of twenty patients was selected and the *TAC3* and *TACR3* genes were sequenced. Ten of these patients were from the primigravidae group with onset of pre-eclampsia before 34 weeks gestation. The other ten patients had pregnancies complicated by abruptio placentae with or without the HELLP syndrome. *TAC3* amplicons representing the complete gene in 20 individuals were subsequently column-purified using the Wizard[®] PCR Preps DNA Purification System (Promega, USA) or QIAquick[®] Gel Extraction Kit (Qiagen) and sequenced.

Three variants in the *TAC3* and two variants in the *TACR3* genes were identified, respectively, by sequence alignment using the BioEdit program and visual inspection of each chromatogram.

The individual sequence variants were identified at the following positions in the *TAC3* gene:

- (i) 5'UTR variant (-25 c-t);
- (ii) Intronic variant IVS3-53 (t-g);
- (iii) 3' UTR variant *TAC3* exon 7 (479, t-c).

The individual sequence variants identified in the *TACR3* gene are as follows:

- (i) Exon 3 variant (nt 857, a-t; K286M);
- (ii) 3' UTR variant, amplicon 5b (nt 1471, t-c).

The variants are discussed individually in further detail.

5.3.3.2.1 *TAC3* gene variants

(i) 5'UTR variant (-25 c-t)

At position -25 relative to the ATG start site, a transition (c-t) was identified (Figure XI) in 6 of 20 patients (allele frequency 8/40 alleles = 0.20).

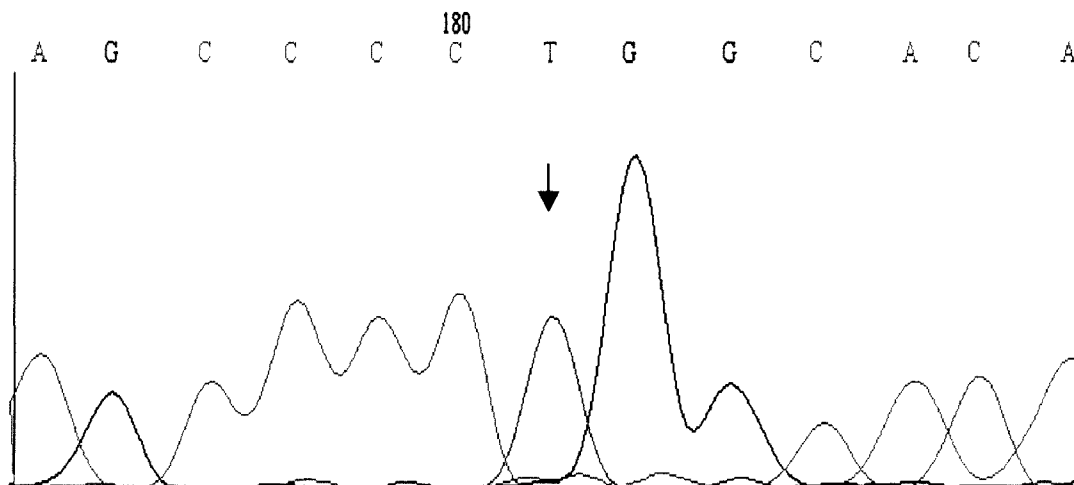


Figure XI: Representation of chromatogram indicating -25(c-t) transition in the exon 1 containing amplicon. This individual is homozygous for the mutant T allele (position arrowed).

Four of the patients presented with pre-eclampsia, of whom one also had a pregnancy complicated by abruptio placentae. The remaining two patients had presented with only abruptio placentae.

Patient 4, a 32 year old G2P1 presented at 29 weeks of uncomplicated pregnancy with an intra-uterine death due to 100% abruptio placentae. She was subsequently diagnosed with hypertension but no proteinuria. The birth weight was 1426 grams. Patient 5, a 27 year-old G3P2, presented at 37 weeks of uncomplicated pregnancy with an intra-uterine death due to 100% abruptio placentae. There was no underlying pre-eclampsia, but the baby only weighed 2050 grams. Patient 10, a 20 year old

primigravida, presented at 34 weeks of pregnancy complicated by pre-eclampsia with an intra-uterine death due to a 100% abruptio placentae. The birth weight was 1250 grams, far below the 10th centile for this gestation. Interestingly, this patient also had another variant, an intronic variant IVS3-53 (g-t) (see below).

Patients 11, 15 and 20 were all primigravidae with pregnancies complicated by early onset pre-eclampsia. Patient 11 presented at 24 weeks gestation with severe pre-eclampsia and reversed end-diastolic flow in the umbilical artery as measured with Doppler. The pregnancy was terminated due to the severity of maternal disease. The fetus weighed 500 grams. Patient 15 presented at 22 weeks with severe eclampsia and the pregnancy was terminated due to the maternal condition. The infant weighed 726 grams and did not survive. Patient 20 presented at 32 weeks with severe pre-eclampsia and an infant of 1956 grams was delivered after induction of labour.

Patients 4 (abruptio placentae only) and 11 (pre-eclampsia only) were homozygous for the -25 c-t variant, while the remaining patients were heterozygotes.

This variant was subsequently confirmed in the SNP database (accession number 2291855). An allele frequency of 0.10 was reported (in 48 alleles) in an unspecified population.

Application of the electronic SMS (Bioedit) utility, provided additional information on alternative methods of detecting the variant form. The mutant t allele abolishes one of the two *MspI* and *HpaII* restriction enzyme sites (cc↓gg). For the purpose of this study, the restriction enzyme, *MspI* was used.

The *MspI* recognition site is: C▼CG G
G GC▲C

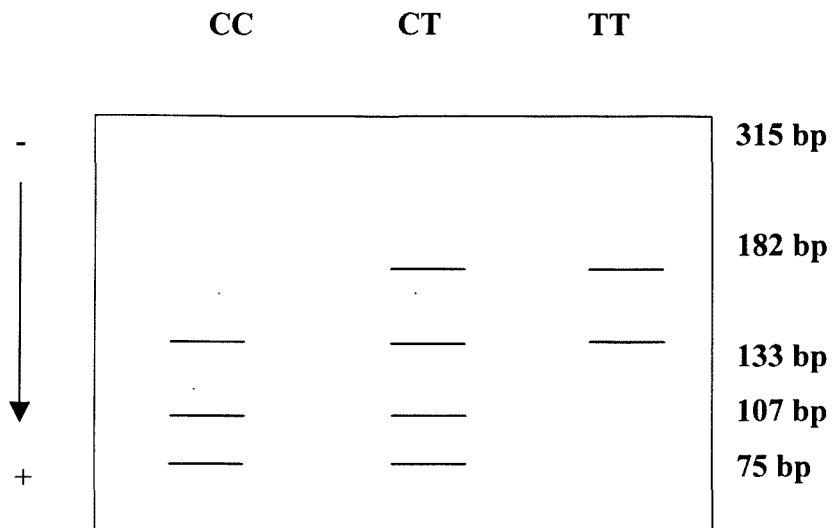


Figure XII: Schematic drawing of *MspI* restriction enzyme map

This restriction site is useful in increasing the throughput of variants rather than the expense of sequencing.

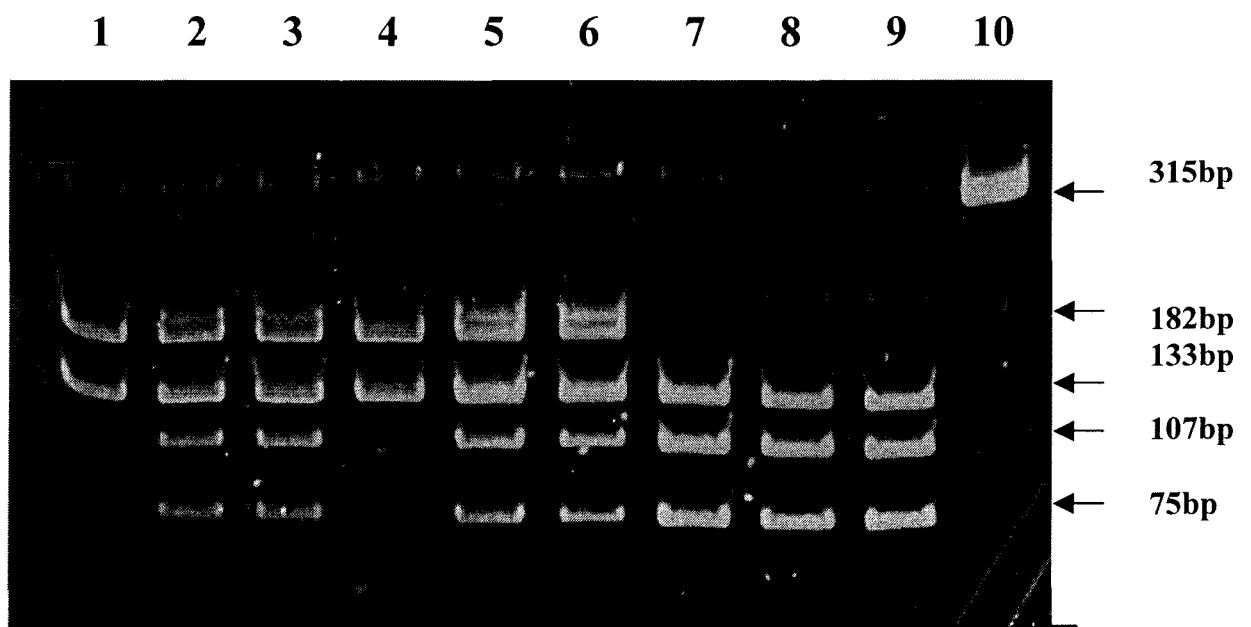


Figure XIII: Photograph of *MspI* restriction enzyme digestion. Lanes 1 and 4 represent homozygote mutants (tt), lanes 2, 3, 5 and 6 represent heterozygotes (ct) and lanes 7, 8 and 9 represent homozygote wild types (cc). Lane 10 represents an uncut PCR product.

The frequency of this promoter variant in 42 control subjects (population-matched) was therefore determined by PCR amplification followed by restriction enzyme analysis (Figure III). There were 12 heterozygotes in this control population of 42, but no homozygous individual. A mutant t-allele frequency of 0.14 was determined.

The allele frequency of the mutant t allele in diseased and controls in this population compared statistically as follows: Odds Ratio (OR) of 1.5, with a 95% Confidence Interval (CI) of 0.5-4.44. The *p* value was non significant at 0.41. When comparing the genotype frequency, the same results were obtained when heterozygotes and homozygotes were combined (OR 1.07, 95% CI 0.28-3.97; *p* value 0.9).

