Characterisation of novel *TAC3* and *TACR3* gene variants and polymorphisms in patients with 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.

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Signature Date

Abstract

In South Africa, pre-eclampsia is the second highest cause of maternal deaths. The incidence of this disease in the Western Cape alone is 6.8% and places a large burden of health care facilities. The placenta and implantation thereof is thought to play the most significant role in the onset of this disease. Among the many theories for its aetiology, is the acknowledged two - stage theory. This is based on evidence that pre-eclamptic placentas demonstrate altered remodelling and invasion into the uterine endometrium and myometrium. The sub-optimal endometrium invasion leads to less oxygenation of the placental environment causing transient hypoxia. Consequently, the placenta is thought to release unknown factors into the maternal circulation which then culminates in clinical features associated with pre-eclampsia. Neurokinin B is thought to be one of these placental factors and subsequently binds to the NKB receptor in the maternal system. Endothelium-derived nitric oxide synthase has recently been shown to activate this receptor.

The aim of this study was to investigate the role of neurokinin B (*TAC3*) and the neurokinin B receptor (*TACR3*) genes in the predisposition of pre-eclampsia and their interaction with eNOS in the South African coloured population together with a matched control cohort.

Fifty infant samples were screened to complete a mother/cordblood pilot study. The maternal sample cohort was subsequently extended to include 124 patients and a cohort of ethnically matched controls. Additionally, 54 patient samples also had NKB levels measured by radioimmunoassay. Samples were genetically screened using multiphor SSCP/HD analysis followed by automated sequencing to confirm variants. Where possible, restriction enzyme analysis was utilised to genotype identified polymorphisms in the patient and control cohorts.

Three novel variants were identified in the *TAC3* gene in the course of this study: an intronic IVS-53g/t transversion, an exonic 295t/c (Ser99Pro) transition and a 479t/c transition in the 3' UTR.

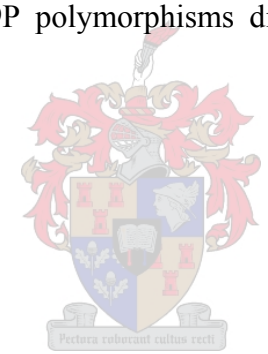
Additionally, four documented SNPs were identified in the *TAC3* and *TACR3* genes. A -25c/t transition was identified in the 5'UTR of the *TAC3* gene. In the *TACR3* gene, a -103t/c transition was identified in the 5'UTR, a amino acid substitution, R286K, was evident in exon 3 and a +75t/c variant was identified in the 3'UTR of the gene. No statistical significant difference was identified in

patient and control groups' allele or genotype frequencies.

None of the variants demonstrated an association with the susceptibility to pre-eclampsia when genotype-phenotype comparisons were performed. However, the *TAC3* -25c/t polymorphism was associated (p=0.02) with lower circulatory NKB levels and the S99P variant demonstrated association (p=0.043) with infant birthweight below 1000g.

In the analysis of interaction between *TAC3*, *TACR3* and *eNOS* alleles, there was a significant difference between patients and controls for the *eNOS* Glu298Asp **g/t**, *TAC3* **c/c** and *TACR3* **t/c** combination. This combination, which was more prevalent in the control cohort, is thought to exercise a protective effect.

Although none of the polymorphisms identified showed an association with the susceptibility to pre-eclampsia, the *TAC3* -25c/t and S99P polymorphisms did show association with aspects of the disease.



Opsomming

Pre-eklamsie is die tweede grootste oorsaak van maternale sterftes in Suid-Afrika. Die siekte toon ‘n voorkoms van 6.8% in die Wes-Kaap alleen en plaas gevolglik ‘n groot las op die provinsiale gesondheidsfasiliteite. Alhoewel die oorsaak van pre-eklamsie nie bekend is nie, geniet die sogenaamde twee-stap hipotese die meeste erkenning. Volgens hierdie hipotese toon pre-eklamptiese plasentas ‘n afname in die mate van indringing in die baarmoederwand wat lei na sub-optimale plasentale suurstof konsentrasies. Gevolglik mag die plasenta deur stadiums van suurstof tekort gaan wat mag lei tot die afskeiding van onbekende faktore. Die afskeiding van hierdie faktore manifesteer as die simptome van pre-eklampsie. Een van die faktore mag die aktivering van Neurokinin B reseptore deur endothelium stikstof oksied (*eNOS*) wees wat Neurokinin B binding tot gevolg het. In hierdie studie is die rol van neurokinin B (*TAC3*) en die neurokinin B reseptor (*TACR3*) in die predisposisie vir pre-eklampsie, asook die rol van *TAC3* en *TACR3* interaksie met *eNOS* in die predisposisie vir pre-eklampsie ondersoek.

In die studie is 50 naelstring monsters geselekteer vir preliminêre studies, waarna 124 maternale monsters sowel as kontrole monsters geselekteer is vir verdere studie. Vier en vyftig monsters se NKB vlakke is gemeet deur radio-immunopeiling analise. Genetiese variasie in die *TAC3*, *TACR3* en *eNOS* gene is ondersoek deur enkelstring konformasie polimorfisme/ hetroduplex (ESKP/HD) analise. Genetiese variasie is bevestig deur DNS volgorde peiling tesame met restriksie ensiem vertering waar moontlik.

In hierdie studie is drie nuwe genetiese variante in die *TAC3* geen lokus geïdentifiseer: ‘n introniese IVS-53g/t transversie, ‘n koderende 295t/c (Ser99Pro) en ‘n 479t/c transisie in die 3’ *TAC3* geen volgorde. Vier voorheen gedokumenteerde DNS variante is voords in die *TAC3* en *TACR3* gene geïdentifiseer. In die 5’ *TAC3* genetiese volgorde is ‘n 25c/t basispaar transisie is opgemerk, terwyl ‘n 5’ -103t/c en ’n 3’ +75t/c genetiese variasie opgemerk is in die *TACR3* geen volgorde. Daar is ook ‘n aminosuur transisie in die *TACR3* ekson 3 (R286K) gevind

Hierdie studie het geen statisties betekenisvolle verband tussen genotipe en alleel frekwensies tussen die pasiënt en kontrole monsters aangetoon nie. Statistiese betekenisvolle assosiasie was ook nie aangetoon tussen die geïdentifiseerde genetiese volgorde variante en vatbaarheid vir pre-eklampsie nie. Voords is betekenisvolle assosiasie (p=0.02) tussen die -25 t/c TAC3 variant en ‘n laer vlak van NKB aangetoon, asook tussen die TAC3 Ser99Pro aminosuur variant en babas met ‘n geboortegewig

laer as 1000g (p=0.043). Die eNOS Glu298Asp g/t; TAC3 c/c en TACR3 t/c alleel kombinasie was teenwoordig in ‘n hoer statistiese betekenisvolle frekwensie in die kontrole groep wat mag aandui dat hierdie alleel kombinasie ‘n beskermende effek teen pre-eklampsie mag hê. Dus, alhoewel geen variasie wat die vatbaarheid vir pre-eklampsie verhoog gevind kon word nie, kon variante in die TAC3 geen (-25 c/t en S99P) wat assosiasie aantoon met sommige aspekte van pre-eklampsie wel aangetoon word.

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List of Abbreviations

~	approximately
%	percentage
°C	degrees Celsius
3’	3’prime
5’	5’prime
µl	microlitre
µmol/l	micromole per litre
µM	micromolar
@	at
a	adenosine
A	Asparagine
ACE	angiotensin-converting enzyme
AGT	angiotensinogen gene
AIDS	Acquired Immune Deficiency Syndrome
APS	ammonium persulphate
ASSP	Alternative splice site predictor
AT	Angiotensin
β	beta
BLAST	Basic Local Alignment of Sequences Tool
bp	base-pair
c	cytosine
C	cysteine
CBS	cystathionine β- synthetase gene
CNS	central nervous system
dbSNP: rs	database single nucleotide polymorphism: reference sequence
dH_2O	distilled water
dHPLC	denaturing High Performance Liquid Chromatography
DNA	deoxyribonucleic acid
dNTPs	2’-deoxy-nucleotide-5’-triphosphates
EDTA	ethylenediaminetetraacetic acid

eNOS	endothelium-derived Nitric Oxide Synthase
ER α	estrogen receptor alpha
ER β	estrogen receptor beta
F	forward primer
F2	prothrombin gene
FVL	Factor V Leiden variant
g	gram
g	guanosine
G	glutamic acid
GFP	green fluorescent protein
hCG	human chorionic gonadotrophin
HCl	hydrochloric acid
HD	heteroduplex analysis
HELLP	haemolysis, elevated liver enzymes and low platelets syndrome
HLA-G	human leukocyte antigen-g
IDT	Integrated DNA Technologies
ILs	interleukins
ISSHP	International Society for the Study of Hypertension in Pregnancy
IUGR	intrauterine growth restriction
IVS	intervening sequence
K	Lysine
kb	kilobases
l	litre
LOD	logarithm of odds
M	moles
mg	milligram
$MgCl_2$	magnesium chloride
mg/ml	milligram per millilitre
MHC	major histocompatibility complex
min	minutes
ml	millilitre
mm	millimetre
mM	milli-molar

mmHg	millimetre of mercury
mmol/l	milli-moles per litre
MMPs	metalloproteinases
mRNA	messenger ribonucleic acid
MTHFR	methylenetetrahydrofolate reductase
n/a	not applicable
NaCl	Sodium Chloride
NCBI	National Centre for Biotechnology Information
ND	none detected
ng	nanogram
NK_1	NKB receptor 1
NK_2	NKB receptor 2
NK_3	NKB receptor 3
NKA	Neurokinin A
NKB	Neurokinin B
NO	nitric oxide
NOS	nitric oxide synthase
p	short arm of chromosome
PAGE	polyacrylamide gel electrophoresis system
PAIs	plasminogen activator inhibitors
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PE	pre-eclampsia
pH	potential of hydrogen
PIGF	placental growth factor
pmol	picomole
PPTP	preprotachykinin B
q	long arm of chromosome
R	reverse primer
R	Glycine
RE	restriction enzymes
REA	restriction enzyme analysis
RIA	radioimmunoassay

rpm	revolutions per minute
s	seconds
S	Serine
sFlt-1	Soluble fms-like tyrosine kinase 1
SDS	sodium dodecyl sulphate
SNPs	single nucleotide polymorphisms
SP	Substance P
SSCP	single strand conformation polymorphism
STS	sequence tagged site
t	thymine
T	threonine
T_a	annealing temperature
TAC3	NKB gene
TACR3	NKB receptor gene
Taq	*Thermus aquaticus*
TBE	tris-borate/EDTA
TDT	transmission disequilibrium test
TEMED	N, N, N' N', -tetramethylethylenediamine
THBD	thrombomodulin
T_m	melting temperature
TNF	tumour necrosis factor
U	units
UTR	untranslated region
UV	ultraviolet
V	volts
VEGF	vascular endothelial growth factor
VLDL	very low density lipid

1 INTRODUCTION

1.1 Pre-eclampsia

1.1.1. Definition and classification

Pre-eclampsia is not simply pregnancy-induced hypertension, but a multisystemic disorder, unique to humans, which is difficult to define and has become known as "the disease of theories". In South Africa, this disorder and other pregnancy related hypertension disorders, represent the second highest cause of maternal deaths (20.7%), with Acquired Immune Deficiency Syndrome (AIDS) as well as other non-pregnancy related infections, taking the lead (Saving Mothers Report 2002-2004). Worldwide, pre-eclampsia is responsible for approximately 50 000-100 000 maternal and ~300 000 perinatal deaths annually (Baker and Kingdom, 2004).

Although pre-eclampsia is clinically recognised by the onset of hypertension and loss of protein in the urine, the disease is phenotypically diverse. As a result of different disease classification systems, as well as different population groups studied, limited data are available to estimate the true incidence of pre-eclampsia despite the fact that studies have been performed worldwide (Baker and Kingdom, 2004).

Internationally, different populations receive different standards of health care and this affects the reported incidence of hypertension in pregnancy (Savitz and Zhang, 1992; Irwin *et al.,* 1994; Knuist *et al.,* 1998). At Tygerberg referral hospital in the Western Cape, the incidence for all forms of pre-eclampsia (late, and early onset; mild and severe) is ~6.8% for non-Caucasian mothers. In the same population, the incidence for early onset severe pre-eclampsia is ~3.6% (Hall *et al*., 2006).

Davey and MacGillivray (1998) devised one of the most commonly used classifications: blood pressure exceeding 140/90mmHg measured on two separate occasions at least four hours apart, occurring only after 20 weeks of pregnancy, coupled with significant proteinuria [defined as measurements of >300mg protein/l in a 24 hour urine specimen or at least +2 on a diagnostic strip on two separate occasions, at least four hours apart]. Generally, the more severe outcomes are due to early onset of the disease, usually between 20 and 34 weeks of gestation.