In the comparison of homozygous patients with combined wild-type and heterozygotes, a Relative Risk (RR) of 3.33 (ranging between 2.26 and 4.91) was obtained with a significant *p* value of 0.037 and implies that variant homozygosity status impacts negatively on health.

(ii) Intronic variant IVS3-53 (t-g)

Three samples each demonstrated a t-g change at position IVS3-53. An allele frequency of 0.075 was determined. This site is not within the conventional splicing branch site and most likely reflects a standard SNP and did therefore not warrant further investigation at the time. No restriction enzyme site is recognised within this particular region.

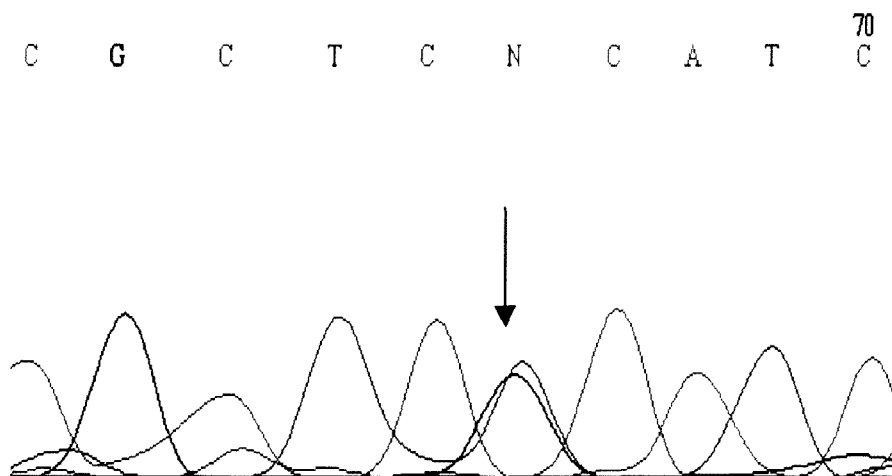


Figure XIV: Representation of chromatogram indicating the intronic variant IVS3-53 (t-g). Heterozygous status is shown (see arrow).

Patient 9, a 22 year old G2P1, presented at 33 weeks gestation with the clinical diagnosis of pre-eclampsia and abruptio placentae and a live infant was delivered, weighing 2050 grams. Patient 10 was discussed earlier. Patient 19 was also a primigravid patient with early-onset, severe pre-eclampsia at 33 weeks. She delivered an infant of 1778 grams.

One of these patients was from the early onset, severe pre-eclampsia cohort while the remaining two patients presented with pre-eclampsia and abruptio placentae. One patient with the IVS3-53 variant was also heterozygous for the -25c-t promoter variant. Her clinical phenotype was described above.

The exon 3 amplicon was subsequently amplified (by PCR) in 25 controls and resolved on multiphor SSCP gels. No variants could be detected (50 alleles).

No restriction site was created or abolished by the IVS3-53T variant. However, the position of this variant requires some discussion due to its proximity to the intron-exon boundaries. Although it appears to be close to the branch site, it cannot be excluded since it may play a role in splicing. Therefore, further characterisation of this variant is required by functional studies.

(iii) 3' UTR variant *TAC3* exon 7 (nt 479, t-c)

A single variant could be discerned in the fragment corresponding to the untranslated exon 7 amplicon. A transition (t-c) was identified at position 479 relative to the ATG start site in patient 13. An allele frequency of 0.025 (1 C allele in 40) was determined. No restriction enzyme site was recognised within this particular region.

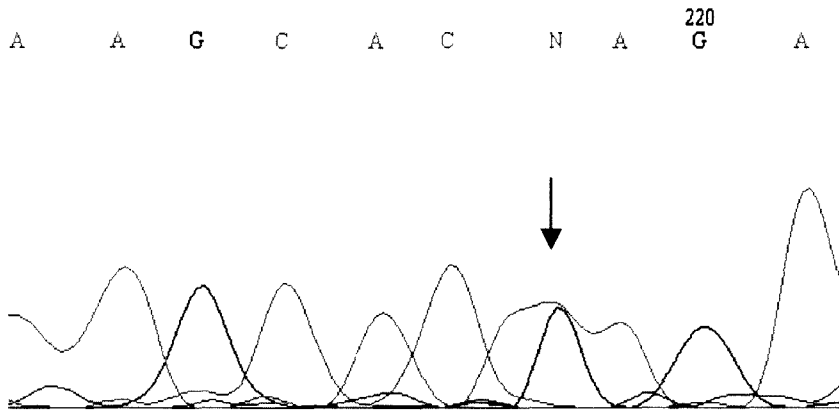


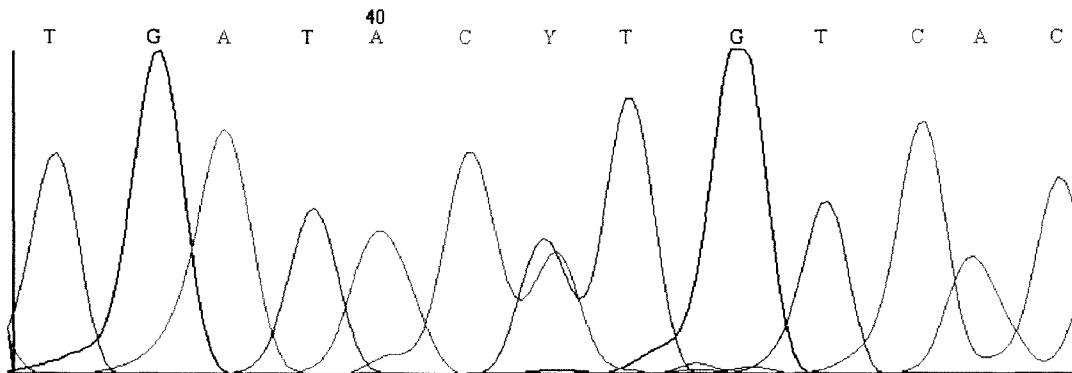
Figure XV: Representation of chromatogram of 3' UTR variant. Reverse complement. A heterozygous individual for the mutant t-c transition is shown (see arrow).

This patient 13 presented at 26 weeks with severe pre-eclampsia and she was admitted for conservative management. The infant was delivered by Caesarean section at 27 weeks due to fetal distress and weighed 892 grams.

Due to its location within the 3'UTR (regulatory) region of the gene, functional studies should clarify the role of this variant.

5.3.3.2.2 *TACR3* Gene variants

(i) Exon 3 variant (nt 857, a-g) / K286M



FigureXVI: Representation of chromatogram indicating the exon 3 variant. A heterozygous status (a-g) is shown. Chromatogram reverse complement.

At nucleotide 857 relative the ATG start site (exon 3), a transition (a-g) was identified in one individual (patient 17) at an allele frequency of 0.025. This patient was a 19 year old primigravida with severe pre-eclampsia. She delivered an infant of 874 grams at 30 weeks gestation.

This variant was subsequently identified in 2 of 21 control individuals (by Multiphor/SSCP gel electrophoresis).

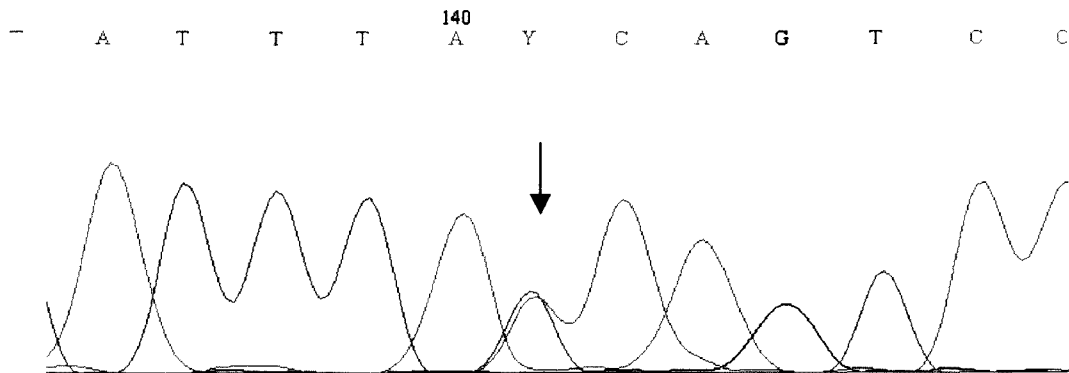
(ii) 3'UTR variant, amplicon 5b (nt 1471, t-c)

Figure XVII: Representation of chromatogram indicating 3'UTR variant. Heterozygous individual shown (see arrow).

At nt 1471 of amplicon 5b, encompassing exon 5 in the 3'UTR region, a substitution (t-c) was identified in 13 of 18 patients. [The reference sequence quoted on NCBI most likely reflects the mutant and not wildtype allele since in our study, we only identified one CC genotype].

There were four individuals in our group that represent the wildtype (tt) and the remaining 13 were heterozygotes (tc). This represents an allele frequency of 0.58 for the t allele and 0.42 for the c allele. All of these patients had severe pre-eclampsia, complicated in five cases by abruptio placentae. Eleven of the thirteen heterozygotes were primigravidae with severe early onset pre-eclampsia.

The clinical statistics of the heterozygous patients identified with this variant were as follows: The mean age of the patients was 21.14 (range 17-27), the median gravidity was 2 (range 1-3) with a parity median of 1 (range 0-2). The mean gestational age was 31.4 weeks (range 28-37 weeks) and the mean infant mass was 1470 g (range 500-2668 g).

This variant was subsequently confirmed on the SNP database (accession number 2765).

In the analysis of control samples, the variant was identified at very similar frequencies.

Table IV is a summary table of the *TAC3* and *TACR3* genes variants.

In the analysis of the sequencing data, several repeat motifs, eg. 'CCTCTCCTCCT' motif in amplicon 1b of the *TACR3* gene, were noted which may be polymorphic. These regions will be further characterised since identification of microsatellite markers may be useful for future association studies.

*TAC3**TACR3*

Patient	5'UTR (-25 c-t)	IVS3-53 (t-g)	3'UTR (479, t-c)	K286M / 857 a-g	3'UTR (1471, t-c)	Features
1					+/-	G2P1; pre-eclampsia; abruptio placentae
2						
3						
4	+/+					G2P1; abruptio placentae
5	+/-				+/-	G3P2; abruptio placentae
6						
7					+/-	G1P0; abruptio placentae
8					+/-	G1P0; abruptio placentae
9		+/-			+/+	G2P1; pre-eclampsia; abruptio placentae
10	+/-	+/-			+/-	G1P0; pre-eclampsia; abruptio placentae
11	+/+				+/-	G1P0; early onset severe pre-eclampsia
12					+/-	G1P0; early onset severe pre-eclampsia
13			+/-		+/-	G1P0; early onset severe pre-eclampsia
14					+/-	G1P0; early onset severe pre-eclampsia
15	+/-				+/-	G1P0; early onset severe pre-eclampsia
16					+/-	G1P0; early onset severe pre-eclampsia
17				+/-	+/-	G1P0; early onset severe pre-eclampsia
18						
19		+/-				G1P0; early onset severe pre-eclampsia
20	+/-				+/-	G1P0; early onset severe pre-eclampsia

Table IV: Summary table of *TAC3* and *TACR3* genes variants and clinical phenotypes

Key: -/- = homozygous wild-type, no mutant allele;
 +/- = heterozygote status;
 +/+ = homozygous mutant.