Due to complications in pre-eclampsia that can be caused by the co-existence of HELLP syndrome (hemolysis, elevated liver enzymes and low platelet count) or abruptio placentae, (premature detachment of the placenta from uterus wall), diagnosis and patient stratification has to be strict in order to obtain the clearest aetiological picture of the disease, and for certain genetic analyses, respectively.

1.1.2. Pathophysiology

The onset and progression of pre-eclampsia are unpredictable and little is known about the aetiology and pathogenesis of the disease (Talosi *et al*., 2000). Table 1 is a summary of the 'concepts' of pre-eclampsia. Among these is the theory proposed by Redman and Roberts (1993) which suggests that pre-eclampsia is a two-stage disorder; this will be dealt with in detail later. Poor placentation and subsequently placental ischemia are thought to be the primary instigators (Redman and Roberts, 1993). Factors such as abnormal implantation, excessive placental size, microvascular disease derived from pre-existing hypertension and diabetes (Combs *et al*., 1993) are thought to result in poor placental perfusion and predispose pregnancies to pre-eclampsia.

Table 1: Summary of proposed causes of pre-eclampsia (taken from Talosi *et al*., 2000).

Concept	Basis of theory
Placental ischemia	Hypoxia/reperfusion in the placenta initiates local oxidative processes and leads to release of factors that consequently cause endothelial damage.
Hyper/dyslipidemia	High serum lipid (VLDL) levels with insufficient antioxidant activity may lead to oxidative processes and consequently to endothelial damage.
Immune maladaption	The placenta may lead to immune processes due to insufficient immune tolerance of the fetus, viz, the release of cytokines, and consequently endothelial damage.
Genetic imprinting	The development of pre-eclampsia may be based on a single recessive gene or a dominant gene with incomplete penetrance.
Mitochondrial defects	Invasion of cytotrophoblasts into the maternal endometrium is a highly energy consuming process. This process may be incomplete in case of a mitochondrial defect.
Disturbance of the invasion of the placental extravillous cytotrophoblasts	Histological observations confirmed incomplete invasion of cytotrophoblasts to the maternal endometrium. This failure may be secondary to any predisposing factor.

1.1.3 Possible Predisposing Factors

Various studies have been performed on pre-eclampsia resulting in numerous outcomes, but as yet, no single disease-marker has been identified. However, some factors have been recognised to contribute to the predisposition of this disorder. These factors may be molecular and/or influenced by genetics as well as environmental factors. Table 2 represents some of the predisposing factors as well as environmental contributions, independent of molecular and genetic mechanisms.

Table 2: Summary of factors possibly predisposing to pre-eclampsia (taken from Talosi *et al*., 2000).

Factors independent of genetic and molecular mechanisms	Poor socio economical conditions Primiparity Young age of the mother Maternal stress Low birthweight (prematurity) of the mother
Factors which may have molecular relationships and may be influenced by inheritance	Previously existing hypertension Diabetes mellitus Clotting abnormalities Hyper-/dyslipidemias
Susceptibility factors which are possibly influenced by genetics	Pre-eclampsia genes? Involvement of mitochondrial dysfunction Interactions between maternal and fetal HLA genes Genetic variability of the renin-angiotensin system Genetic variability of endothelial nitric oxide synthase

1.1.4 Molecular and Genetic Pre-disposing Factors

Various molecular and Genetic factors contribute to pre-eclampsia. A selected few include:

a) Previously existing disease

It has been shown that women with a medical history of certain underlying diseases have an increased risk of developing pre-eclampsia. A recent study showed that women who suffer from chronic hypertension have an 11-fold higher risk of developing pre-eclampsia compared to normotensive patients (Samadi *et al*., 2001). The same applies to patients whom have diabetes mellitus. Garner *et al*., (1990) reported a study in which diabetic women had a 9.9% increased risk of developing pre-eclampsia. Thrombophilia, whether acquired or inherited can

also be associated with an increased risk although this evidence is contradictory (Baker and Kingdom, 2004).

b) Clotting abnormalities

Inadequate placental perfusion may be caused by intervillous or spiral artery thrombosis and therefore associate with pre-eclampsia (Kupferminc *et al*., 1999). Thrombotic lesions are characteristic of pre-eclamptic placentas and patients have been shown to have increased coagulation ability. Platelets are an integral part of the coagulation cascade and show decreased levels in full blood counts of pre-eclamptic patients (Lachmeijer *et al.,* 2002).

Genes that are therefore likely to be candidates for pre-eclampsia susceptibility include those that have been involved in thrombophilia and coagulation pathways.

Extensive studies have been performed on the methylenetetrahydrofolate reductase (*MTHFR*) variants C677T and A1298C and factor V Leiden (*FVL*) variant G1691A; these results have however, proven to be very controversial. Most of these studies have failed to prove an association between these variants and pre-eclampsia (Lachmeijer *et al.,* 2002). Differing diagnostic criteria for defining pre-eclampsia, combined with the inconsistencies thereof, could lead to these conflicting results. Combining late and early onset forms of the condition and the inclusion or exclusion of patients exhibiting HELLP syndrome symptoms could result in varying forms of association or lack thereof (Baker and Kingdom, 2004).

No compelling evidence has been provided to support associations between the coagulation and thrombophilia genes, viz, plasminogen activator inhibitor-I (*PAI-I*), prothrombin (*F2*) variant G20210A, cystathionine β-synthetase (*CBS*) and thrombomodulin (*THBD*), and the pathogenesis of pre-eclampsia, although they have been extensively researched (De Maart *et al*., 2004).

c) Hyper- /dyslipidemias

Normotensive and pre-eclamptic pregnancies are associated with hyperlipidemia. In pre-eclampsia, by 15 to 20 weeks of gestation, circulating fatty acids are increased before the onset of the disorder (Lorentzen *et al*., 1994). When placental factors, such as lipid peroxides

and trophoblastic components are released into the maternal circulation, their effects on the endothelium may be enhanced by the hyperlipidemia- mediated activation or "sensitisation" of the endothelial cells (Lorentzen *et al*., 1998). Common coding sequence variations in the lipoprotein lipase gene have also been shown to substantially increase the risk of pre-eclampsia (Hubel *et al*., 1999).

d) Mitochondrial dysfunction

Tobergsen *et al*, (1989) observed a high incidence of pre-eclampsia in a family with mitochondrial dysfunction. It has also been reported that the mitochondrial transfer ribonucleic acid genes contain mutations which are associated with pre-eclampsia (Folgero *et al*., 1996). This is not surprising as the formation of the placenta as well as the process of implantation are both processes which require high levels of energy, and therefore any defects in the energy-producing system could result in poor placentation. However, population based studies have not supported this hypothesis as mitochondria are transmitted maternally only and pre-eclampsia clearly has a paternal contribution (Trupin *et al*., 1996; Lie *et al*., 1998).

e) Human Leukocyte Antigens (HLA)

Many inconsistencies as well as contradictions have been identified in the findings concerned with the role of HLA in the development of pre-eclampsia.

Due to the multisystemic nature of pre-eclampsia, as well as an increased occurrence in primigravidae mothers, immune mechanisms cannot be ignored in the aetiology of the disorder. Pre-eclampsia is unlikely to be the simple result of excessive HLA antigen sharing between mother and fetus, as was first thought, but more likely, a complex mechanism involving feto-maternal compatibility (Hunt and Orr, 1992). HLA-G, a non-specific HLA I group antigen is expressed by trophoblast cells (Kovats *et al*., 1990). A more recent study revealed that an absence/reduced level of HLA-G expression, in extravillous cytotrophoblasts, is associated with pre-eclampsia. According to this study, trophoblasts lacking HLA-G may be vulnerable to attack by the maternal immune system (Goldman-Wohl *et al*., 2000).

f) Genetic Variability in the Renin-Angiotensin System

Since the placenta is thought be the primary instigator of this disorder, all molecules and components present in the placenta are possible candidates. For this reason, the renin-angiotensin system (renin, prorenin, angiotensinogen, angiotensin I, angiotensin II, angiotensin-converting enzyme (ACE), and angiotensin receptors) which is present in the human placenta has been investigated (Morgan *et al*., 1998; Cooper *et al*., 1999). The area around the spiral arteries which undergoes remodelling, is exposed to the expression of renin, ACE, and AT receptor 1 (Morgan *et al*., 1998). What is known thus far about the actions and presence of the renin-angiotensin system suggests that the local spiral artery renin-angiotensin system may play a role in the pregnancy-induced remodelling of these vessels (Cooper *et al*., 1999). During the last decade, the possible role of genetic variability in the members of renin-angiotensin system in the pathogenesis of pre-eclampsia has been extensively examined (Shah, 2003; Laskowska *et al*., 2004; Slatineanu, 2005) resulting in many conflicting results.

g) Endothelium-Derived Nitric Oxide Synthase (eNOS)

Endothelium-derived Nitric Oxide Synthase (eNOS), which is one of the producers of nitric oxide, is widely distributed in placental tissue (Buttery *et al*., 1994). In a study performed by Postovit *et al,* (2001), cross-sections of pre-eclamptic tissues were taken and subsequently characterised by high numbers of macrophages and a low degree of trophoblastic invasion compared to normal third trimester tissue which had a high degree of trophoblastic invasion and low number of macrophages (Reister *et al*., 1999). This high number of macrophages has been revealed to lead to embryo loss due to their increased production of nitric oxide (Haddad *et al.,* 1995; Baines *et al*., 1997).

In the study performed by Postovit *et al.*, (2001) they demonstrated that NO mimetic agents inhibit trophoblastic invasion. It was also postulated that NO production leads to trophoblast cell death since NO mediates apoptosis in numerous cell types (Keller *et al*., 1990, Garban and Bonavida, 1999; Duffield *et al*., 2000). Therefore there is a possibility that NO can lead to apoptosis of the trophoblastic cells and by this means, prevent adequate remodelling. This has been shown in studies where pre-eclamptic tissues have demonstrated high levels of extravillous trophoblast cell apoptosis (Genbacev *et al*., 1999; DiFederico *et al*., 1999).

1.1.5 Familial predisposition

Risk for the development of the disease is increased in the case of primiparity, work related psychosocial strain during pregnancy, poor social background, the mother's own low birth weight, prematurity, and young age (Abi-Said *et al*., 1995). These factors are however, not enough to predict pre-eclampsia accurately and therefore a genetic- and/or bio-marker is needed.

A significant locus was identified on human chromosome 2p13 when Icelandic families, representing 343 women, were investigated (Arngrimsson *et al*., 1999), while in another study, linkage had been assigned to chromosome region 4q (Harrison *et al*., 1997). Linkage studies using pre-eclamptic families, have also reported a susceptibility locus on the eNOS region of chromosome 7q36 (Arngrimsson *et al*., 1997). However, since pre-eclampsia is a complex multisystemic disorder it is unlikely that only one gene is responsible for its pathogenesis. It is more likely to be caused by a combination of polymorphisms together with environmental factors, as well as a fetal-maternal genetic component. Paternally-derived genetic contribution is also thought to be a component, since there is an increased risk of pre-eclampsia in women who become pregnant by a man who has already fathered a pre-eclamptic pregnancy with another woman (Lie *et al*., 1998).

There has been limited success in the search for maternal and fetal genes involved in pre-eclampsia. Some previously investigated genes are listed in Table 3.

Despite numerous studies and investigations of many candidate genes, no susceptibility profile has been established for the development or onset of pre-eclampsia. Since evidence implicates the placenta as well as implantation in this disease, it may be more relevant to study the genes that are involved in these processes. Mutations in these genes could be responsible for disruptions in these processes and subsequently lead to pre-eclampsia (Arngrimsson *et al.,* 1994).

Table 3: Summary of genes that have been investigated in the search for a predictive marker for pre-eclampsia (adapted from Lachmeijer *et al.,* 2002).