Chapter 6

Discussion and Future Prospects

The *TAC3* and *TACR3* genes were found to harbour several sequence variants. These variants were distributed across the genes, encompassing the 5'UTR, exonic, intronic and 3'UTRs. The majority of these variants appear to be novel, with only the *TAC3* 5' UTR -25(c-t) and *TACR3* 1471(t-c) variants documented in the SNP database. Data on the remaining variants will be deposited in the SNP and relevant mutation databases and so contribute to the growing knowledge of the human genome.

Each of the variants identified in this study represents a single base pair substitution. No deletions, insertions or obvious rearrangements were identified. Of the five variants, four are transitional changes: *TAC3* -25 c-t, 3' UTR nucleotide 479 t -c and *TACR3* exon 3 nucleotide 857 a-g and amplicon 5b nucleotide 1471 t-c. The only transversion change was the *TAC3* IVS3-53 (t-g).

The variants were detected by automated sequencing, which although costly, is regarded as one of the most accurate mutation detection methods. The Multiphor SSCP/HA did initially not reveal any variants at a constant running temperature of 9°C, and on a standard gel matrix. Since most SSCP protocols recommend adjusting the running conditions, this was done concurrently with direct automated sequencing of selected samples.

Conformational variants were then identified by Multiphor analysis, after piperazine had been added to 'tighten' the gel matrix and the running temperature altered to 12°C. Amplicons of close to 400 bp could then be distinguished with the adjusted protocol and the two variants identified in the *TACR3* gene by automated sequencing, could be confirmed by the presence of SSCP conformational variation. The sequence variation was not reflected in the heteroduplex bands on the gels.

Selecting a subgroup of patients to be processed by automated sequencing proved to be a difficult task. It was decided to select groups that would include patients with (i)

only early, onset severe pre-eclampsia, (ii) with only abruptio placentae and (iii) those with a combination of both, since it is believed that these conditions may share a common pathological pathway. So, although the patient groups analysed in this study were originally small (due to expense involved in automated sequencing), these patient groups can now be extended to further characterise the positively identified variants.

Only one variant was identified in the 5' untranslated region of the genes. The c-t change at position -25 relative to the ATG start codon of the *TAC3* gene, is suggestive of a promoter-region variant, with classic Eukaryotic regulatory motifs such as TATA boxes often residing at such locations. It is believed that the 5'UTR ~30 nt region immediately upstream from the start codon of most genes is highly conserved and therefore may play a critical role during transcription, and consequently, translation (Shabalina *et al.*, 2004). Detailed bioinformatic searches are currently underway to characterise the 5'UTR and specifically, the complete promoter region of the *TAC3* gene. Variation within a promoter, is expected to impact significantly on the expression of that gene, with or without sequence variation elsewhere in the gene (or the modulating receptor). The conservation "profile" of the gene will also be analysed phylogenetically.

However, since the frequency of this variant has already been established at 0.10 in a control population (SNP database), matched closely with our own control panel (0.14), it would seem unlikely that variation at this site is strongly detrimental. Do the observations of (i) an allele frequency of 0.2 in the patient cohort and (ii) homozygosity for the variant restricted to patients, suggest otherwise? An answer is not immediately clear since one homozygous patient (patient 4) presented clinically with a pregnancy complicated by abruptio placentae and another (patient 11) with pregnancy complicated by early onset severe pre-eclampsia, while two of the heterozygotes (patients 15 and 20) presented with early onset severe pre-eclampsia, one heterozygote (patient 5) with pregnancy complicated by abruptio placentae and the remaining one (patient 10) with early onset severe pre-eclampsia further complicated with abruptio placentae. The difference in genotype frequency between patients and controls (0.20 vs 0.14) may indicate that this variant impacts slightly on

susceptibility to “placental vasculopathy”. Its effect may also be context-dependent in that expression of the variant allele may depend on the presence or absence of specific sequence variants at other loci (possibly like the *TACR3*).

An intronic variant was identified in the exon 4-encompassing amplicon of the *TAC3* gene and designated IVS3-53 (t-g). This novel variant was identified in the heterozygous state in three individuals. Although this variant appears not to be at a critical position w.r.t. the intron/exon junction (it is located 53bp before exon 4), it may impact on splicing and possibly generate alternative splicing forms of the *TAC3* transcript. This possibility can be addressed by functional analysis where this mutant allele is cloned into a suitable vector and its splicing potential assessed (Zaahl *et al.*, 2004).

Two variants were located in the 3’UTR of the individual genes, viz., *TAC3* exon 7 and *TACR3* amplicon 5b. Both these variants were single base substitutions in single individuals. Sequence variation in the 3’UTR is thought to influence transcript stability (Kuersten *et al.*, 2003), therefore further characterisation of these variants are required.

The only exonic mutation identified in the study (a-t change at nucleotide position 837) resulted in the substitution of Lysine (K) for a Methionine (M) residue in exon 3 (K286M) of the *TACR3* gene in a single patient with severe pre-eclampsia. Lysine is a positively charged, basic hydrophilic amino acid and Methionine is a non-polar hydrophobic molecule. The location of this amino acid substitution within the NKB receptor protein is critical since it may affect the structure/conformation (and consequently function).

When further analysing the patients with the *TAC3* 5’UTR -25 variant, it was interesting to note that the two homozygotes for this variant (patients 4 and 11), had a very similar genetic profile with regards to 5 candidate genes screened (Appendix H). The only difference in their genetic profiles was that patient 4 was heterozygous for both the MTHFR c677t and the a1298c variants, while patient 11 was only heterozygous for the a1298c variant. This may be significant in the cumulative effect of gene variants.

Five patients (patients 5, 10, 11, 15, 20) were identified as combined heterozygotes for the *TAC3* 5' UTR -25 variant and the *TACR3*, 3'UTR amplicon 5b variant. Further analysis of this combination would be significant since the 5'UTR and 3'UTR are considered to be modulators and thereby contribute to the regulation of transcription (Sonenberg 1994 and Shabalina *et al.*, 2004). These patients did not show any obvious differences in their gene profile of the candidate genes (Appendix H). Patient 10 was identified with the *TAC3* IVS3-53 variant as well, so this individual's sample should definitely be included in functional studies. This patient presented with early onset severe pre-eclampsia further complicated by abruptio placentae, therefore heterozygosity for the abovementioned variants may be a susceptibility status for these conditions.

One patient (patient 13) with early onset severe pre-eclampsia was a combined heterozygote for the *TAC3* 3'UTR exon 7 and the *TACR3* amplicon 5b variants, both confined to the 3' untranslated regions. Another primigravida patient (patient 17) presenting with severe pre-eclampsia was a combined heterozygote for the *TACR3* exon 3 and 3'UTR amplicon 5b variants. Interestingly, both these patients were homozygous wildtype for 5 other gene variants screened (Appendix H), except that they were homozygous mutant carriers for the AGT variant. These samples would be useful for the functional studies.

This leaves the question whether these variants could alter "normal" NKB function and so predispose an individual to placental vasculopathy like pre-eclampsia and abruptio placentae? Page and Lowry (2000) first proposed a potential role for NKB in the pathogenesis of pre-eclampsia: when an ischaemic placenta develops due to the defective trophoblastic invasion of the spiral arteries, the placenta starts to secrete increased levels of NKB. This then stimulates the NK3 receptors (the preferred receptor of NKB), which in turn leads to the constriction and contraction of the mesenteric and hepatic portal veins. This ultimately results in an increase in the blood pressure and potential damage to the liver and kidneys, in order to satisfy the greater demand for blood to the uterus and placenta. This mechanism of action by NKB would also explain the low or undetectable levels of NKB in normotensive pregnancies (D'Anna *et al.*, 2002 and Page *et al.*, 2000).

We speculate that when the *TAC3* and *TACR3* genes are not functioning “normally” due to variants in the genes, then the NKB levels may be increased/decreased to compensate for this lack of function. It may be that the affinity of NKB for its receptor becomes diminished, or the receptor itself loses specificity. This question could only be answered following functional studies.

Since patients have been identified with variants in the both the *TAC3* and *TACR3* genes, it may be essential to further elucidate the interaction between the two genes/gene products. Neurokinin B seems to be the only tachykinin produced by the placenta that interacts with the peripheral tachykinin receptors (Page and Bell, 2002). It will be interesting to watch future microarray studies that may identify any other gene/gene products that may interact with NKB.

Neurokinin B has been found to be expressed in higher concentrations in older rats (Cintado *et al.*, 2001) and it is further believed that NKB expression may be hormonally controlled (Page *et al.*, 2001). Oestrogen response elements have been identified in the 5'UTR of the *TAC3* gene (Page *et al.*, 2001) and therefore future functional studies (like Luciferase-reporter transfection studies) should include environmental variables such as hypoxic conditions (mimicking the pre-eclamptic placenta) and possibly also fluctuating oestrogen concentrations in the media to gauge the influence of these stimuli on the expression of the particular gene construct.

When comparing the mutation detection methods, it becomes apparent that even though variants were not identifiable on the Multiphor system, they were identified by direct sequencing. Although direct sequencing has been described as the ‘gold standard’ (Kirstensen *et al.*, 2001) in mutation detection methods due to its high sensitivity, it is extremely costly for use in routine screening. Since the Multiphor system yielded some variants when the gel matrix and environmental factors were adjusted, one has to include these variables when using this mutation detection system in order to fully appreciate its significant advantages such as high throughput and cost effectivity.

Extensions of this project should include the following:

- i) Extending the study cohort (patients and controls) by further characterising the variants in the *TAC3* and *TACR3* genes that have been identified.
- ii) Identifying alternative methods for detecting the variants that do not create or abolish restriction enzyme recognition sites in larger control and patient cohorts. Sequencing is generally too expensive for large-scale screening. Alternative SSCP/HA protocols and systems should be evaluated.
- iii) Functional studies of the variants. The 5' and 3' regions of the *TAC3* and *TACR3* genes would be significant in this regard. Constructs with a variety of alleles can be generated and these could be expressed in tissue culture in classical transfection studies. A variety of environmental factors can be introduced including hypoxia, oxidative stress, and fluctuating hormone concentrations and the influence thereof measured on gene expression..
- iv) Individuals who were identified with more than one variant should be followed up and family studies performed in order to track the transmission patterns of the variants and evaluate NKB levels in the non-pregnant state.
- v) Variant combinations (like the *TAC3* 5'UTR -25 / *TACR3* 3'UTR) should be investigated on a biochemical level, including receptor/ligand kinetic studies.

In conclusion, the aims of the study have been met. These data have contributed to knowledge of the Human Genome and have led to the documentation of novel sequence variants, which may be associated with altered NKB levels. The link between NKB and pre-eclampsia remains to be established, and requires further investigation. Ultimately, it is hoped that studies such as this will facilitate the development of a predictive test for pre-eclampsia, and lead to “tailored” treatment for this life-threatening condition.

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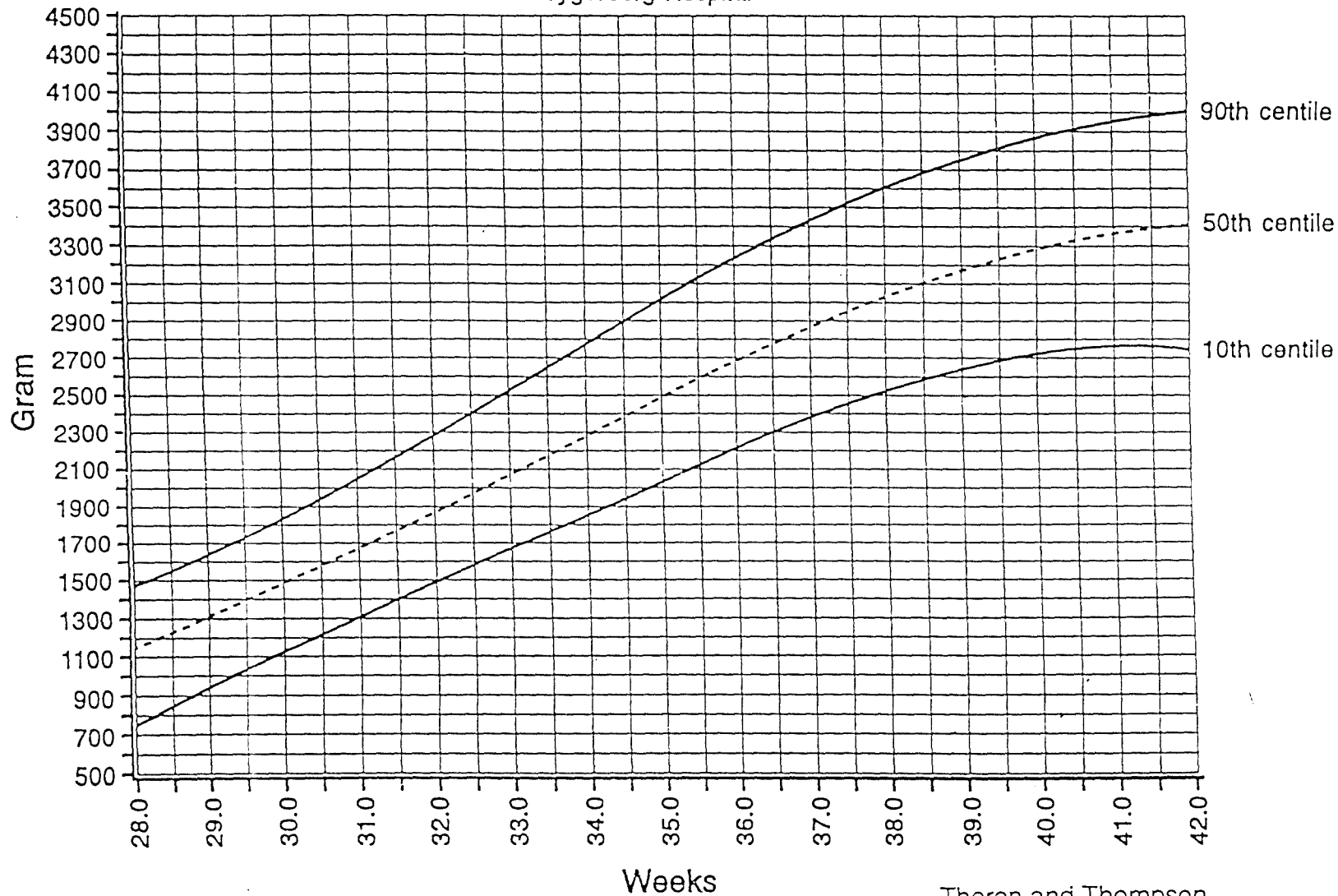
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CENTILE BOUNDS : WEIGHT FOR GESTATIONAL AGE

Tygerberg Hospital



Theron and Thompson

STELLENBOSCH UNIVERSITY
FACULTY OF HEALTH SCIENCES
**INFORMATION AND INFORMED CONSENT DOCUMENT FOR
DNA ANALYSIS AND STORAGE**

TITLE OF THE RESEARCH PROJECT:

REFERENCE NUMBER:

PRINCIPAL INVESTIGATOR:

Address:

.....

DECLARATION BY OR ON BEHALF OF PARTICIPANT:

I, THE UNDERSIGNED, (name)

[ID No:] the participant/*in my capacity as of the participant [ID No:]
..... of (address).

A. HEREBY CONFIRM AS FOLLOWS:

1. I/*The participant was invited to participate in the above-mentioned research project of the Department of, Faculty of Health Sciences, University of Stellenbosch.
2. The following aspects have been explained to me/*the participant:
 - 2.1 **Aim:** Worldwide new causes of certain diseases or conditions are continuously being discovered by research on the cells and molecules of the body. This project aims to Additionally, this project aims to collect genetic material (blood and/or tissue from surgical procedures) to analyse for certain defects (mutations) and to store excess material for future research. When a large group of patients with similar diseases has been collected, meaningful research into the disease processes may become possible.
 - 2.2 **Procedures:** I/*The participant will be requested to provide information about my/*his/*her medical history. Depending on the underlying problem or disease, blood (10ml, about 2 teaspoonfuls) and/or tissue (obtained only when surgery for the disease is deemed essential; the sample needed is 100mg, about the size of a match head) will be collected from me/*the participant. After my/*the participant's and my/*the participant's immediate family's written informed consent has been obtained, blood (also 10ml) or a mouth wash sample may be requested from my/*the participant's immediate family to investigate whether there is an underlying familial (genetic) cause for this disease.
 - 2.3 **Genetic considerations:**
 - The blood or tissue may be used to create a cell line, which can grow indefinitely and can be used to synthesize more of my/*the participant's DNA at any time in the future;
 - The DNA may be stored for several years until the technology for meaningful analysis becomes available;
 - The results of the analyses carried out on this material in the current study will be made known to me/*the participant in accordance with the relevant protocol, if and when it becomes available. In addition, I/*the participant authorise(s) the investigator(s) to make the information available to (doctor's name), the doctor involved in my/*the participant's care, as well as to the following family members (names);
 - The DNA will be maintained indefinitely, unless I/*the participant request(s) to have it and/*or the stored clinical data destroyed by contacting the investigator conducting the

- 8. I/*The participant have/*has received a copy of this document for my/*his/*her records.
- 9. The Research Subcommittee C/Ethics Committee, Faculty of Health Sciences, Stellenbosch University, has approved recruitment and participation of individuals in this study on the basis of:
 - Guidelines on Ethics for Medical Research of the SA Medical Research Council;
 - Declaration of Helsinki;
 - International Guidelines : Council for International Organisations of Medical Sciences (CIOMS);
 - Applicable RSA legislation.

B. HEREBY CONSENT VOLUNTARILY TO PARTICIPATE/*ALLOW THE POTENTIAL PARTICIPANT TO PARTICIPATE IN THIS STUDY:

Signed/*Confirmed at on20.....
(place) (date)

.....
*Signature or right thumb print of participant/*representative of participant*

.....
Signature of witness

DECLARATION BY OR ON BEHALF OF INVESTIGATOR(S):

I, (name) declare that

- I explained the information in this document to (name of the patient/*participant) and/or his/her representative (name of the representative);
- she/*he was encouraged and afforded adequate time to ask me any questions;
- this conversation was conducted in Afrikaans/*English/*Xhosa/*Other and no translator was used/*was translated into (language) by (name).

Signed at on20.....
(place) (date)

.....
Signature of investigator/representative of investigator

.....
Signature of witness

DECLARATION BY TRANSLATOR:

***Delete where not applicable**

..... (name) confirm that I

- translated the contents of this document from English into (language);
- explained the contents of this document to the participant/*the participant's representative;
- also translated the questions posed by (name) as well as the answers given by (name), and
- conveyed a factually correct version of what was related to me.

Signed at on20.....
(place) (date)

.....
Signature of translator

.....
Signature of witness

IMPORTANT MESSAGE TO PARTICIPANT/*REPRESENTATIVE OF PARTICIPANT:

Dear participant/*representative of participant,

Thank you very much for your/*the participant's participation in this study. Should, at any time during the study,

- an emergency arise as a result of the research, or
- you require any further information with regard to the study, kindly contact (name) at (telephone number/address).

present study, (name) (telephone number)
or the Chairperson of the Research Subcommittee C/Ethics Committee at
..... (telephone number) if the former cannot be located;

- The analyses in the current study are specific to the condition or disease mentioned above and cannot determine the entire genetic make-up of an individual;
- Genetic analyses may not be successful in revealing additional information regarding some families or some family members;
- Even under the best conditions, current technology of this type is not perfect and could lead to unreliable results.

2.4 **Confidentiality:** My/*The participant's identity will be kept confidential throughout. Information will not be associated with my/*the participant's name. The research staff will use only a coded number, access will be limited to authorized scientists and any scientific publications, lectures or reports resulting from the study will not identify me/*the participant by name.

2.5 **Voluntary participation:** Participation is voluntary and I/*the participant may decline participation, or withdraw from the study at any time without any loss of benefits to which I am/*the participant is otherwise entitled. Future management at this or any other institution will not be compromised by refusal or withdrawal.

2.6 **Risks:** There are no more than minimal medical or psychological risks associated with this study:

- I/*The participant may feel some pain associated with having blood withdrawn from a vein and may experience discomfort, bruising and/or slight bleeding at the site;
- As some insurance companies may mistakenly assume that my/*the participant's participation is an indication of a higher risk of a genetic disease which could hurt my/*the participant's access to health or other insurance, no information about me/*the participant or my/*the participant's family will be shared with such companies as this investigation cannot be regarded as formal genetic testing for the presence or absence of certain genes.