Implicated in	Gene	References	
		Association	No Association
Hemodynamics	AGT	Ward *et al.,* 1993 Arngrímsson *et al.* 1993 Takimoto *et al.,* 1996 Kobashi *et al.,* 1999 and 2001 Morgan *et al.,* 1999 (a+b) Hefler *et al.,* 2001 Levesque *et al*., 2004	Morgan *et al.,* 1995 Wilton *et al., 1995* Guo *et al.,* 1997 Harrison *et al.,* 1997 Arngrímsson *et al.,* 1999 Suzuki *et al.,* 1999 Moses *et al.,* 2000 Curnow *et al.,* 2000 Lachmeijer *et al.,*2001(a) Bashford *et al.,* 2001 Roberts *et al*., 2004 GOPEC Consortium, 2005
	REN	Maryuyama *et al*., 2005	
	NOS3	Arngrímsson *et al.* 1997 Guo *et al.,* 1999 Yoshimura *et al.,* 2000 Bashford *et al.,* 2001 Hefler *et al.,* 2001 Savvidou *et al.,* 2001 Kobashi *et al.,* 2001 Tempfer *et al.,* 2001	Harrison *et al.,* 1997 Lewis *et al.,* 1999 Arngrímsson *et al.,* 1999 Lade *et al.,* 1999 Landau *et al*., 2004 GOPEC Consortium, 2005
	EDNI	Barden *et al*.,2001	
	ACE	Lavesque et al., 2004 Kim *et al*., 2004 Fatini *et al*., 2006	Roberts *et al*., 2004
	AGTR1	Lavesque et al., 2004 Plumer *et al*., 2004	GOPEC Consortium, 2005 Roberts *et al*., 2004
Thrombophillia	MTHFR	Gandone *et al.,* 1997 Sohda *et al.,* 1997 Kupferminc *et al.,* 1999 Pegararo *et al*., 2004 Komas *et al*., 2004	Powers *et al.,* 1999 Chikosi *et al.,*1999 O'Shaughnessy *et al.,* 1999 de Groot *et al.,* 1999 Kaiser *et al.,* 2000 Laivuori *et al.,* 2000b Kobashi *et al.,* 2000 Rajkovic *et al.,* 2000 Raijmakers *et al.,* 2001 Kim *et al.,* 2001 Lachmeijer *et al.,*2001(b) Livingston *et al.,* 2001 (a) Ozcan *et al.,* 2001 Kaiser *et al*., 2001 Morrison *et al*., 2002

Table 3: continued:

Implicated in	Gene	References	
		Association	No Association
Thrombophillia (continued)	FVL	Dizon-Townson *et al.,* 1996 Nagy *et al.,* 1998 Mimuro *et al.,* 1998 Krauss *et al.,* 1998 Kupferminc *et al.,* 1999 Rigo *et al.,* 2000 Ozcan *et al.,* 2001 Watanabe *et al.,* 2002 Komas *et al*., 2003 Dudding *et al*., 2004	Lindqvist *et al.,* 1998 and 1999 de Groot *et al.,* 1999 Van Pampus *et al.,* 1999 Kim *et al.,* 2001 Livingston *et al.,* 2001 (a) Hillermann *et al.,* 2002
	F2	Kupferminc *et al.,* 1999	Higgins *et al.,* 2000F Livingston *et al.,* 2001 (a) Ozcan *et al.,* 2001 Hillermann *et al.,* 2002 Morrison *et al*., 2002
Oxidative stress	LPL	Hubel *et al.,* 1999 Kim *et al.,* 2001	Kim *et al.,* 2001
	GST	Zusterzeel *et al.,* 2000	Kim *et al*., 2005
Immunogenetics	IL-1	Faisel et al., 2003 Markovic *et al*., 2005	Hefler *et al.,* 2001 Lachmeijer *et al.,*2002(b) Haggerty *et al*., 2005
	HLA-G	O'Brien *et al.,* 2001	Humphrey *et al.,* 1995 Aldrich *et al.,* 2000 Bermingham *et al.,* 2000
	TNF	Chen *et al.,* 1996 Daher *et al*., 2006	Dizon-Townson *et al.,* 1998 Lachmeijer *et al.,*2001(c) Livingston *et al.,* 2001 (b) Daher *et al*., 2006
Lipid metabolism	LPL	Kim *et al*., 2001	Chikosi *et al*., 2000
	APOE		Belo *et al*., 2004

1.2 Pre-eclampsia as a genetic disease

1.2.1 Genetics

The completion of the human genome project has brought about a new aspect in the search for candidate genes. Vast numbers of polymorphisms have been identified and submitted to

databases which facilitate genotyping and haplotype analysis. Polymorphisms may or may not have functional consequences, which allows them to be classified as disease-causing or "inert". These variants can be present in the form of single nucleotide polymorphisms (SNPs), tandem repeats or other forms of microsatellites. Numerous methods as well as technological advances have been developed to identify novel as well as known polymorphisms. These methods include, among others, single strand conformation polymorphism (SSCP, Orita *et al.,* 1989), heteroduplex analysis (HD, Keen *et al.,* 1991), Multiphor SSCP/HD gel electrophoresis (Liechti-Gallati *et al.,* 1999), denaturing high performance liquid chromatography (dHPLC; Oefner and Underhill, 1995) and automated sequencing (Myers *et al.,* 1985). Throughput and sensitivity may vary according to the technique. Techniques such as SSCP and dHPLC have the potential to be used as high throughput methods but have lower sensitivity levels than sequencing. Characterisation of sequence variants can be performed by restriction enzyme analysis (REA), where a site is created or abolished by a specific variant allele. This method however only facilitates characterisation of known variants and can not aid in the detection of novel sequence variations.

In some cases, a single polymorphism appears to be inert when occurring "alone" but when examined in combination with another polymorphism the two may contribute to disease expression (Melen *et al*., 2006). It is therefore important to genotype multiple variants within a given gene and analyse their functional consequences individually. Follow up analysis with the loci typings in combinations will reveal whether they have "modifier" effects on each other.

1.2.2 Genetic Investigations

There are four main methods that have been used to genetically map complex diseases, viz, linkage studies, positional and functional cloning, candidate gene searches, as well as case control association studies.

Linkage analysis is used to identify a region on a chromosome that may be linked to a disease phenotype. This approach is based on the assumption that loci which are positioned close to each other on the chromosome will be inherited together. LOD (logarithm of odds) scoring is then used to determine whether the region is linked or not, and if so, provides a "map position" for the locus. Positional cloning relies on the basis of identifying genes, using the

map position, with no prior knowledge of the gene function. Functional cloning, on the other hand, relies on the biological basis of the disease. Candidate genes are selected and subsequently characterised. Patient samples are then collected and the appropriate gene is screened for the presence of any pathogenic mutations (Brown, 1999). Association between the variant and the disease can be sought by performing case control association studies and identifying whether a particular allele is more prevalent in only one of the two sample cohorts.

However, in the mapping of complex diseases, none of these strategies is very successful. In pre-eclampsia, the mode of inheritance is complex and unclear and most studies assume models which may be incorrect, leading to limited success with linkage mapping (Baker and Kingdom, 2004).

Case-control association studies have proved to be more successful. This allows for investigation of genes thought to play a role in the pathogenesis of the disorder by comparing the allele and genotype frequencies of the genes in affected and control populations (Campbell and Rudan, 2002).

This approach was used in this study and included a number of different techniques which will be dealt with in detail later.

1.3 Placental-derived circulation factors

1.3.1 Proposed two-stage model

Due to the multisystemic nature of the disease, the primary cause of pre-eclampsia has been difficult to elucidate. However, with the symptoms disappearing soon after delivery or following the termination of the pregnancy, the most compelling evidence points towards the placenta (Palma Gamiz, 1998). This evidence is more convincing in the case of hydatidiform mole, where the uterus contains only discarded placental tissue and pre-eclampsia occurs at a high frequency (Scott, 1958). Redman and Sargent (1991) suggested that a two-stage model is responsible for pre-eclampsia.

a) Stage one

At the onset of pregnancy, the uterine spiral arteries undergo physiological change including the extravillous cytotrophoblast invasion of the uterine endometrium and myometrium. Spiral arteries are then transformed into low-resistance vessels to allow for easy flow of blood (Pijnenborg *et al*., 1983). If however, defective invasion occurs, the spiral arteries retain their muscular-elastic properties and responsiveness to vasoactive substances (Lim *et., al* 1997). This is thought to lead to placental ischemia, the observed endothelial dysfunction and eventually pre-eclampsia (Roberts *et al.,* 1989). While this process of remodelling is essential for successful pregnancy, pre-eclamptic pregnancies have been documented to have poor or no transformation (Pijnenborg *et al*., 1998). Only 50-70% of spiral arteries involved in the remodelling process have been shown to be transformed in pre-eclamptic pregnancies (Meekins *et al.,* 1994). This lack of transformation subsequently leads to a lack of invasion. With fewer arteries invading the endothelium, there is a lower supply of oxygen resulting in a transient hypoxic environment for the placenta (Redman and Sargent, 1991).

b) Stage two

The ischemic placenta, resulting from a defective invasion, is thought to release unknown factors into the maternal circulation. This, along with maternal risk factors, is then responsible for the multisystemic complications which culminate in pre-eclampsia (Higgins & Brennecke 1998, Roberts 1998, Taylor *et al.,* 1998, Van Wijk *et al.,* 2000). Figure 1 is a schematic representation of the suggested pathophysiological two-stage mechanism of pre-eclampsia.

The molecules released can be, among others, oxygen free radicals and cytokines such as TNF-α and IL1-α and -β (Benyo *et al.,* 1997). These molecules cause endothelial dysfunction, which is the central theme of pre-eclampsia (Dekker and Sibai, 1998).

1.3.2 Influence of maternal risk factors on the two-stage model

In the two-stage model, it is thought that maternal risk factors could influence the presence of poor trophoblast invasion. The suggested maternal risk factors have been extensively

reviewed; they include immune maladaptation (Dekker & Sibai, 1999), genetic predisposition (Broughton and Pipkin, 1999), underlying diseases (Dekker *et al.,* 1995) and environmental factors (Neela & Raman, 1993).

a) Immune maladaptation

With first time pregnancies accounting for 75% of all pre-eclampsia cases, it is evident that immunity plays an important role in the development of the disease in primiparous women (Chesley, 1984). Eskenazi *et al.* (1991) stated that multiparous women have a decreased risk of developing pre-eclampsia compared to nulliparous women who have a five to ten times increased risk. A pregnancy resulting from the same partner (even if not carried to term) reduces the risk, while multiparity with different partners results in similar risk levels as primiparous women (Robillard *et al.,* 1993). The risk of developing pre-eclampsia is inversely related to the duration of cohabitation (Robillard *et al.,* 1994). This evidence strongly implicates molecules involved in immunity in pre-eclampsia aetiology.

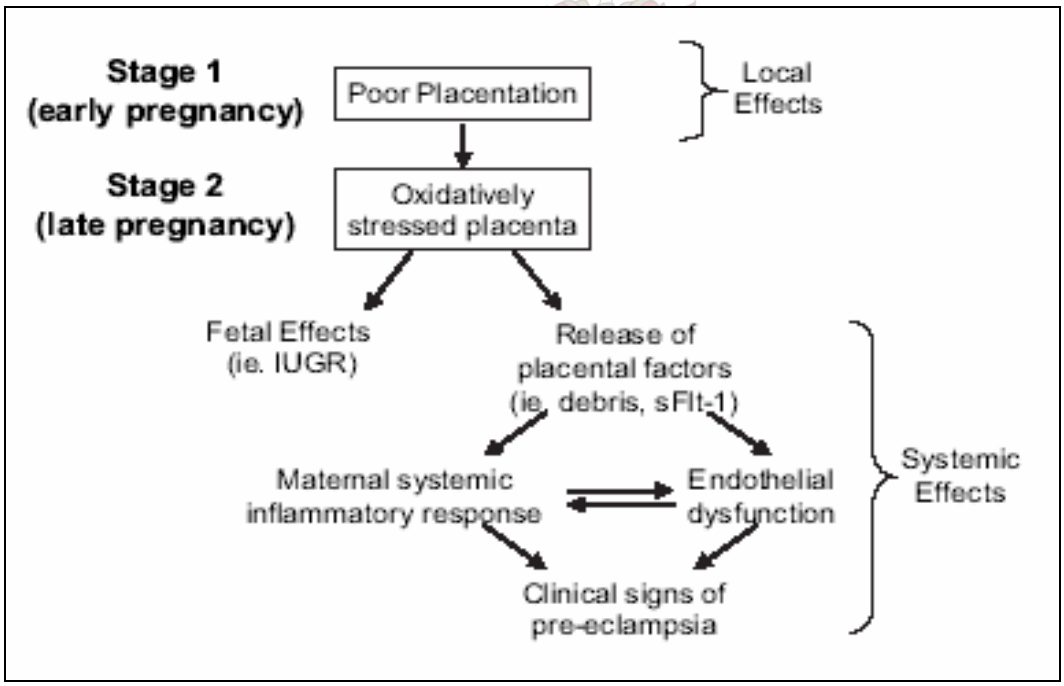


Figure 1: Schematic representation of the two stage development of pre-eclampsia. In the first stage, in early pregnancy, insufficient trophoblast invasion leads to poor placentation which then results in transient placental hypoxia. The second stage occurs when the oxidatively stressed placenta releases factors into the maternal system which subsequently results in clinical signs of pre-eclampsia (taken from Page *et al*., 2001)

For successful invasion to occur, the invading trophoblast cells need to interact with the major histocompatibility complex (MHC) of the maternal decidual tissue. Numerous molecules, viz,

cytokines (ILs and TNF), proteolytic enzymes (such as the MMPs) and oxygen free radicals (which induce lipid peroxidation) may be increasingly released and subsequently contribute to endothelial cell dysfunction (Dekker and Sibai, 1998; Baker and Kingdom, 2004).