2.7 **Benefits:**

- Although there may not be any direct benefits to me/*the participant by participating at this stage, family members and future generations may benefit if the researchers succeed in scientifically delineating certain disorders further. Thereby the rational approach to the clinical diagnosis and therapy of its manifestations may be facilitated. The identification and location of the genes involved in such disorders, could in the end lead to the development of methods for prevention and to forms of new treatment aimed at curing or alleviating these conditions;
- In the unlikely event that the research may lead to the development of commercial applications, I/*the participant or my/*the participant's heirs will not receive any compensation, but profits will be reinvested into supporting the cause of further research which may bring benefits to my/*the participant's family and to the community, such as health screening, medical treatment, educational promotions, etc;

2.8 **Permission for further studies:** Before my/*the participant's material is used in further projects in the future, the written approval of the Research Subcommittee C/Ethics Committee, Faculty of Health Sciences, will be obtained.

3. The information conveyed above was explained to me/*the participant by (name) in Afrikaans/English/Xhosa/Other* and I am/*the participant is fluent in this language/* was translated and explained by (name).

4. I/*The participant was afforded adequate time to pose any questions and all questions were answered to my/*the participant's full satisfaction.

5. I/*The participant was not pressurized to participate.

6. I/*The participant will not be paid for participation, but reimbursement of travel costs will be considered (if applicable).

7. I/*The participant will not incur any additional costs through participation.

Appendix C

Questionnaire

GENETIC ASPECTS OF PREECLAMPSIA:

NAME:

HOSPITAL NO:

BIRTHDATE:

1. AGE:

2. RACE:

3. GRAVIDITY:PARITY: MISCARRIAGES:ECTOPIC:

PREVIOUS PREGNANCIES:

- | | | | | | | |
|--------------------------|--|--|--|--|--|--|
| 4. YEAR: | | | | | | |
| 5. PARTNER : (NO) | | | | | | |
| 6. G.A. COMPLICATIONS: | | | | | | |
| 7. COMPLICATIONS:(LIST) | | | | | | |
| 8. METHOD DELIVERY: | | | | | | |
| 9. BIRTHWEIGHT: | | | | | | |
| 10. G.A.-DELIVERY: | | | | | | |
| 11. OUTCOME: (LIST) | | | | | | |
| 12. NICU: YES=1,NO=2 | | | | | | |
| 13. SMOKE: YES =1, NO =2 | | | | | | |
| 14. ALCOHOL: YES=1,NO=2 | | | | | | |

CURRENT PREGNANCY:

15. VDRL: NEG =0, POS TREATED =1, NOT TREATED =2
16. BLOOD GROUP:
17. CERVICAL CYTOLOGY: NORMAL =1 ABNORMAL =2
18. CERVICAL CULTURES:
NOT DONE =1, NEG =2, GONO =3, CHLAMYDIA =4, GBS =5, OTHER =6
19. URINE MCS: NEG =0, ASYMP.BACTURIA TREATED =1, NOT TREATED =2,
NOT DONE =3, UTI =4
20. SF GROWTH: <10 th =1, NORMAL =2, >90th =3
21. PROTEINURIA: (GESTATION)
22. BP:(ADMISSION)
23. AMNIOCENTESIS FOR KARYOTYPING: YES =1.NO =2(GESTATION)
24. FETAL MOVEMENTS: NORMAL =1, DECREASED =2
25. ULTRASOUND: YES =1, NO =2
26. DOPPLER: N =1, >95TH =2, AEDV =3, REDF =4
27. G.A. WHEN COMPLICATIONS DEVELOPED:
28. COMPLICATIONS: _____
29. SMOKE: YES =1, NO =2
30. ALCOHOL: YES =1, NO =2
31. MEDICATION AT ANY TIME DURING PREGNANCY: _____ |
FOLATE =1,Fe =2, ASPIRIN =3, ANTIHYPERTENSIVES =4, PYRIDOXIN =5 , OTHER =6

AT DELIVERY:

32. GESTATION:
33. BIRTH WEIGHT:

33A MALE =1, FEMALE =2

33B DELIVERY TYPE: _____

34. APGAR SCORE:

35. OUTCOME: (LIST)

36. MORBIDITY:(LIST) _____

37. NICU: YES =1, NO =2

38. DAYS:

39. REASON: _____

40. SPECIAL INVESTIGATIONS:

UREUM: |_____| LDH: |_____|

KREAT: |_____| WCC: |_____|

AST: |_____| HB: |_____|

ALT: |_____| HKT: |_____|

GENERAL
INFORMATION:

ADDRESS:

BORN AND RAISED:

41. YOURSELF: _____

42. YOUR MOTHER: _____

43. YOUR FATHER: _____

44. CURRENT PARTNER:(FATHER OF YOUR CHILD) _____

45. YOUR PARTNERS MOTHER: _____

46. HAS SHE HAD TROMBOTIC EPISODES:

47. ANY COMPLICATIONS DURING YOUR PARTNER'S BIRTH: _____

48. PREVIOUS PARTNER(S) -CHILDHOOD AND PLACE OF BIRTH:

49. DO YOU HAVE SISTERS OR HALF SISTERS WHO HAVE THE FOLLOWING COMPLICATION
PRETERM BIRTH =1, DEAD BABY =2, BLEEDING LATE IN PREGNANCY =3,HIGH BLOOD

PRESSURE =4

ADDRESS OF SISTER:

50. DID YOUR MOTHER HAVE ANY OF THE FOLLOWING:

PRETERM BIRTH =1, DEAD BABY =2, BLEEDING LATE IN PREGNANCY =3,HIGH BLOOD

PRESSURE =4, THROMBOSIS =5

Appendix D

The GAP participants were subdivided into 9 groups (Groups A-H), consisting of at least 50 patients.

- (i) Group A: control group of patients with pregnancies uncomplicated by any hypertensive condition in the index pregnancy or any previous pregnancy;
- (ii) Group B: primigravidae patients with early onset severe pre-eclampsia;
- (iii) Group C: primigravidae patients with late onset pre-eclampsia;
- (iv) Group D: multigravidae patients with early onset severe pre-eclampsia;
- (v) Group E: multigravidae patients with late onset pre-eclampsia;
- (vi) Group F: primigravidae patients with pregnancy-induced hypertension only;
- (vii) Group G: multigravidae patients with pregnancy-induced hypertension only;
- (viii) Group H: pregnancies complicated by placental abruption and some with the HELLP syndrome;
- (ix) Group I: pregnancies complicated by IUGR.

Appendix E

TAC3 Gene Annotation

Annotated sequence file:

LOCUS NT_029419 10502 bp DNA linear CON
19-FEB-2004

DEFINITION Homo sapiens chromosome 12 genomic contig.

ACCESSION NT_029419 REGION: complement(19545092..19555593)

VERSION NT_029419.10 GI:29803948

KEYWORDS

SOURCE Homo sapiens (human)

ORGANISM Homo sapiens

Eukaryota; Metazoa; Chordata; Craniata; Vertebrata;
Euteleostomi;

Mammalia; Eutheria; Primates; Catarrhini; Hominidae; Homo.

REFERENCE 1 (bases 1 to 10502)

AUTHORS International Human Genome Sequencing Consortium.

TITLE The DNA sequence of Homo sapiens

JOURNAL Unpublished (2003)

FEATURES Location/Qualifiers

source 1..10502

/organism="Homo sapiens"

/mol_type="genomic DNA"

/db_xref="taxon:9606"

/chromosome="12"

ORIGIN

1 tggctcactg aaacctccgc ccctggggtt caagccattc tctgcctta gcctcctgag

61 tagctgggat tacaggggtg tgccaccag cccagctaatt tttgtattt ttagtacaga
121 cagggtttca ccttgttggc caggctggc tcaaattcct gaccttga tccacctgcc
181 tcggcctccc aaagtctag gattacagc gtgagccact gtgcccgcc tttttttt
241 tttttttt ttaggaagt gtatttggg cttttaact agcttgttt ttaattagat
301 tattgccttt aggggtggc ctttaataa aaagggggaa gaaaacatag gtttagggc
361 ctcatattta aatgggtaaa gcaggcatag ctggaaggca gaatacagaa cccccctaat
421 caaggatctc attttatat tgaatcctag gcccccaaa agagggaaat gtcattggac
481 gagatgtgtg gcattttat cgagtcccc actgtaaaga tgctcccca aggctggcag
541 gcagcccagt gccgattagc ccactctgtg cttagtctt tttttttt tttttgag
601 gtggagtctt gctctgttc ccaggctgga gtgcaatggc gtgatctcg ccaatgcaa
661 tctctgtctc tgggttcaa gcgattctc tgcctcagc tccaagtag ctgagattac
721 aggcaccagc cactatgctc agctaattt ttgtatttt agtagagatg gggttcaac
781 atgttgcca ggtgtgtc gaactctga cccaagtga tccgccgcc tcggcctccc
841 aaagtctgg gattacagc gtgagccacc atgcctggcg tcttagcct attttaatg
901 ggagttcat cctcaatgt gtagcttc atgtctta ggtgcccag accatgttt
961 taaaattta aatgcacgaa gaaataagta gcctgtata gtagtaatac tttgttga
1021 ataactgca taagtcatc ctaaaactgt atttttatc tagttattat atatgactag
1081 ctatatgtc agtttttaa ataatacaa gtaattatt tttgcatcc tcaaaaacca
1141 aagagattag gtaatgtag gtagaagaga gcagagctt agacctgaga agaactgcc
1201 catgactcgt gaaactccac aacgaaagta ggagaccca aaaagggt gagtgcac
1261 tttctgaat tttttttt ttttagatgg agtctgtc tgcaccagg ctggagtga
1321 gtggtgcaat ctggctcag cctccgagt agctaggatt acaggcacgc gccaccatga
1381 ccagctaatt tttgtattt tagtagagac agcgttcac catgttgcc aggatggtc
1441 cg^atctcttg acctcgtgat cggccgcct cggcctcca aagtctggg attacaagc

(G/A)(dbSNP:695934)

1501 tgagccactg cactcggccg gtcagataat tttttggcc agttttaca tagagtaatt
1561 ttaggtttta tggctggcct tggggcaaag gggttctggt tttatagct ggtcttgggg
1621 gagaatggaa ccgagtgaca agaggacaag agagggtcag agaaaaactt ctgcttctga
1681 ggcggctatt gaggccttca ttttgagta ttgtcctcta agccccagca gtgtcaaact
1741 gtacacaaac catacacagc agccagctcg ggtgctgta ggaaatggtc tcaactgctgg
1801 gtctgtgggg tatgtgtgtg tctgggtgtg tggctactgt ctgcatcctc ctcccccta
1861 cagcctcccc gcctcccctc cagccaccci gggctgggtg acctcagcc cctcccctca
1921 gctcccctag acctcccag agcctttatc agggagctgg gactgagtga ctgcagcctt

>TAC3-Exon 1(2001-2117 -> 117bp)

1981 cctagatccc ctccactcgg **TTTCTCTCTT TGCAGGAGCA CCGGCAGCAC
CAGTGTGTGA**

2041 **GGGGAGCAGG CAGCGGTCCT AGCCAGTTCC TTGATCCTGC
CAGACCACCC AGCCCC^CGGC**

(T/C)(dbSNP:2291855)

2101 ACAGAGCTGC **TCCACAG**gta ggcaagtggg agaatgctgg atggaccaga
gctggcacca

2161 ggggacagga gccagcgtca ggagggaata aagcagatgg cagcctctga taggggagca

2221 ggggactggg aa^ggtgagca caaagcacct gtagggccga gagctggttg gtgttggag

(G/C)(dbSNP:2291856)

2281 cctgtggcta cagactcatt cttcatacc agaaagtttt tgcctaagtc ttgggattat

2341 ctagtactgg aaaatagcat ccagatccc tctccagct gactgaggaa acagaccagt

2401 ccatgtceta caaatctate atctttcttg ggagctagag tctcctggc accactatag

2461 cattgcacat ctctgggga gatatctgat ggggtagcag gaaactaag cccaagggt

2521 gtaccccctt ctcaaaata cttccacc tctctccaga ccagggttg gacagtggag

2581 ttgggggctg gggaagcagg gtc^{aaagccaa} gctgctggta atgaatgtct cttgtgtctt

2641 caccatgct gtatctctct cttctctct ttacctgagt cctgtccctt tgetctccca

>TAC3-Exon 2(2702-2820 -> 119bp)

2701 gGCACC^■■■■A GGATCATGCT GCTATTCACA GCCATCCTGG
CCTTCAGCCT AGCTCAGAGC

■■■■

2761 TTTGGGGCTG TCTGTAAGGA GCCACAGGAG GAGGTGGTTC
CTGGCGGGGG CCGCAGCAAG

2821 gtaagtctcc cctggcagag tactggggac atcacgggaa cttgggactc tgctgtctg

2881 gcaagvtgta ggggzaaac tggggtgggg gggttgtccg tcagagggca tttgcctcc

2941 ctttgattt cttgtttct ctggctctt catgttcca ctgtctccag gtgtgttgt

3001 gtctctgtat ctctgcatgt cttgacacc ttgtacataa aaggtgccct acaaatatgt

3061 tgtttggtgg gttgattgat gggagacttg gtgattggat ggtactgtga ggggtgagct

3121 aggggtgtct aaggctctct atagtctacc tcaggtccct ttgcaaggga cagatctctt

3181 ctatttctg gatggtatga aacagtcaga atttcttcc caaatggtta tttgtgtct

3241 atttaccta tcagttatgt gtattgttt atttcaaaa tgcaataaa ttccttatc

3301 ttttctcat ccacccccag taacctcagg tgcttctaag atcccaacce cttccttctt

3361 ctctttctc cctgcccgc ctctatctc tgcttagtca ggataggaaa acaacaacag

3421 caaaaaaac agattgagcc tcgattcca cagttccttt acgaaaaaga ataggaattg

3481 tcaggtagg ggtacagggg gaggataggg aggaagtctt ttcaaggttt tgaatgaca

3541 gcaattacat cgttacaat gctttaaga tgattgcggg tgggacttat tacaattca

3601 atgtgtgaag ttaactgcc tctcagctc aaatctgttc agcatctcat tataggaggt

3661 gggcagagta ttcaacaatt tgggaaaagt ggctgcctga acaccacatg ctgggccaag

3721 ggagttatca ccagggcagc cttgcaggtg gcagcagttg tgccatatcc aaaaggccag

3781 aaccgttaa aaaaaaaca cccaggggag tgccaagtat gggctggaca ccgttgag

3841 ccacaaagt ccagcccagg atagttagag tatctgagtt ctctgagac aaactgttt

3901 caagacctg gccaatgaga tgcccctct gccctcttg gtcaatgaat gaga^gggatt

(G/A)(dbSNP:2122694)

3961 gccatctac cccttctct tgagagtctg tgaggatgag ggaaattggg gcaggaagag

4021 ggtagtacct aggtgtgcct aggcaactgg gttggtatgt gtgggggtgt gttctgtga
4081 aatgcacttc tgtgttgca caacagccga aggatgcctg gttctggaa agagaggcgc
4141 tgctgagact tgagattga gatgaaaac tccagccatg atcattgta ttgtctctct
4201 gcagctgcaa ttaactggct gtgtggtg tgcccaccac cctgctgtac gcaagttgct
4261 aaaaaaaaa aaaaaatcac agggacaatc aagagcccgt gctgggcaac agctctagaa
4321 cttgggattc agttgtggag agaagaagac gtgcctctg agcatgtgc ctctctggaa
4381 ttctagacct agggccaaaa gggagaggga gagaaaacta gaggcggaaa gccatggaga
4441 atagagaaag aggtggtgga aaacaggag agaaacatcc atggacatcg tgcagagtgg
4501 gggaatcaca ggtgcagatg tgtgcctcca atccacatc gcatgtgaat cacctggggg
4561 gctgcttaa atgcagattc tgtctcagga ggtctggggg aggaacaaga gtctgcattt
4621 ctaacaggct ctgtgtatg ctggtgtgc tgttggtcca caggtcactc ctggagcacc
4681 actatagct ccagtgtgaa ccagaggaaa ctctgaaaga aatagggtgt cggattcagg
4741 atgggctcag gaagaggctg tttctgtgg gaaaaggatg agtggatccg ggtgggagcc

>TAC3-Exon 3(4833-4926 -> 94bp)

4801 tctgcctca cccctctttg tttctcct agAGGGATCC AGATCTCTAC
CAGCTGCTCC

4861 AGAGACTCTT CAAAAGCCAC TCATCTCTGG AGGGATTGCT
CAAAGCCCTG AGCCAGGCTA

4921 GCACAGtag gaggcggccc taggggagag gggaatgagg ggcaggatic tgaagataag

4981 aggtctgg ga gatecctcca gatgggagag agatgggga tagcttagtg aatcggtag

5041 ggttgtgac tgaacccgc tctcatcact ^ttccaactc actccccatt tagacatcg

(5071)(branch site - YNYRAY)

>TAC3-Exon 4(5116-5145 -> 30bp)

5101 ttcttggtt cacagATCCT AAGGAATCAA CATCTCCCGA GAAACgtaag
tacctcttc

5161 tcctccta tctcttgcca cttgccaga gctctgtggg gcattgggcc caggggcat

5221 ttgtccagc ccttctcac ctggtaaaa caalatgcca gctccc actg ctcagccaac

5281 ctttcctgaa agggagaggc catccagaac taggaggaag ctggtgtgag gggcatggtg

5341 ggctctccct ctgctggctg gtccttgaa aacaagggga tctctctg gacctgaaaa

5401 ttccaaatca ggcacctgct agagcagaaa attctgaaa tgtggaggaa gaaaggtga

5461 gcagagagag tgggttagg ggaggcactt gctaacctgaggagtcattgacaag

5521 aaaaaggaac agagaccaga aaccagtct cagaagtgt gacctatgc tgggagatg

>TAC3-Exon 5(5629-5682 -> 54bp)

5581 cttcacttc tcatcatcac ^tgctgacaat gttggccctt tctgcagGT GACATGCATG

(5601)(branch site - YNYRAY)

5641 ACTTCTTTGT GGGACTTATG GGCAAGAGGA GCGTCCAGCC
AGgtaggagt gttggagggt

5701 acagtggaag ggcttaggt actggcagag tatgacagaa gtcacgtgcc tcatattgt

5761 caccaatggg aagacagga ccttcttac cttcagttag gttcctcgg cccttcac

5821 ccaatcagct tggatccaca gga^aagtctt cctgggaac agaggagcag agaccttat

(G/A)(dbSNP:733629)

5881 aaggtagtc tttgcagct gggaggaagg ataggagac tctgctcca cccagtctc

5941 ccaactctgt ctggaacac tgc^ccgtcat agccagccct ttgctgttg atcagggtg

(T/C)(dbSNP:2270734)

>TAC3-Exon 6(6052-6126 -> 75bp)

6001 agttcacatt cagaaagatc cctcttactt acactgttcg cttacccta gACTCTCCTA

6061 CGGATGTGAA TCAAGAGAAC GTCCCCAGCT TTGGCATCCT
CAAGTATCCC CCGAGAGCAG

6121 AA^■■■■Ggtaa ggattgtca ttagagagg gagaggggac tgggagggg gctgtgggg

■■■■

6181 ttgccagctg tgcattctt cccatgctac aggtattaaa gctcatagat ttgcctgaa

6241 atacactgcc aatgcccage aactgtcgg ccaaacacaa agacacttag aggcactgt

6301 gttgtacac atccccctc tttcatctt ttctctgga tcatggacgg cagctgacta

6361 ttgagcagga gtgagtgtg ggagatgagg agagaggggc tccccgatg ggcaattct
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6481 aattacaaat accacaacca atccacagge aagcatttgc ctcccaggca ggctgagcct
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6961 gcctgagtgg gctgacaaat tctcacttg gtatgcgagt gtaagagtcc cccagggaag
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7141 ggagagggag agagattggg aaggctcgtat atcatggaga gcctcctgct tttctctctg
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7321 tttagtgtg ggtgagaggg cacaagagc tataataact gtaacttct gattacatg
7381 tagtactgt atcatttgc tctcattaga tggttattc agtctgccc acggccagat
7441 aattatacga gcagctatat ctggatgaca tactctgctc cagcgttatg cactggccat
7501 aaagataatt acagtgcaat ttgctatag tattttatac aaatggcaaa aacaagtga
7561 ttgtggaat ctactttta tgcttgttg tcatccagg ctcttcaga gggaccata
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7921 aacttggttc ctacatcttt ggtccacatg ggccccactg gccatgcaca caggtgtgta
7981 gagtaatgta aatatggcag ctgggaaggt gcaagtacct gcggctagga gagttccatc
8041 ctcaggccca aagcctggag ggcaggetga gggtaagac ttgttcttc ctcttcaca
8101 gagcctctc ccttctctc ctgtgccac agcaggtttt cagtgggact ttttacagg

>TAC3-Exon 7(8216-8502 -> 287bp)