1.3.3 Circulating factors

Candidate molecules are substances of placental origin which enter the maternal system. These molecules circulate within the maternal circulation in excessive or decreased amounts in pre-eclampsia compared to normotensive pregnancy. These factors may also be molecules that are able to induce effects on the utero–placental boundary as well as peripheral sites. Numerous factors, that are thought to be released by the placenta as a result of poor invasion and a transient hypoxic environment, have been proposed. These molecules have previously been divided into two groups, namely non-vasoactive peptides and vasoactive peptides.

a) Non-Vasoactive peptides

i Leptin

It has previously been reported that leptin plasma levels are increased in pregnancy-induced hypertension and pre-eclampsia (Vitoratos *et al*., 2001). For this reason it was thought that leptin may be a predictive marker for pre-eclampsia. One study reported that leptin levels were increased in maternal plasma levels before the onset of pre-eclampsia (Anim-Nyame *et al*., 2000). A controversial study reported that this was not the case in their population and that leptin concentrations were similar in patients with pre-eclampsia and in normotensive pregnancies (Martinez-Abundis *et al*., 2000).

Friedman and Halaas, (1998) showed that several transcription factors of the leptin gene promoter are upregulated in pre-eclamptic samples. This in turn, leads to an increase in the expression of leptin protein. This has remained a very controversial topic which could be influenced by differing diagnostic criteria as well as health care facilities.

ii β - human chorionic gonadotrophin (β-hCG)

β-hCG secretion is used as a basis for pregnancy tests and is the most clear-cut test involving peptide markers during pregnancy. It is secreted by the blastocyst and early placenta, to prolong the life of the corpus luteum. Numerous studies have reported an association of this molecule with pre-eclampsia. A study performed by Vaillant *et al*, (1996) showed that β-hCG was a positive predictor for pre-eclampsia (by measuring concentrations at 17 weeks gestation). They found these results to be comparable to the abnormalities of the Doppler waveforms of the uterine arteries which is thought to be the best and earliest testing method for pre-eclampsia. Results from a study performed by Ashour *et al*, (1997) showed that multiparous women have increased concentrations in the second trimesters. Only when hCG was incorporated into a multifactorial model (including body mass index, parity and age) did the sensitivity of the test prove effective with a specificity of 71% (Lee *et al*., 2000).

iii Inhibin

Inhibins are glycoprotein hormones which are produced in the human placenta by cytotrophoblasts and subsequently released during pregnancy (Petraglia *et al*., 1987). Hamasaki *et al*, (2000) reported that pre-eclamptic patients had higher concentrations of inhibins compared to their matched control group. They concluded that this may reflect hyperplasia of trophoblastic cells. This hyperplasia is thought to be due to the certain degree of hypoxia which is the original step in the two-stage theory. Petraglia *et al*, (1987) has previously shown that the synthesis and storage of inhibin occurs in the cytotrophoblasts and so it is thought that hyperplasia of these cells results in increased inhibin levels.

iv Soluble fms-like tyrosine kinase 1 (sFlt-1)

Recent studies have suggested that sFlt-1, an anti-angiogenic protein, may be implicated in the onset of pre-eclampsia. It has been shown to be present at increased levels in the placenta (Zhou *et al*., 2002; Maynard *et al*., 2003) and serum (Maynard *et al*., 2003; Koga *et al*., 2003; Tsatsaris *et al*., 2003) of women with pre-eclampsia. It is suggested that this molecule induces endothelial dysfunction by binding to the placental growth factor (PIGF) and vascular endothelial growth factor (VEGF) receptors. This subsequently inhibits the interaction with the receptors on the cell surface. By binding these receptors, circulatory levels of PIGF and

VEGF are lowered, these decreased levels are evident during pre-eclampsia and have even been noted before the onset of the disease (Poliottie *et al*., 2003).

b) **Vasoactive peptides (Vasoconstrictors)**

Due to the hypertensive nature of pre-eclampsia, it is no surprise that vasoconstrictive peptides have been considered possible contributors. Of these vasoconstrictors, a few will be discussed below. The endothelins and angiotensins, which help control the functions of vascular smooth muscle cells and circulating blood cells, have received the most attention as vasoconstrictors.

i Endothelins

Endothelins belong to a family of three polypeptides and are potent vasoconstrictors which serve an important role in the process of placentation and throughout gestation (Yanagiswa *et al*., 1988). Concentrations in amniotic fluid have been found to be 10-100 times higher than in serum, confirming its origin (Germain *et al*., 1997). A study performed by Margarit *et al.* (2005) investigated endothelin concentrations in women undergoing amniocenteses for antenatal screening and compared that to the incidence of pre-eclampsia in later pregnancy. They demonstrated a statistically significant increase in the endothelin levels of women who later developed pre-eclampsia. Shaarawy and Abdel-Magid (2000) suggested that first trimester endothelin concentrations be combined with mid trimester blood pressure readings to increase the predictive value.

ii Angiotensins

Pre-eclampsia has most consistently been associated with renal involvement (Shah, 2005). Therefore renin-angiotensinogen genes have been identified as candidate genes for pre-eclampsia in the placenta (Nielson *et al*., 2000). Normal pregnancy is characterised by an increase in renin as well as angiotensin. Zunker *et al,* (1998) showed that the concentrations of angiotensin I increased and those of angiotensin II decreased in the maternal system in the first week after birth. This is thought to be the reason why the health of women with pre-eclampsia is sometimes prone to deteriorate during this period (Zunker *et al*., 1998).

iii Neurokinin B

Evidence indicated that neurokinin B (NKB), previously not found in the periphery, fulfils the criteria for a specific pre-eclampsia marker (Page *et al*., 2000a).

1.4 Neurokinin B (NKB)

NKB is a member of the tachykinin family, a group of structurally related peptides with the capacity to contract smooth muscle. The three documented mammalian peptides include Substance P (SP) and Neurokinin A and B. The receptors for each peptide are termed NK_1, NK_2 and NK_3, respectively (Maggi 1995; Patak *et al*., 2003). These receptors mediate processes such as activation of the immune system (Ansel *et al*., 1993), vasodilation (Brownbell *et al.,* 2003), vascular reactivity (D'Orleans-Juste *et al*., 1991) and smooth muscle contraction (Patak *et al*., 2000). Page *et al*. (2000a) reported that NKB causes potent contraction of the hepatic portal vein, venoconstriction of the mesenteric beds and increased heart rate observed in pre-eclampsia (Page *et al*., 2000a).

Human NKB mRNA (NM_013251), expressed in the placenta, is encoded by seven exons and spans a genomic region of 5.4 kb. Exons 1 and 7 correspond to the 5' and 3' untranslated regions of the mRNA, respectively (Page *et al*., 2001). These 7 exons encode a precursor molecule, preprotachykinin B (PPTB) and NKB is the only tachykinin derived from this precursor, being encoded by exon 5 only (Pennefather *et al*., 2004).

1.4.1 Regulation of NKB

The arcuate nucleus of the hypothalamus shows increased levels of NKB gene expression in postmenopausal women; however, when supplemented with estrogen, there is a decrease in NKB mRNA-expressing neurons and the level of expression within individual cells. Two estrogen receptors, namely estrogen receptor α (ER α) and estrogen receptor β (ER β) mediate the actions of estrogen. In one reported study, knockout mice were used (ER α and ER β) to determine which receptor is responsible for the regulation of NKB. They found that NKB expression level decreased significantly when ER β knockout mice were treated with estrogen (Dellovade and Merchenthaler, 2004).

The regulation of NKB could occur at various levels of gene expression. At the DNA level, NKB could be up- or down-regulated during transcription in the presence of a specific promoter region variant (Greenwood *et al*., 2002); polymorphisms in close proximity of the splicing domain could create or abolish splice variants resulting in alternative splice variants which subsequently alter expression (Cartegni *et al*., 2002). Post-translational modifications such as glycosylation (attachment of sugar units to a peptide) and phosphorylation (attachment of amino acids such as serine, threonine and tyrosine) could also be responsible for influencing the peptide and in doing so, vary levels of NKB in the systemic circulation.

1.5 NKB Receptor (NK3)

Three tachykinin receptors are present, namely NK_1, NK_2 and NK_3. Expression of NK_3 occurs primarily in the central nervous system (CNS) but has also been identified in certain peripheral tissues such as the human and rat uterus, the human skeletal muscle, lung and liver, the rat portal and mesenteric vein, and certain enteric neurons from the gut of different species (Tsuchida *et al*., 1990; Massi *et al*., 2000; Page and Bell, 2002; Fioramonti *et al*., 2003; Lecci and Maggi, 2003; Patak *et al*., 2003).

Compared to the other tachykinin receptors NK_3 is 465 residues longer extending at the amino-terminal region. NK_3 is encoded by the *TACR3* gene (NM_001059) which, localised to human chromosome 4, contains 5 exons. This is represented by Figure 2 (Pennefather *et al.,* 2004).

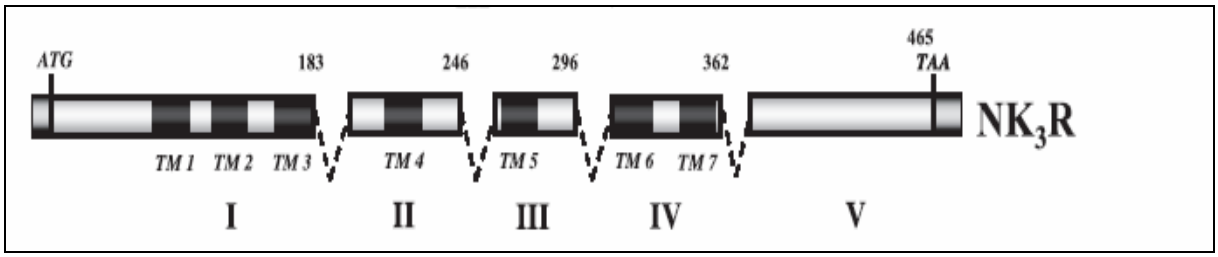


Figure 2: Schematic representation of the NK_3 gene. *TACR3* is made up of 5 exons with the ATG codon in exon 1 and the stop codon (TAA) in exon 5 (adapted from Pennefather *et al.,* 2004).

The tachykinins recognise all three receptors, although they have preferential binding to specific receptors (Mussap *et al*., 1993; Regoli *et al*., 1994; Maggi, 2000; Lecci and Maggi, 2003). The rank order of potency for the NK_1 receptor is SP > NKA > NKB; while it is NKA > NKB > SP for the NK_2 receptor and NKB > NKA > SP for the NK_3 receptor (Regoli *et al*., 1994; Maggi, 2000).

1.6 Biological functions of NKB

1.6.1 NKB and pregnancy

It is has been reported that the activation of NK3 causes the hepatic portal vein to contract (Mastrangelo *et al.,* 1987), venoconstriction of the mesenteric beds (D'Orleans-Juste *et al.* 1991) and increased heart rate. Therefore, it is thought that the secretion of NKB, from the placenta, activates the receptor which in turn is responsible for the maternal adaptation. Very little detail is known about the mechanisms underlying this process (Thornburg *et al.* 2000). When high levels of NKB were infused into female rats it indicated that NKB may be involved in these hemodynamic events (Cintado *et al*., 2001).

Throughout gestation, maternal blood volume and red cell mass increase gradually. Along with this, stroke volume and heart rate increase as well as venous compliance and venous blood volume, whereas systolic and diastolic blood pressures decrease (Thornburg *et al.* 2000).

The above data has resulted in speculation w.r.t. the direct function of NKB in pregnancy. It was subsequently presumed that NKB could be responsible for causing the vascular changes. It has been suggested that the placenta starts to release NKB when the need for a greater blood supply arises (Page *et al*., 2000b). This NKB release in turn activates the NK3 receptor on the venous side of the maternal system which results in an increase of blood pressure by the contraction of the large veins of the mesenteric beds and the hepatic portal vein (Mastrangelo *et al.* 1987) . Consequently, the blood flow to the uterus is increased and therefore the blood flow to the liver is decreased (D'Orleans-Juste *et al.* 1991).

During this time of low blood supply and low oxygenation, the trophoblasts are prevented from differentiating into invasive phenotypes (Genbacev *et al.,* 1999) which further prevents

optimal placental perfusion, required for the controlled invasion of the trophoblasts. At this critical period, a transient surge of NKB secretion may be required to improve perfusion in the newly established placental bed.

NKB levels, measurable from the ninth week of gestation, increase gradually throughout normal pregnancy (Page 2000; D'Anna, 2002). This finding created expectations that NKB measurement, if altered early in pre-eclampsia, could be used as an early biomarker for the condition, before clinical symptoms manifest and complications become established.