8161 ~~ctcagctctc ccttctctc ctgtgccac agcaggtttt cagtgggact ttttacagg~~ gttttttt gttttgttt gtttttgc ctcagTACTC

(8169)(branch site - YRYRAY)

8221 CACTTCCGGA CTCCTGGAC^T ^GCATTAGGAA GACCTCTTTC
CCTGTCCCAA TCCCCAGGTG

(8240-8377)(STS:G11486), (8241-8429)(STS:TIGR-A004P28)

8281 CGCACGCTCC TGTTACCCTT TCTCTCCCT GTTCTTGTA
CATTCTTGTC CTTGACTCC

8341 TTCTCCATCT TTTCTACCTG ACCCTGGTGT GGAAACTGCA
TAGTGAATAT CCCCCACCCC

8401 AATGGGCATT GACTGTAGAA TACCCTAGAG TTCCTGTAGT
GTCCTACATT AAAAATATAA

8461 TGTCTCTCTC TATTCCTCAA CAATAAAGGA TTTTGCATA
TGaatgatgt ggtgtgtg

8521 tttactgtt tgggtggtg gttttctgt tcctgac~~ctc ctcagctac atggtatata~~

8581 cacacatact tatgatacac acattcata ttaaataa aataactta catatcttt

8641 tgtatatatc tatttctga acagtgcctt acacagtgtc tgcacgatg agtatcagat

8701 ttatttagt attaaaataa atacacgaat ttggaagatg gtttctaaca cacaaagatt

8761 ttacagacc agtttagat aaagaaaaa caggccgggc ccggtggctc acgctgtaa

8821 tcccagcact ttgggaggcc gaggcgggtg gatcagagg tcaggagtc gagaccagcc

8881 tgaccaacat ggtgaaacc ctctctact aaaaataca aaattagcca ggcattggtg

8941 cgcattcctg taattccagc tacttgggag gctgaggcag gagaatcgtt tgaaccagg

9001 aggcaggggt tgactgagc cgagatcac cactgcact ccagcctggg caacaagagc

9061 aaaaaactcc gtctcaaac aaaacaaca acaaaaaaa acaataaaa aagaaaaaga
9121 aaaagaaaa aaatattcag aatgacttgt attactagga tgggtctggg agatattcat
9181 tctgaaact gaccctact aattagagaa ggaggtgggg atcaaggctg tccggagacc
9241 cagccacaga ggaggacaaa tctatgacc tatacaattt tttgtctcc aaatgctgag
9301 cctgggttct gtgacagatc ctggggatga aatgatgact catacacaga gtttacagt
9361 tagcagggtc gtggacaagc aaacagaact tgatccagct aggatgggat gtggacaggg
9421 aagttactac cgaggccaag aaagagagga gcagatatct tcaccgtaa ctggctgct
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9661 ttctgtacc taatggaaca gccagtgaaa tctcaggct cttatctta gcgtgtaca
9721 ggggcctttg ttatgccct gaattgcact gataaacat caacacatag atttccaag
9781 gcagtgaag gacaggcca cagagccaga ggccacttc tgcagtcctt tcattctagt
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10081 ggctttctct gggcatgaa agctacaaga gttggttctg ctgacttg gtgggagta
10141 ggcttatagg ctgagatgag acaattgctt tgcaagtagg aacattaagt gcagaaagat
10201 tgctctctag tgggactgac aaaaattgca gtactgggga ctccagaaaa aaatgaagac
10261 aaatgtaag ttagattcct gtgtttgtac ttgaagaatg tgtgaaggga tctgaccct
10321 cccttctctg ttgtaaaaca gttgatgctt aaagatatct ggtccacaag acctgacta
10381 aattcctggc cctttctct ccatttaact ttgtatatgt ttgtattgt gactatatg
10441 tgattactt taaaagact tcagtataag tggatatac tttcacctgc gtcttttga
10501 tg

**Key: exon = light grey highlight; start and stop codons = [redacted];
primers = grey.**

Appendix F

TACR3 Gene Annotation

Annotated using mRNA sequence:

LOCUS NM_001059 1755 bp mRNA linear PRI 20-DEC-2003

DEFINITION Homo sapiens tachykinin receptor 3 (TACR3), mRNA.

Annotated sequence file:

LOCUS NT_016354 136349 bp DNA linear CON 19-FEB-2004

DEFINITION Homo sapiens chromosome 4 genomic contig.

ACCESSION NT_016354 REGION: complement(29002755..29139103)

VERSION NT_016354.16 GI:37539910

KEYWORDS .

SOURCE Homo sapiens (human)

ORGANISM Homo sapiens

Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
Mammalia; Eutheria; Primates; Catarrhini; Hominidae; Homo.

REFERENCE 1 (bases 1 to 136349)

AUTHORS International Human Genome Sequencing Consortium.

TITLE The DNA sequence of Homo sapiens

JOURNAL Unpublished (2003)

FEATURES Location/Qualifiers

source 1..136349
/organism="Homo sapiens"
/mol_type="genomic DNA"
/db_xref="taxon:9606"
/chromosome="4"