1.6.2 NKB and pre-eclampsia

NKB is believed to play a role in the two-stage model. It was proposed that if the defective trophoblast invasion is not rectified after the 10th to 12th weeks of pregnancy, the placenta will start to release NKB into the maternal circulation (Page *et al*., 2001). In normal pregnancies, the surge measured during this time could be responsible for the period of correction. In a study performed by Page *et al*, (2000a) high levels of NKB were detected in third trimester patients with pre-eclampsia who never had satisfactory trophoblast invasion.

The reason for believing that NKB is a key factor in the initiation of the clinical disorder of pre-eclampsia is due to the fact that NKB could account for many if not most of the diverse symptoms portrayed in this disease. Constriction and contraction of the mesenteric and hepatic portal veins could be due to activation or stimulation of NK3 and this could result in damage to the liver and kidneys. In turn, reduced blood flow to these organs would lead to an accumulation of toxic metabolic products such as lipid peroxides and this product contributes to endothelial cell damage and dysfunction.

In the cases where NKB is present in excessive amounts, the other tachykinin receptors may be activated. Stimulation of NK_1, found on platelets (Gecse *et al.,* 1996) and neutrophils (Perianin *et al.,* 1989) may induce complications common to pre-eclampsia. These high levels of NKB could also be responsible for cerebral complications as high intravascular levels have been shown to dilate the blood vessels via the NK_1 receptors (Jansen *et al.,* 1991, Kobari *et al.,* 1996) located in the endothelium.

NKB plasma levels were low or undetected in most normotensive pregnancies studied and also in those of males and non-pregnant females (Page *et al.,* 2000*b*; D'Anna *et al.,* 2002). Page and Lowry (2000a) proposed a theory that the increased secretion of NKB, due to the hypoxic environment, leads to the stimulation of NK_3 receptors on the venous side of the maternal system which leads to the reported contractions of the portal veins and mesenteric beds. The "re-directed" blood flow from the liver would result in increased blood flow to the uterus, and may explain the maternal liver damage observed in pre-eclampsia (Pennefather *et al*., 2004).

1.7 NKB and eNOS

Pre-eclampsia is associated with reduced placental blood flow (Lunell *et al*., 1982). Nitric oxide (NO) acting as a potent vasodilator, released by endothelial cells, is a major contributor to the maintenance of low basal vascular tone in the human placenta. Various studies have suggested that low NO production may be of importance in pre-eclampsia (Yallampalli *et al*., 1993).

Increased NKB production from placental syncytiotrophoblasts occurs in pre-eclampsia, and causes hypertension when injected into rats, suggesting it is an important factor in the pathophysiology of the disease (D'Anna et al., 2002). It is also known that the placenta of pre-eclamptic women display multiple abnormalities representative of oxidative stress.

Nitric oxide is important in pregnancy for physiological vascular adaptation which involves increased blood volume, vascular output and decreased vascular resistance in many tissues including the uterine arteries and placental vessels (Hambartsoumian *et al*., 2001). In normal pregnancy, these changes are accompanied by increased endogenous NO production (D'Anna *et al*., 2004). Blocking the production of NO in mice (by administering NOS-inhibiting agents) causes them to present with pre-eclamptic-like symptoms, suggesting a direct relationship between the physiological vascular adaptation and NO (Duane, 2000).

Altered NO production in pre-eclampsia has also been demonstrated by the measurement of NO metabolites (nitrates and nitrites) in patients' peripheral circulation (Norris *et al*., 1999). In a recent study, D'Anna *et al* (2004) reported higher NO metabolite levels in the peripheral

circulation of pregnancies complicated with pre-eclampsia compared to levels in normal pregnancies.

As previously mentioned NKB, which is the only tachykinin expressed by the placenta (Page *et al.*, 2000a), acts predominantly through the NK_3 receptor. The activation of this receptor is mediated by production of NO (Maggi *et al*., 1993; Mizuta *et al*., 1995) and has been shown to determine the activation of nitric oxide synthase (NOS) (Linden *et al*., 2000). This was also described by Page *et al*. (2001) when he suggested that NK3 receptors may play a role in NO release (Page *et al.*, 2001).

1.8 Aim and Objectives

The aim of this project was to investigate the role of the Neurokinin B (*TAC3*) and Neurokinin B receptor (*TACR3*) genes in predisposition to pre-eclampsia.

This would be achieved by:

1. Bioinformatic characterisation of the *TAC3* and *TACR3* genes.
2. Screening the *TAC3* and *TACR3* genes in South African non-Caucasian pre-eclamptic maternal and infant samples to identify the spectrum of sequence variants compared to that in normotensive pregnancies, and
3. Case-control association studies comparing;
    - Sequence variants and circulating NKB concentrations
    - Sequence variants and clinical outcome
    - *TAC3*, *TACR3* and *eNOS* variants and risk of pre-eclampsia.
4. Performing appropriate statistical analyses to determine whether any gene variants analysed contribute to the pre-eclampsia disease profile in both mothers and infants.

2. MATERIALS AND METHODS

2.1 Materials

Institutional and ethical approval (Appendix 1) were obtained from the Ethics and Research Committee of the Faculty of Health Sciences, University of Stellenbosch (C99/025). Written informed consent (Appendix 2) was also obtained from all participants.

2.1.1 Patient Cohort

i.) For the completion of a pilot study, the genes encoding NKB and its receptor, NK_3, viz. *TAC3* and *TACR3* respectively, were screened in 50 cord blood samples which corresponded to the 50 maternal samples screened in the original pilot study (Carelse-Tofa *et al*., submitted). This was to track the transmission of observed sequence variants between pre-eclamptic mothers and their infants.

The cordblood samples were divided in the same manner as the original maternal samples of the pilot study: 20 control individuals who had uncomplicated pregnancies; 20 Primigravidae samples with early onset severe pre-eclampsia and 10 patients with pregnancies complicated by abruptio placentae.

ii.) Once the pilot screen was complete, the pre-eclamptic maternal cohort was extended to 120 samples and the characterising restricted to variants identified in the pilot study**.**

iii.) For the NKB/eNOS case-control association study; 124 samples were screened. Of these 124 samples, 54 samples were prepared for radioimmunoassay (RIA) analysis which was performed by collaborator Dr Rene Moser at IBR Biopharmaceuticals, Matzingen, Switzerland.

The pre-eclamptic cohort consisted of mostly primigravidae mothers, of mixed ancestry with severe, early onset pre-eclampsia (<34 weeks gestation) together with their infants cord-blood samples which were collected at delivery (by an appointed research sister). Blood samples were stored in EDTA Vacutainers (Becton, Dickinson and Company, New Jersey, USA) and

kept at -20°C until DNA extractions were subsequently performed. Although fathers of the babies were encouraged to participate, they were mostly absent due to varying circumstances. Samples were collected at Tygerberg Hospital in the Western Cape, South Africa from individuals who largely represent the major ethnic group in this catchment area.

A diagnosis for pre-eclampsia was established according to the International Society for the study of Hypertension in Pregnancy (ISSHP) guidelines (Davey and MacGillivray, 1988). This was defined as a blood pressure measurement of 140/90mmHg or higher on at least two separate occasions, four hours apart, by means of the Korotkoff phase V heart sounds (disappearance of pulse sounds). Together with this, the presence of significant proteinuria of 300mg protein/l or more in a 24 hour urine collection or a persistent +2 on a diagnostic stick occurring after 20 weeks of gestation (Dekker *et al*, 1995).

Detailed questionnaires (Appendix 3) were completed by the participants. Clinical records were also attached to the questionnaires for further patient stratification. Patients who had a medical history of hypertension or miscarriage were not included in the study.

This study concentrated mainly on samples from 30 newborns, delivered to mothers who had severe, early onset pre-eclampsia: onset after 20 weeks but before 34 weeks of gestation and 120 additional pre-eclampsia maternal samples.

2.1.2 Control Cohort

A group of 20 ethnically matched samples, from uncomplicated pregnancies and healthy pregnancy outcomes (term deliveries and babies weighing >10 percentile according to gestational age (Appendix 5) for this specific population group), were used as controls. Women with a history of hypertensive disease or recurrent miscarriage or congenital abnormalities were excluded from the study. Cordblood samples were collected from healthy pregnancies as mentioned above, although they were not infants of the control mothers.

Blood samples were collected by a registered nurse or clinician on duty and stored as previously mentioned until DNA extractions were performed. Ethical approval was obtained

(Appendix 4) for construction of a control patient database. Patients completed consent forms and remained anonymous throughout the study.

2.2 Methods

ALL WET BENCH TECHNIQUES THAT HAVE BEEN DESCRIBED, HAVE DETAILED PROTOCOLS IN THE APPROPRIATE APPENDICES.

2.2.1 BIOINFORMATICS

To date, there is no evidence in the literature of a mutation screen involving the *TAC3* and *TACR3* genes.

Any alterations in gene expression could be as a result of genetic alterations in the structure of the gene; therefore, it was important to examine the gene in its composite form. Complete bioinformatic characterisation of the gene was performed, which included examining intron-exon boundaries, positioning all previously identified SNP's and other polymorphisms as well as an attempt to characterise the promoter region.

a) Gene Structure

Genomic annotations of the *TAC3* (Appendix 6) and *TACR3* (Appendix 7) were collected from NCBI (http://www.ncbi.nlm.nih.gov/) and Ensembl (http://www.ensembl.org/index.html) database websites and further annotated with Pearl Script (George Rebello). Intron-exon boundaries, start codons and stop codons were defined and annotated on the relevant sequences.

b) Sequence variations

SNPs and STSs encompassing the coding and non-coding sequences of the genes as well as the sequence 1kb up- and downstream of both the genes were annotated on the sequences. The SNPs and STSs in *TAC3* and *TACR3* have been highlighted in purple on the annotations of the genes in Appendix 5 and 6, respectively. Tandem Repeat Finder (Benson, 1999) was

used to identify any potential repeats within the genomic span of each gene, including 1kb up- and down-stream.

c) Promoter analysis

Due to the limited knowledge available on the *TAC3* and *TACR3* gene and promoter regions, various programs, viz, CpG detector (http://www.ebi.ac.uk/emboss/cpgplot/), MIT MC Promoter MM II (http://www.genatlas.org) and Cluster Buster (http://zlab.bu.edu/cluster-buster/) were used to perform promoter prediction.

d) Oligonucleotide primers

Primers for *TAC3* and *TACR3* were largely used as previously described by Carelse-Tofa *et al.,* (submitted). Primers for *eNOS* Glu298Asp were used as previously described by Hillermann *et al.,* (2005).

Primers were designed to amplify appropriate exons while encompassing flanking intronic regions. The *TAC3* gene contains 7 exons of which only exons 2 to exon 6 are coding. Exons 1 and 7 correspond to the 5' and 3' untranslated regions, respectively (Page *et al*., 2001). These particular exons were also screened as they may contain regulatory motifs. The *TACR3* gene consists of 5 exons of which are all coding. Only one primer set was used for genotyping the *eNOS* Glu298Asp variant (Hillermann *et al*., 2005).

Reference sequences were subjected to Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) primer design software and analysed for hairpin, homo- and hetero-dimer formation using the IDT® (Integrated DNA Technologies, Inc, Coralville, IO, USA) online oligonucleotide Analyser (http://scitools.idtdna.com/Analyzer/). Following analysis, NCBI Basic Local Alignment Tool (BLAST) (http://ww.ncbi.lm.nih.gov/BLAST/) search was utilised to ensure primer specificity and integrity. All oligonucleotide primers were synthesised by IDT® or by the University of Cape Town's Synthetic DNA laboratory (Cape Town, ZA).

Primer sequences as well as relevant Tms are shown in Table 4 and Table 5 for *TAC3* for *TACR3,* respectively. These sequences have also been highlighted on the appropriate annotations to show where they are located in relation to the exons.

2.2.2 DNA Extractions

DNA extractions were performed on whole blood, using the GENTRA™ **PureGene®** genomic purification kit (Minneapolis, USA) (Appendix 8). This method required 300µl of whole blood, to which cell lysis solution was added to lyse the red blood cells. The nuclear membranes of white blood cells were then lysed and proteins precipitated. DNA was precipitated from the remaining aqueous phase with isopropanol and then washed in 70% ethanol to remove excess salt. Pellets were dissolved in DNA hydration solution and stored at 4°C until needed.

DNA from remaining whole blood samples was extracted using a variation of the "salting out" method originally described by Miller *et al* (1988) (Appendix 9 shows step by step methodology). For this method, a maximum of 10ml whole blood was used per extraction. Cells were lysed with lysis buffer, placed on ice, and subsequently centrifuged to form a pellet. The supernatant was discarded and the pellet was re-suspended in phosphate buffered saline solution (PBS) and centrifuged. Following centrifugation the pellets were incubated overnight at 55°C in a solution of nucleic lysis buffer; sodium dodecyl sulphate (SDS) and proteinase K. Following overnight incubation, saturated NaCl was added and the solution was shaken vigorously for 1 minute. After centrifugation, the supernatant was transferred to a clean centrifugation tube and spun again. The supernatant was again transferred to a clean tube and two volumes of ethanol was added to precipitate the DNA. DNA was rinsed with 70% ethanol and centrifuged, followed by careful removal of DNA which was subsequently dissolved in distilled water and mixed overnight. DNA was resolved on a 1% agarose gel to assess concentration and quality. Using the NanoDrop® ND-1000 Spectrophotometer (NanoDrop Technologies, USA) the DNA sample's concentration was determined and then stored at 4°C until further use.

Table 4: *TAC3* gene primer names, exon and amplicon sizes, melting temperatures and sequences utilised in the study.

TAC3

Exon	Amplicon	Exon size (bp)	Amplicon size (bp)	T_m (°C)	T_a (°C)	Primer Sequence (5' - 3')
1	*TAC3* - 1	117	315	58	52	F: TGGGATTGGTGACTCTCAG
				56		R: GAATAAAGCAGATGGCAGC
2	*TAC3* - 2	119	297	60	55	F: AAGCCAAGCTGCTGGTAATG
				60		R: GACAGCTGTAGTGAGGAAAC
3	*TAC3* - 3	94	341	62	57	F: AGCACCTACTTCTTCTCGTCCAG
				66		R: CCTTTCAGATGGGAGAGAGATG
4	*TAC3* - 4	30	282	60	55	F: TCTGAAGATAAGAGGCTGG
				60		R: CAAACAATATGCCAGCTCCC
5	*TAC3* - 5	54	290	60	55	F: CTGTGAGGAGTCATGCTTG
				60		R: GAGGAAAGACAGGACCTTT
6	*TAC3* - 6	75	265	60	55	F: TTGAACACTGCCCGTCATAG
				60		R: CCTCCCATGCTACAGGTATT
7	*TAC3* - 7	287	420	64	55	F: AGGATATAAGATGTGATTTCAGTG
				60		R: CTCCTCCAGCTACATGGTAA

Key	
T_m (°C)	Melting Temperature
T_a (°C)	Annealing Temperature

Table 5: *TACR3* gene primer names, exon and amplicon sizes, melting temperatures and sequences utilised in the study.

TACR3

Exon	Amplicon	Exon size (bp)	Amplicon size (bp)	T_m (^oC)	T_a (^oC)	Primer Sequence (5' - 3')
1a	*TACR3* - 1a	691	354	60 60	55	F: ATTCTTTCTGCCTGCCAGAG R: AGCCACCCAGTCRCAACTG
1b	*TACR3* - 1b	691	354	68 64	59	F: TGAACCTGACCGCCTCGCTA R: ACGTGTTGAAGGCGGCCATG
1c	*TACR3* - 1c	691	351	60 58	53	F: TACTTCCTTGTGAACCTGGC R: CACTCGAGGGCTACAAATG
2	*TACR3* - 2	190	342	66 66	55	F: CCTTTGAAATACCTTTGAAACTCC R: GTTGCTCCTAATCTGTAGTTTCC
3	*TACR3* - 3	153	307	60 64	55	F: AAGCTGAGCCAATTCAGTCC R: TTAACATGCCATGACTAGATTGC
4	*TACR3* - 4	199	345	64 64	59	F: ATTGGCAGAAAAGCATGATTTGC R: ATTGTATGTTTCCAGTGAAGGTG
5a	*TACR3* - 5a	529	335	62 60	55	F: GGTAGAATTTTCTGTGGCAGC R: GCAGATTTGGAATTCCTGCG
5b	*TACR3* - 5b	529	384	60 60	55	F: ACCAAGTTTCAATGGCTGC R: CGAGTTTACAAGTGTTTTCCTGAC

Key	
T_m (°C)	Melting Temperature
T_a (°C)	Annealing Temperature

2.2.3 The polymerase chain reaction (PCR)

a) PCR amplification

All PCR reactions were carried out in a total volume of 50µl using the GeneAmp® PCR System 2700 from Applied Biosystems (California, USA). Each reaction consisted of either 10µl 5X reaction buffer (including 1.5mM $MgCl_2$), 15pmol of each primer, 200µM dNTP's and 0.5U goTaq® DNA polymerase (Promega, WI, USA) or 10µl 5X reaction buffer, 15pmol of each primer, 200µM dNTP's, 2mM $MgCl_2$, and 0.5U goTaq® DNA polymerase and ~40ng of genomic DNA. Amplicon sizes, primer sequences and amplification reactions for the *TAC3* and *TACR3* genes are given in Table 4 and 5, respectively. Reaction profiles for the amplification of the *TAC3* and *TACR3* amplicons are given in Table 6 and 7 respectively.

b) Gel electrophoresis

To verify amplification, PCR products were resolved on a 1.5% agarose gel in a 1 X TBE buffer (90mM Tris-HCl, 90mM Boric acid and 1mM EDTA, pH 8.0). Equal volumes of PCR product and loading dye (95% Formamide, 20mM EDTA, 0.05% Xylene Cyanol, 0.05% Bromophenol Blue up to a total volume of 20ml with dH_2O) were mixed and resolved by electrophoresis. Ethidium bromide (Sigma, Missouri, USA) staining (0.05%) was used for UV fluorescence visualisation of the PCR products on a Multigenius Bio Imaging System (Syngene, Cambridge, UK).

2.2.4 Mutation Detection

a) Multiphor SSCP/HD electrophoresis

The Multiphor Electrophoresis technique first described by Liechti-Gallati *et al* (1999) was used to detect sequence variants according to the protocol (Appendix 10 provides step by step methodology). This method combines Single Strand Conformation Polymorphism (SSCP) and Heteroduplex (HD) analysis and therefore increases the mutation detection rate. Following electrophoresis, silver staining was used to visualise the various banding patterns. This method of mutation detection was performed on all the *TAC3* and *TACR3* amplicons to identify previously described as well as novel polymorphisms.

Table 6: Reaction profiles for amplification of the *TAC3* gene amplicons.

	T_a (°C) (a)	Time at Temperature (b)	(c)	(d)	(e)	N° of Cycles (f)	[primers] (pmol)
***TAC3* ex1**	52	2 min	30s	30s	30s	x30	10
***TAC3* ex2**	55	2 min	20s	30s	30s	x30	15
***TAC3* ex3**	57	2 min	30s	30s	45s	x30	15
***TAC3* ex4**	55	2 min	20s	30s	30s	x30	10
***TAC3* ex5**	55	2 min	20s	30s	30s	X30	15
***TAC3* ex6**	55	2 min	20s	30s	30s	X30	15
***TAC3* ex7**	55	3 min	45s	30s	45s	X30	15

Table 7: Reaction profiles for amplification of the *TACR3* gene amplicons

	T_a (°C) (a)	Time at Temperature (b)	(c)	(d)	(e)	N° of Cycles (f)	[primers] (pmol)
***TAC3* ex1a**	55	3 min	30s	45s	45s	x30	10
***TAC3* ex1b**	59	3 min	30s	45s	45s	x30	10
***TAC3* ex1c**	53	3 min	30s	45s	45s	x30	10
***TAC3* ex2**	51	3 min	30s	45s	45s	x30	15
***TAC3* ex3**	55	3 min	30s	45s	45s	x30	10
***TAC3* ex4**	59	3 min	30s	45s	45s	x30	10
***TAC3* ex5a**	55	3 min	30s	45s	45s	x30	15
***TAC3* ex5b**	55	3 min	30s	45s	45s	X30	10

Index:

Temperature	Time	no. of Cycles
95 °C	(b)s	
95 °C	(c)s	
(a)	(d)s	(f)s
72 °C	(e)s	
72 °C	5 min	
4 °C	∞	

b) Multiphor methodology

A back plate and the well-plate were cleaned thoroughly with ethanol, whereafter plate glue was rubbed onto the back plate until resistance was felt and then wiped down with ethanol again. Spacers were cleaned with ethanol and then laid down on the well plate. The two plates were then clamped together firmly and the gel mix poured in. After allowing the gel to polymerise for one hour, the well-plate was carefully removed avoiding any damage to the gel. The back plate with the gel was then placed on the multiphor platform using a little distilled water to create hydrostatic tension.

Gel products were prepared by adding 3µl Multiphor loading dye to ~2µl PCR product and denaturing at 95^{o}C for 5 minutes before quenching on ice. Once 3µl of the combined, denatured product was loaded, strips of chromatography paper which had been soaked in Tris Borate Buffer were placed on the top and bottom areas of the gel. The electrode plate was then placed over the gel and the gel resolved at 355V for 105 minutes at 11^{0}C.

c) Automated sequencing

Samples showing conformational changes were re-amplified and resolved by electrophoresis on 1.5% agarose gel to ensure amplification. The PCR products were purified with the Bioline SureClean (Bioline, UK) (Appendix 11) and then checked using the NanoDrop® ND-1000 Spectrophotometer (NanoDrop Technologies, USA). Automated sequencing was outsourced to the Division of Human Genetics, University of Cape Town and the University of Stellenbosch's Core DNA Sequencing facility, SA.

Sequencing results were obtained in the form of electropherograms and subsequently analysed using BioEdit Sequence Alignment Editor (Isis Pharmaceuticals, California, USA). Sequences were checked individually for any variation and subsequently aligned with corresponding reference sequences, by using BioEdit Multiple Alignment Tool (http://mbio.ncsu.edu/Bioedit/bioedit.html) to identify any sequence variants.

d) Restriction enzyme analysis.

Restriction enzyme recognition sites were identified and characterised for observed conformational variants using the enzyme recognition site locator program in BioEdit Sequence Alignment Editor (http://mbio.ncsu.edu/Bioedit/bioedit.html). This method of genotyping was used to verify any variant detected by Multiphor analysis once automated sequencing was completed. All remaining samples in patient and control cohorts were subsequently screened by this method. Table 8 shows a summary of the exons in which conformational variation was detected, as well as the corresponding restriction enzymes used to genotype these variants.

In each case, 15µl of the corresponding PCR product was digested with 5-10U of the appropriate restriction enzyme and 1X buffer in a total volume of 20µl. Each reaction was incubated overnight according to manufacturer's temperature recommendations. Depending on the size of the various fragments, restriction products were either resolved on 2% agarose gels, in 1 X TBE buffer, for 90 minutes at 70V or on 5% mini polyacrylamide gel electrophoresis systems (PAGE) for 40 minutes at 250V (Appendix 12).

2.2.5 Biochemical analysis

Single plasma samples from 54 patients with early-onset, severe pre-eclampsia (from the cohort of 120) were collected during pregnancy and stabilised with Aprotinin B. NKB levels were determined by radioimmunoassay (RIA) according to manufacturer's recommendations (Penninsula Laboratories, Bachem Ltd, UK) by IBR Biopharmaceuticals, Matzingen, Switzerland.

2.2.6 Statistical analysis

Five types of statistical analysis were performed on the generated data, including:

- **Hardy-Weinberg Equilibrium** for genotype distribution at each locus
- Comparisons of **genotype** distributions between the sample groups, using Chi-squared analysis (personal program); and

- Analysis of the **allele** frequencies, compared in all the cohorts using StatCalc (EpiInfo 2000, version 6, Centers for Disease Control and Prevention, USA).
- Genotype-phenotype correlations between subdivided goups using StatCalc (EpiInfo 2000, version 6, Centers for Disease Control and Prevention, USA).
- Allelic interaction between patient and control cohorts for various allele combinations (Butt *et al*., 1979)

Sub-analysis was performed by grouping genotype frequencies together. These groupings have been referred to as recessive models (homozygous wildtype and heterozygous genotypes compared to the homozygous mutant genotype); and dominant models (homozygous mutant and heterozygous genotypes vs. the homozygous wildtype genotype). Analysis was performed using StatCalc and then compared in the various cohort groups (model nomenclature from Hager *et al.,* 1998).

Table 8: Mutation screen summary showing amplicon name, variant detected, restriction enzyme used in detection if any and the variant reference..

Amplicon name	Variant	Restriction enzyme	Temperature	Recognition site	Reference
TAC3 – 1	-25 (c-t)	*Msp* I (NEB)*	37 °C	C ▲C G _G	dbSNP: 2291855
TAC3 – 4	IVS -53 (g-t)	ND			NOVEL
TAC3 – 6	417(t-c) / S99P	*Hinf* I (NEB)*	37 °C	G ▲A N T _ C	NOVEL
TAC3 – 7	479 (t-c)	ND			NOVEL
TACR3 – 1a	-103 (t-c)	*Nci* I (NEB)*	37 °C	C C ▲S_G G	db:SNP: 3733632
TACR3 – 3	857 (a-g) / K286M	ND			db:SNP: 2276973
TACR3 – 5b	1471 (t-c)	*BseN* I (NEB)*	65°C	A C T G ▲G N_	db:SNP: 2765

* New England Biolabs, Ipswich, USA

ND= none detected

3. Results

3.1 Bioinformatics

In silico characterisation of the *TAC3* and *TACR3* genes was performed using databases and internet-based programs. Figures 3 and 4 demonstrate intron-exon boundaries, all previously recorded SNPs and other polymorphisms as well as the position of the primer sets utilised in this study.