ORIGIN

```

1 gcaggagaat caggtaggga ggttcagtg agccgagatg gtggcagtac agtccagctt
61 cggctgggca tcagagggag accgtggaga gagagggaga gggagacggt ggggagaggg
121 agacggtggg gagagggaga gggagagggg gagggagagg gctaaagtac tttcatctca
181 ggacttctca gatcctttt ttccttcta ttcttaatg tgaaaaaata ctgctagact
241 ctaggcttca agaatagaat tcatatataa ctgcttcatt gctatactcc cagtatctgg
301 catagcacct gaaagaagt atgctaatac atagctaaga aatgatagac aatgaattat
361 cactgatccc tcttgggctc aaggagatt ctcagtaggt aaggcagatt gagaaggaaa
    
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421 gccatgcctt gaatgcctcc cttcctctga aaggaataaa ttttaaact tctttcatta
 481 tatctaattc catcattatc cttttttcc ttgtcatatc cctctactt tgtataagcc
 541 caatgaaggc aaaactgaaa aattaaatg tttgttata gtattacaag gaaagcaaca
 601 gtaaatgag tgtatttcta cacaactaag taattttca gcttcaaaaa tctgatcaaa
 661 acatgcttaa ttaccaaacc accacataac ctattttgta attttgagta agcaggtgtg
 721 tcatatggag atatattttt aatgtcttc taattgcatt gggaaaggaa gaggtttca
 781 tttgtaaca gcaatttcta gatgatgaat tagaacaag aagtaatctt tcaaaagaaa
(continuous)

2821 gcaggagcct cagttctcca gcctgtgccg ggcagctggc accctccta ^cgtgtgatgt
 (G/C)(dbSNP:3733631)

2881 gactcgataa cccttaatct cgggaggatc tcattcttcc tgcctgcccag ac>ccgcaagc

2941 gcctctgaga aagtgcagct gttgccctt agccccagct gcatc^cccta accagcaggg
 (2986)(branch site - YNYRAY)

>TACR3-Exon 1(3001-3689 -> 689bp)

3001 ATTGCAGTAT CTTTCAGCTT CCAGTCTTAT CTGAAGAC^TC
 CGGCACCAAA GTGACCAGGA

(T/C)(dbSNP:3733632)

3061 GGCAGAGAAG AACTTCAGAG GAGTCTCGTC TTGGGCTGCC
 CGTGGGTGAG TGGGAGGGTC

3121 CGGGACTGCA GACCGGTGGC G^█ GCCACT CTCCCAGCAG
 CAGAAACCTG GATAGACGGG

█
 3181 GGTGGAGGCG TGGGTGCAGA CGCCG TGAAC CTGACCGGCT
 CTTCTA>GCTGC CGGGGCGGCC

3241 ACGGGGG<CAG TTGAGACTGC GTGGCTGCAA CTGCTGGACC
 AAGCTGGCAA CCTCTCCTCC

3301 TCCCCTCCG CGCTGGGACT GCCTGTGGCT TCCCCGCGC
 CCTCCCAGCC CTGGGCCAAC

3361 CTCACCAACC AGTTCGTGCA GCCGTCCTGG CGCATCGCGC
 TCTGGTCCCT GGCATATGGT

3421 GTGGTGGTGG CAGTGGCAGT TT^TGGGAAAT CTCATCGTCA
 TCTGGATCAT CCTGGCCAC

(3443-3620)(STS:RH70865)

3481 AAGCGCATGA GGACTGTCAC CAAC TACTTC CTTGTGAAAC
 >TTTCTC CGACGCCTC<
 3541 ATGACCGCCT TCAACAGCTT GGTCAATTTC ATCTACGCGC
 TTCATAGCGA GTGGTACTTT
 3601 GGCGCCAACT ACTGCCGCTT CCAGAACTTC TTTCCTATCA
 CAGCTGTGTT CGCCAGCATC
 3661 TACTCCATGA CGGCCATTGC GGTGGACAGg tgaggagagg acagacagag
 aggaaagagg
 3721 gagaagggaa agaaaagaac tggcggtgg gataagatta caaaggaaat agtaacgtaa
 3781 cggggtctat aaaagtaaag agactaagaa ttttctga aaggaggaca ctaacc<eat
 3841 ttagccctc gagtggctga acaagttct gcttccgtgg cctccaacac tcaagctca
 3901 aatcattgag cttattaag gatattaga gggactgaag aaggtgaatg agggacaggt
 3961 gaaaatgcc ttcaaagtag ggcaaactta gctagaaacg ttaag^tacc agcaaacttt
 (T/C)(dbSNP:3796950)
(continuous)
 64261 tacgctgct ctactcaagc agtccttacc aactggtt^c ccaaagagc aatgactgta
 (A/C)(dbSNP:10516505)
 64321 actattgaaa cgccttga atacttga actccattt caaactctgt tataaagaaa
 >TACR3-Exon 2(64414-64602 -> 189bp)
 64381 accaatgaaa tggtttgg ttgtttata tagGTATATG GCTATTATTG
 ATCCCTTGAA
 64441 ACCCAGACTG TCTGCTACAG CAACCAAGAT TGTCATTGGA
 AGTATTTGGA TTCTAGCATT
 64501 TCTACTTGCC TTCCCTCAGT GTCTTTATT CAAAACCAA
 GTCATGCCAG GCCGTACTCT
 64561 CTGCTTTGTG CAATGGCCAG AAGGTCCCAA ACAACATTTC
 ACgtaagta attctctatt
 64621 atggtttca atcagttta tcaaacttt agaaaactac agattaggag caac<aattaa
(continuous)
 65281 taacaaatgg tgcaaa^taat ggtgcccatt taaagcagtc atttatcta cctttgctct
 (T/G)(dbSNP:3822290)
 65341 acccaaagt cagattttg aacaaaata ttaggaggca cttattaag gaaaatcaga
 65401 cagctcatat taaactgagt accctattca tattattaat acatcttaatt tgtatgataa
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65521 cttagtaatc tactgagaaa tggaaaggca aggatattta agattcactt tacaaaaaac

65581 aaaacaaaa^c aaaaaaaa^ca ctggtatgga gaagaccgtt ctaacaaact ttttaattac

(A/-)(dbSNP:11445973), (C/-)(dbSNP:3839188)

.....(continuous)

66361 atgtagaaat aactcttggg agttttaatt atgatttaaa gctgagccaa tttagt^c cc

(66417)(branch site - YNYYRAY)

>TACR3-Exon 3(66473-66623 -> 151bp)

66421 aatatttcat tccaataagg tcttcagcat gtgttttct tatttttcat agTTACCATA

66481 TTATCGTCAT TATACTGGTG TACTGTTTCC CATTGCTCAT
CATGGGTATT ACATACACCA

66541 TTGTTGGAAT TACTCTCTGG GGAGGAGAAA TCCCAGGAGA
TACCTGTGAC A^AGTATCATG

(G/A)(dbSNP:2276973)

66601 AGCAGCTAAA GGCCAAAAGA AAGgtactgg tccatgtgt ttacctagca
tttgtatagg

66661 ttatggtata tcagaaagaa aagcaatcct gtcctggcct gtaatacaa tatgatctga

.....(continuous)

131041 ttaactgtat tggcagaaaa gcatgatttg caataagcta ttggaactat tatgagtatt

>TACR3-Exon 4(131134-131330 -> 197bp)

131101 atatttgta aaaaatgactt tttttctta tagGTTGTCA AAATGATGAT TATTGTTGTC

131161 ATGACATTTG CTATCTGCTG GCTGCCCTAT CATATTTACT
TCATTCTCAC TGCAATCTAT

131221 CAACAATAA ATAGATGGAA ATACATCCAG CAGGTCTACC
TGGCTAGCTT TTGGCTGGCA

131281 ATGAGCTCAA CCATGTACAA TCCATCATC TACTGCTGTC
TGAATAAAAAG gtaaaaacaa

131341 aacta^cgaaa tgcaagtgc ttgtcacacc caacttcaact ggaaacatac aatggtgttc

(T/C)(dbSNP:6822961)

131401 cattttcttg gtcaaattc aaaatgcaga aggaaggaa aatttaagaa ttactgaga

131461 gaggcacagg ctttatcttt caagaccccc atacacactt tataaagcat cccacattaa

131521 ta^attagatg ccccaaataa tgatcttggg gttctaactg gatgattctt ctatgggcca

(G/A)(dbSNP:6847994)

131581 acttcttctc ccaaaatctc agaattttac ctgccaaaga aaaagaagtg cccctcatgat

.....(continuous)

132601 ctgggaatca atgcttctca ttaatgggaa attaagattc aattcaaaca agaactaaac

132661 gactaacaac agtactgtga cataaattct aagagtctgg ctaaaaacca aactcaaaaa

132721 ctgagttgtt ataactgcct taagtagaat ttctgtggtc agctgaaaga catatctgct

>TACR3-Exon 5(132823-133349 -> 527bp)

132781 tgaaaaaata acttttctt tctgtggcct gcttttctc agATTTCGAG CTGGCTTCAA

132841 GAGAGCATTT CGCTGGTGTC CTTTCATCAA AGTTTCCAGC
TATGATGAGC TAGAGCTCAA

132901 GACCACCAGG TTTCATCCAA ACCGGCAAAG CAGTATGTAC
ACCGTGACCA GAATGGAGTC

132961 CATGACAGTC GTGTTTGACC CCAACGATGC AGACACCACC
AGGTCCAGTC GGAAGAAAAG

133021 AGCAACGCCA AGAGACCCAA GTTTCATGGT CTCC TCTGGC
AGCAATGCCA ATCTGCTC

133081 CGCCACTTCA AGTTTCATAA GCTCACCTA TACCTCTGTG
GATGAATATT CT^■■■■■TTCCA

133141 TTTCTGAGG TAAAAGATTA ^GTGTGAGACC ATCATGGT^GC
CAGTCTAGGA CCCATTCTC

(133161-133273)(STS:WI-9174), (133179-133328)(STS:WI-18877)

133201 CTATTTA^CCA GTCCTGTCCT ATATACCCTC TAGAAACAGA
AAGCAATTTT TAGGCAGCTA

(T/C)(dbSNP:2765)

133261 TGGTCAAATT GAGAAAGGTA GTGTATAAAT GTGACAAAGA
CACTAATAAC ATGTTAGCCT

133321 CCACCCAAA TAAAATGGGC TTAAATTTa ttcttgaaa actctaaatt
attatatgca

133381 atgaacaaaa atatgtcagg aaaatacttg taaactgccc agtctatctc attfacaat

133441 tgcaatatac atttgtgaca ttaaaatgat atatagttt tccaagagat taaagaatct

133501 ttaaacata atattgtaag tgaagggaaa caaaatctgt ataaaatctg tataaaatag

133561 gcttttttgc ctagagatat aaaatgggaa aaaaagttaa atgattcatt ttcccactag

133621 agtaatggaa aattaacctc aagaagtagg aactgaaaat ctttgctcaa gaaaactcat

133681 tttgtagaat gtaaagattt aaagatttaa agatttcttc catggcattt aactgaaaag

133741 gaaatctagt taaatcatta ggcaaatgta tatttcattc tcttgattt tttctgaga

133801 aagtgaaatt taaaaaatt atatacttt tgaccattac ttattttga ataccaagct
133861 aaaaagtagt cagttaagac cctaaaaata tgaagaaat tcctatcatt tttattttt
133921 cacagatttt ttaaatagct agattataaa acagtaaagg tacattcaa taaatagagg
133981 aagaatacta tttatgttct cttcctctcc cctcactgaa aatagaataa ggactaggag
134041 aaaatatctt caaaggcaac tccttaggga aaaaaagtca caagtgtgac aaaag^actat
(A/C)(dbSNP:7438251)
134101 atggttgaaa aataccaata tatgttcat atgagtaaac taggcaaggt gtagacaagt
.....(continuous)
135841 atcagaaaaa ttcaaaatat ttttaaaca atataaaaga atcaaaaagg cttacatcat
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(A/C)(dbSNP:4642251)
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(G/A)(dbSNP:6533099)
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136261 acaaaataaa tgtgaaaaat aaatgctggc cagctcaaca tttgaattta gttttgcaa
136321 atgcaaaatt tcctctaaaa atttatgat
//

Key: exon = light grey highlight; start and stop codons = black highlight; primers = grey.

Appendix G

Recipes and Protocols

PCR primer dilutions

20.0 μ l 100pmol/ μ l stock

180.0 μ l dH₂O

200.0 μ l 10pmol/ μ l primer

dNTP mix

5.0 μ l each stock solution (10mM G, A, T, C)

980.0 μ l dH₂O

1000.0 μ l

PCR Protocol

Key: F = Forward

R = Reverse

5.0 μ l 10xbuffer

10.0 μ l dNTPs

3.0 μ l MgCl₂

1.5 μ l F primer

1.5 μ l R primer

0.1 μ l BioTaq

1.0 μ l DNA

50.0 μ l

PCR cycle conditions

95°C 2 min.

94°C 30 sec.		x 40 cycles
T _a 15 sec.		
72°C 1 min.		

72°C 5 min.

Multiphor Solutions

3.4% C, 40% stock

58.8g Acrylamide

1.2g bis-Acrylamide

Make up to a final volume of 200ml with distilled water.

0.75M Tris Formate buffer

90.8g Tris

pH to 9.0 with Formic acid

Tris Borate buffer

125.9g Tris

17.3g Boric acid

Make up to 950ml with distilled water and pH to 9.0.

Add 50µl of 4% Bromophenol Blue solution and make up to a final volume of 1L with distilled water.

41% Glycerol

41ml Glycerol

Make up to final volume of 100ml with distilled water.

10% Ammonium Persulphate

1g APS

10ml SABAX water

Restriction Gel Mix

5.3ml 3.4% PAGE mix
8.5ml Tris formate buffer
3ml 41% glycerol
200µl APS
20µl TEMED

Loading dye

47.5ml 95% Formamide
0.16g 100mM NaOH
0.125g 0.25% Bromophenol Blue
0.125g 0.25% Xylene Cyanol
Make up to a final volume of 50ml with distilled water.

Silver Staining Solutions

Fixing Solution **per 300ml**

Ethanol	30ml
acetic acid	1.5ml
distilled water	268.5ml

Staining Solution **per 300ml**

Silver nitrate	0.3g
Distilled water	300ml

Developing Solution **per 300ml**

Sodium hydroxide	4.5g
Distilled water	300ml
Formaldehyde	1215µl

Appendix H**Table of candidate genes already screened in study patient cohort.**

Sample No.	MTHFR C677T	MTHFR A1298C	AGT M235T	eNOS G298A	Prothro G20210A	fVL G1691A
1	1	1	3	2	1	1
2	2	1	3	2	1	1
3	1	1	3	1	1	1
4	2	2	3	1	1	1
5	1	1	3	2	1	1
6	1	1	3	1	1	1
7	1	2	1	2	1	1
8	1	1	3	1	1	1
9	2	1	2	1	1	1
10	1	2	2	1	1	2
11	1	2	3	1	1	1
12	2	1	3	1	1	1
13	1	1	3	1	1	1
14	1	2	3	1	1	1
15	1	1	3	1	1	1
16	1	1	2	1	1	1
17	1	1	3	1	1	1
18	1	1	3	1	1	1
19	1	2	3	1	1	1
20	1	2	3	1	1	1

Key: AGT = Angiotensinogen; eNOS = endothelial nitric oxide synthase; Prothro = Prothrombin; fVL = factor V Leiden.

1 = - / - (homozygous wild-type, no mutant allele); 2 = + / - (heterozygote status);

2 = + / + (homozygous mutant).

Ten of these patients belonged to the Group H and the other ten were from the Control group (as previously described).

Appendix I

These data have provisionally been presented at the:

- i) South African Genetics Society conference (April 6-9th April, 2004) and the
- ii) University of Stellenbosch Health Faculty's "Academic Yearday" (August 19th, 2004) and

Data will be presented at the forthcoming:

- iii) International Society for the Study of Hypertension in Pregnancy (ISSHP) in Vienna, Austria (November 14-17th, 2004) and
- iv) South African Society for Human Genetics (SASHG) conference scheduled for April, 2005.
- v) A manuscript describing the findings of the study and incorporating the biochemical measurements of NKB is currently in preparation.