3.1.1 Gene structure

The *TAC3* gene on chromosome 12q13-q21 which encodes neurokinin B, spans a genomic region of 6501bp and consists of 7 exons of which exon 1 represents the 5' untranslated region, and exon 7 the 3' untranslated region. The start codon (ATG) is 5bp into exon 2 and the stop codon (TAG) is 71bp into exon 6 (Figure 3). The exons of the *TAC3* gene range in size from 30bp to 287bp; this range is shown in Appendix 6 which represents a genomic annotation of the *TAC3* gene.

Figure 4 is a schematic representation of the *TACR3* gene (encoding the neurokinin B receptor), which consists of 5 exons and spans a genomic region of 40 348bp on human chromosome 4q25. Exon 1 and 5 represent the 5' and 3' untranslated regions, respectively. In this gene, start (ATG) and stop (TAA) codons are 141bp into exon 1 and 310bp into exon 5, respectively. Appendix 7 is a full genomic annotation of the *TACR3* gene and shows the sizes of the exons which range from 151bp to 689bp.

3.1.2 Sequence variation

The *TAC3* gene annotation [NM_013251] collected from the NCBI database, revealed 9 SNPs (Appendix 6 purple highlight) (Table 9), and no STS variants in the region of the gene (at this time - August, 2006). Utilisation of the Tandem Repeats Finder program revealed no repeats in the sequence of the *TAC3* gene, including the regions 5kb upstream and downstream.

Table 9: A composite table of all the SNPs that span the *TAC3* gene, including SNP reference number, nature of the SNP as well as the frequency in which it occurs in different populations according to the NCBI HapMap data (http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs).

dbSNP	Nature and position	Population	Frequency
dbSNP: rs2291855	c/t 5'UTR	Asian	c/c (0.76) c/t (0.24) t/t (0)
		Sub-Saharan African	c/c (0.93) c/t (0.073) t/t (0)
dbSNP: rs2291856	g/c 3' of exon 1	No detail	No detail
dbSNP: rs17119330	a/g 3' of exon 2	European	a/a (0.02) a/g (0.17) g/g (0.82)
		Asian	a/a (0) a/g (0.17) g/g (0.82)
		Sub-Saharan African	a/a (0) a/g (0.11) g/g (0.89)
dbSNP: rs17119327	a/g 3' of exon 2	European	a/a (0.02) a/g (0.17) g/g (0.82)
		Asian	a/a (0) a/g (0.18) g/g (0.82)
		Sub-Saharan African	a/a (0) a/g (0.10) g/g (0.90)

Table 9: continued

dbSNP: rs2122694	a/g 3' of exon 2	European	a/a (0) a/g (0.09) g/g (0.91)
		Asian	a/a (0) a/g (0) g/g (1.00)
		Sub-Saharan African	a/a (0) a/g (0) g/g (1.00)
dbSNP: rs733629	c/t 3' of exon 5	Asian	c/c (0) c/t (0.23) t/t (0.77)
		Sub-Saharan African	c/c (0) c/t (0.12) t/t (0.88)
dbSNP: rs2270734	a/c 3' of exon 5	European	c/c (1.00) c/t (0) t/t (0)
		Asian	c/c (0.93) c/t (0.07) t/t (0)
		Sub-Saharan African	c/c (1.00) c/t (0) t/t (0)
dbSNP: rs11609825	c/g 3' of exon 6	European	c/c (0) c/g (0.05) g/g (0.95)
		Asian	c/c (0) c/g (0) g/g (1.00)
		African American	c/c (0) c/g (0) g/g (1.00)
dbSNP: rs3759138	t/c 3' of exon 7	No Detail	No Detail

The *TACR3* gene annotation from NCBI, revealed that the gene is encompassed by approximately 356 SNPs as well as 12 STS's. Table 10 is a summary of the SNPs present in the coding regions only.

Table 10: A composite table of SNPs that span the coding region of the *TACR3* gene, including SNP reference number, nature of the SNP as well as the frequency in which it occurs in different populations according to the NCBI HapMap data.

dbSNP	Nature and position	Population	Frequency
dbSNP: rs3733632	c/g 5' of exon 1	European	c/c (0.55) c/g (0.43) g/g (0.02)
		Asian	c/c (0.49) c/g (0.47) g/g (0.04)
		Sub-Saharan African	c/c (0.05) c/g (0.13) g/g (0.82)
dbSNP: rs2276973	a/g exon 3	European	a/a (0.99) a/g (0.01) g/g (0)
		Asian	a/a (0.97) a/g (0.03) g/g (0)
dbSNP: rs6822961	g/a 3' of exon 4	European	g/g (1.00) g/a (0) a/a (0)
		Asian	g/g (1.00) g/a (0) a/a (0)

Table 10: continued

dbSNP: rs17033889	a/g exon 5	European	a/a (0) a/g (0.01) g/g (0.99)
dbSNP: rs2765	t/c exon 5	European	t/t (0.45) t/c (0.43) c/c (0.12)
		Asian	t/t (0.27) t/c (0.43) c/c (0.29)
		Sub-Saharan African	t/t (0.38) t/c (0.53) c/c (0.08)

No repeats were identified in the gene (by the Tandem Repeats Finder) other than in the 5' UTR region. Repeat motifs identified can be seen in Table 11.

Table 11: Results generated by Tandem Repeat Finder for the *TAR3* gene, showing position of repeat relative to ATG start site, the repeat motif (sequence that is repeated) as well as the number of times it is repeated.

Position relative to ATG start site	Repeat sequence	Times repeated
-27527 bp	TTTTTTTA	6
-12949 bp	ATATATACGTGC	23
-2905	GGGAGAGGGAGACGGTG	4

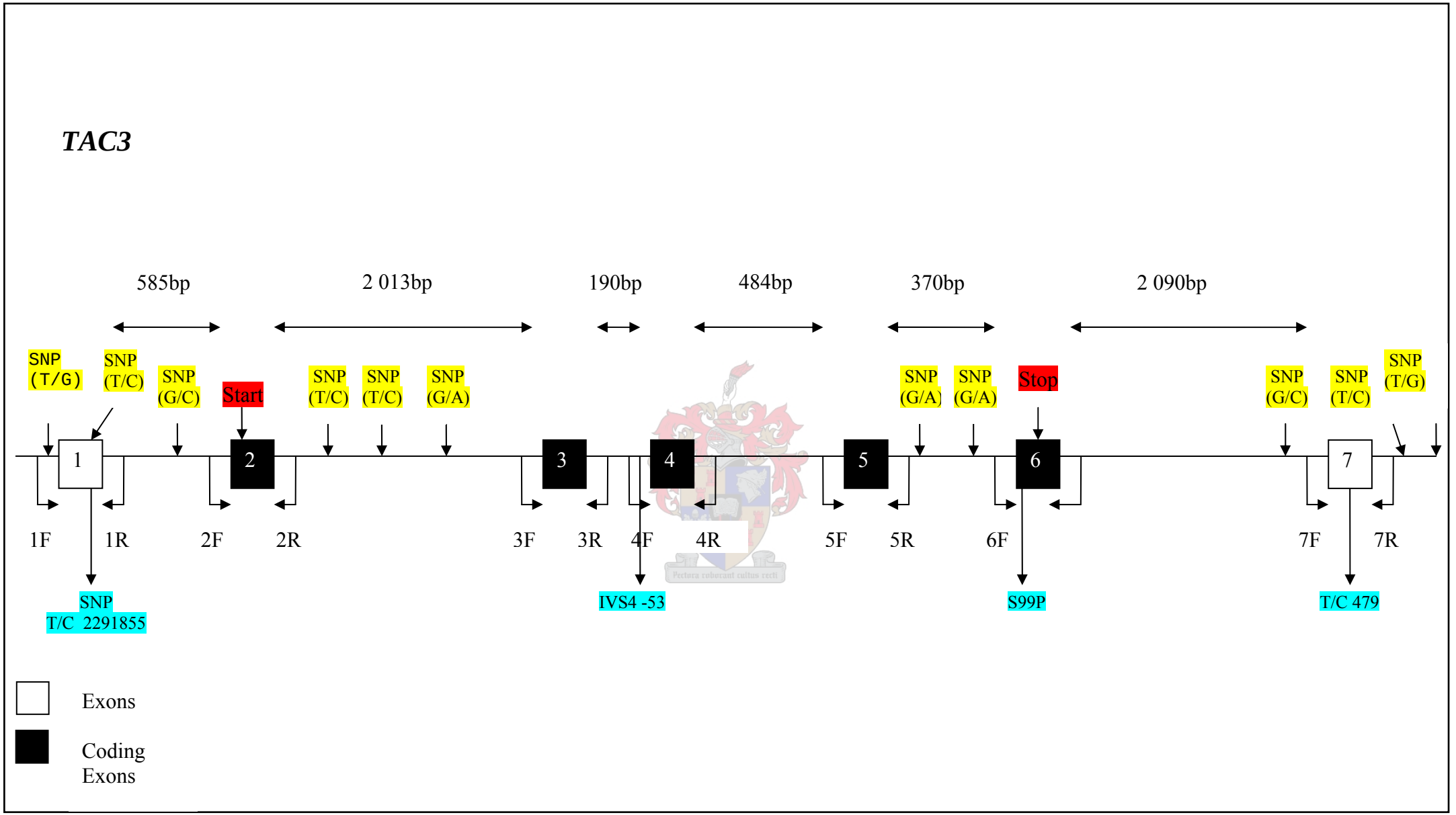


Figure 3: Schematic representation (not drawn to scale) of the *TAC3* gene, indicating positions of the 5' and 3' untranslated region, exons and primer sets utilised (F and R) in this study. The start (ATG) and alternative stop (TGA, TAA) codons are indicated. The SNP's highlighted in blue have been identified in this study.

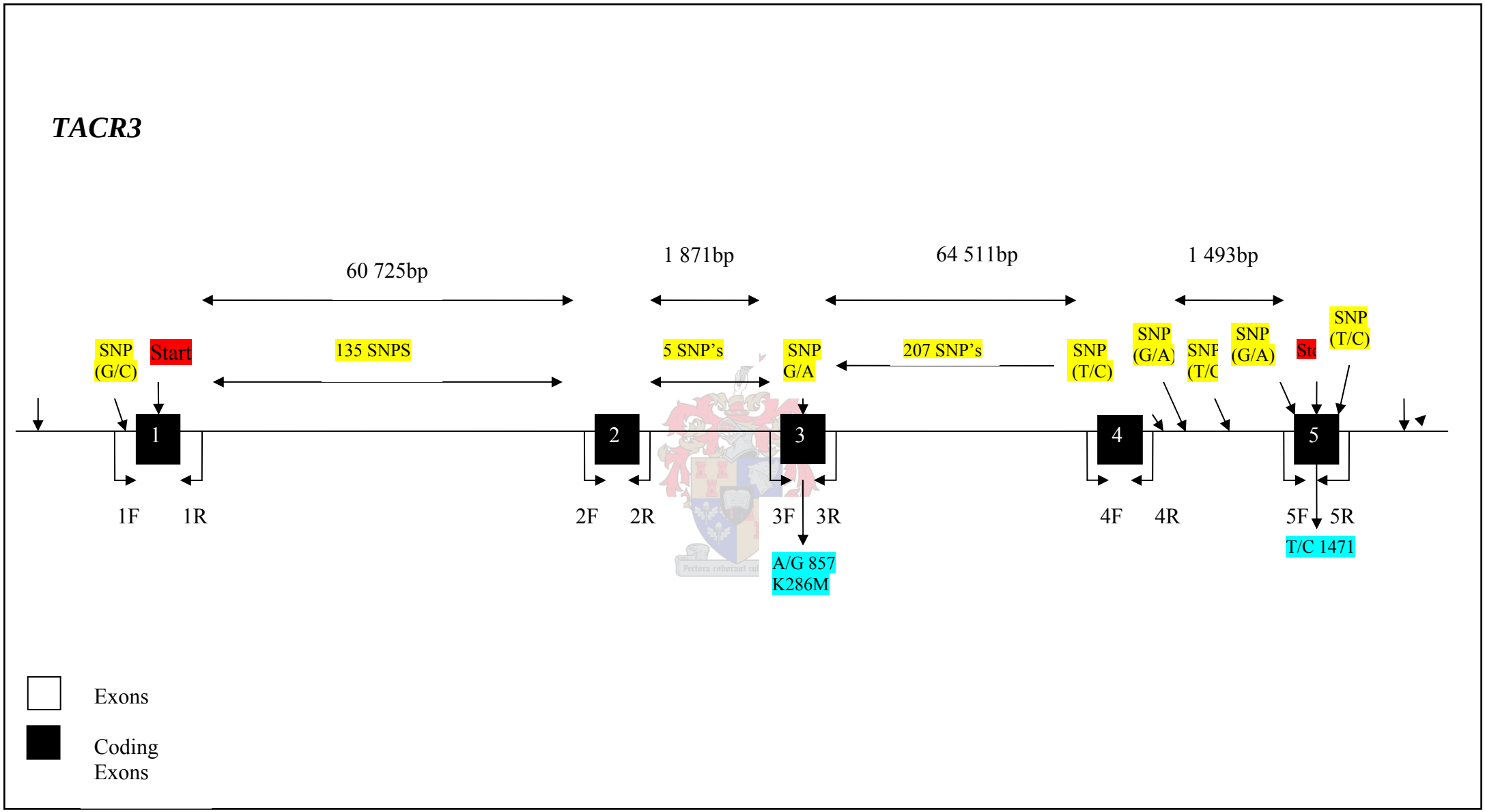
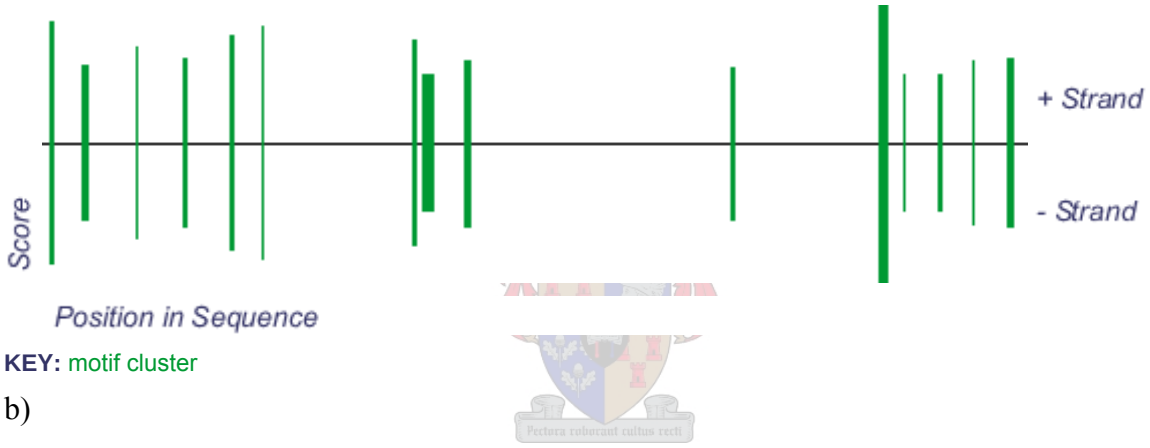


Figure 4: Schematic representation (not drawn to scale) of the *TACR3* gene, indicating positions of the 5' and 3' untranslated region, exons and primer sets utilised in this study. The start (ATG) and alternative stop (TGA, TAA) codons are indicated. The SNP's highlighted in blue have been identified in this study.

3.1.3 Promoter region

Due to the limited knowledge available on the *TAC3* gene as well as the promoter region, promoter prediction programs, viz, CpG detector, MIT MC Promoter MM II and Cluster Buster, were utilised for the characterisation of the promoter region. No common motifs were identified in any of the program outputs examined. Figure 5 is an example of the output generated by Cluster Buster. However, none of these motifs were identified by CpG detector and MIT MC Promoter MM II, such that no consistency could be observed in the analysis and hence, no conclusions could be drawn at this time.

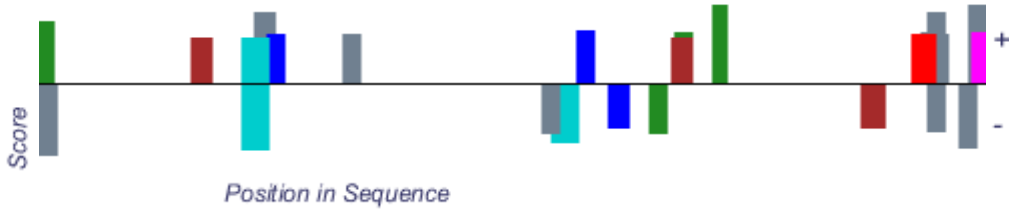
a) *TAC3*



KEY: motif cluster

b)

Cluster: 84792 to 85474 Score: 10.4



Motif	Position	Strand	Score	Sequence
Ets	84792 to 84802	+	8.38	gagaggaatgg
Tef	84792 to 84803	-	9.7	cccattcctctc
LSF	84902 to 84916	+	6.01	acatgtttggacttg
NF-1	84938 to 84955	+	6.05	atttggtacctagccaac
NF-1	84939 to 84956	-	9.07	tgttggctaggtaccaaa
Myf	84949 to 84960	+	9.64	agccaacagctg

Figure 5: a) Diagram generated by Cluster Buster to show where motif clusters are present throughout the given *TAC3* sequence, and b) cluster detail showing the top ten clusters at the a specific position in the entered sequence.

In summary, the genes contain several polymorphic motifs of which the SNPs are the most common and widely distributed. This should facilitate any future association studies, as well as haplotype analyses. The lack of consistent promoter motifs is disappointing since each gene harbours at least 1 sequence variant within a potential promoter region. Investigations into the potential influence of these variants on gene transcription, may consequently be limited since there will be uncertainty as to where to position the primers relative to essential regulatory motifs. An option would be to generate a large corresponding genomic fragment, and selectively reduce it, all-the-while assessing it by transcription analysis (Lang *et al*., 2004).

3.2 Patient Demographics

The demographic details of the study participants were obtained from the completed demographic questionnaires (Appendix 3) which were obtained from pre-eclamptic mothers, recruited to this study. Clinical data was retrieved from clinical notes by authorised personnel. Table 12 summarises the relevant data.

Table 12: Demographic and clinical data of pre-eclamptic patients (n = 120) recruited for this study.

	Mean	**Range**	**Normal values ***
Age (years)	25	15-43	n/a
Gravidity	1.8	1-6	n/a
Parity	0.71	0-5	n/a
Systolic BP (mmHg)	163.42	120-220	n/a
Diastolic BP (mmHg)	107.23	80-160	n/a
Gestation at Delivery (weeks)	31.04	20-41	~38-40
Birth Weight (g)	1517.69	324-3704	Appendix 5
NKB concentration (ng/l)	73.578	9.6-267.8	n/a

* = general values for individuals from all races and genders

n/a = not applicable

The median maternal age which was relatively low (~25 years) was consistent with the observation that most patients are likely to be primiparous. Systolic and diastolic blood pressures were consistently higher in patients, as expected, compared to normal pregnancies. Normal gestation completes at ~38-40 weeks compared to pre-eclamptic pregnancies which tended to deliver earlier, mostly due to clinical intervention. This was also evident in the birthweight of infants (Table 13) which was usually lower in pre-eclamptic pregnancies

Table 13 Clinical data of gestational age and birthweight of pre-eclamptic infants (n = 30) recruited to this study.

	Mean	**Range**	**Normal values ***
Gestation at Delivery (weeks)	29.20	22-36	~38-40
Birth Weight (g)	1297.21	392-2650	Appendix 5

* = general values for individuals from all races and genders

3.3 Genetic Analysis

Seven sequence variants were identified in this study, of which three are novel. These variations and their characterisations are shown in Table 14.

Table 14: A composite table, including the name, nature and references of all *TAC3* and *TACR3* variants identified in this study.

	Exon/amplicon	**Variant**	**Nature**	**SNP ref#**	**Reference**
***TAC3* gene**	1	-25 (5' UTR)	c / t	rs2291855	NCBI SNP database
	4	IVS3 -53	g / t	-	**NOVEL**
	6	Ser99Pro	t / c	-	**NOVEL**
	7	+113 (3' UTR)	t / c	-	**NOVEL**
***TACR3* gene**	1a	-103 (5'UTR)	t / c	rs3733632	NCBI SNP database
	3	R286K	a / g	rs2276973	NCBI SNP database
	5b	+75 (3'UTR)	a / g	rs2765	NCBI SNP database

UTR = untranslated region
IVS = intervening sequence
Nomenclature based on annotation used in this study

These variants will now be discussed in further detail.

3.3.1 *TAC3*

Seven amplicons were designed to cover the complete coding region of the gene, as well as flanking intron-exon boundaries. Of these seven amplicons, four presented with conformational variations, of which three were novel.

a) Exon 1 -25 c/t

Multiphor SSCP/HD gel electrophoresis of the exon 1 amplicon revealed two conformational variants in the pilot study (Figure 6a). Following automated sequencing, these conformational variants were shown to represent the wildtype sequence and a known SNP (dbSNP: rs2291855) at position -25c/t relative to the ATG start site. An *Msp I* (New England Biolabs, Ma, USA) restriction enzyme recognition site was then identified in the C allele. Genotype and allele frequencies are shown in Table 15.

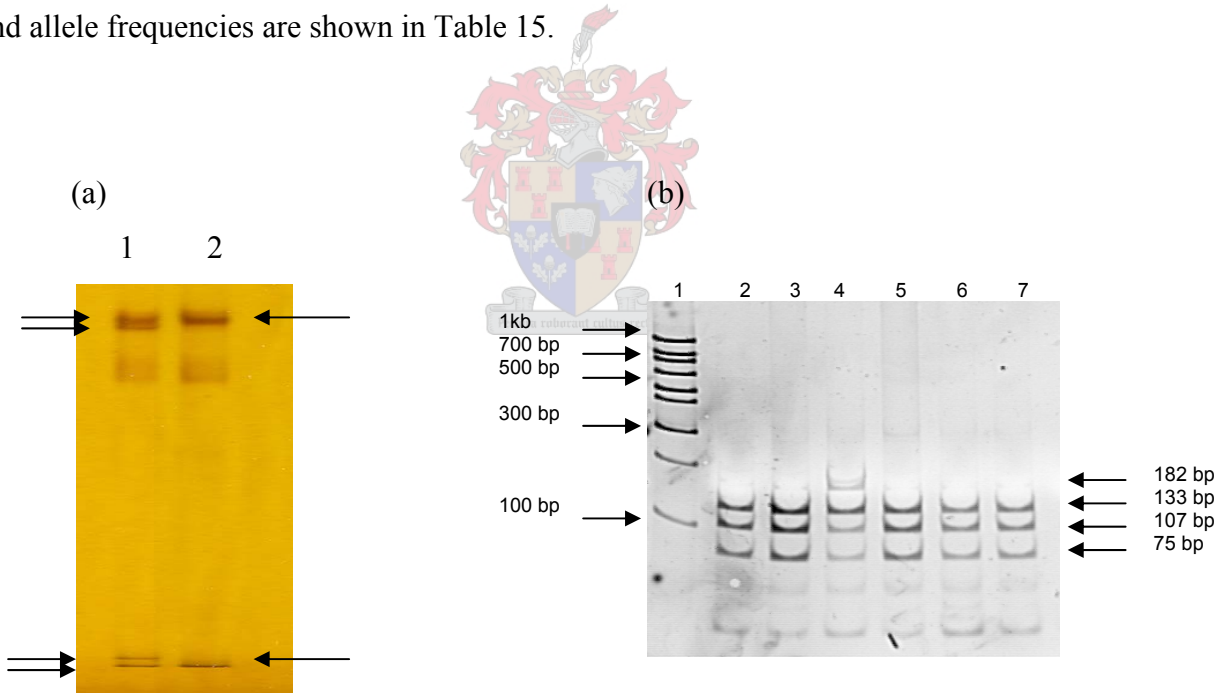


Figure 6: (a) Multiphor SSCP/HD electrophoresis reflecting the conformational changes indicated in the SSCP component as well as the heteroduplex component of the multiphor gel and (b) a photograph of the *MspI* restriction digest on a polyacrylamide gel. Lane 1 shows a 1kb ladder, lane 4 represents a heterozygote c/t genotype and the remaining lanes represent homozygous c/c genotypes.

Table 15: Genotype and allele frequencies for the -25c/t variant identified in cordblood samples of the pilot study and the extended cohort of 120 maternal samples.

		Pilot study			Extended cohort	
		Cordblood	**Mothers**	**controls**	**Mothers**	**Controls**
TAC3	*No of samples*	*25*	*20*	*11*	*120*	*42*
Exon 1	c/c	17 (0.68)	14 (0.70)	8 (0.73)	103 (0.86)	30 (0.72)
-25 c/t (5'UTR)	c/t	7 (0.28)	4 (0.20)	3 (0.27)	16 (0.13)	12 (0.28)
	t/t	1 (0.04)	2 (0.10)	0	1(0.008)	0
	c	41 (0.82)	32 (0.8)	19 (0.86)	222 (0.93)	72 (0.86)
	t	9 (0.18)	8 (0.2)	3 (0.14)	18 (0.07)	12 (0.14)

One of the cordblood samples, representing an infant of one of the homozygous maternal samples previously screened in the pilot study, demonstrated homozygosity for the t allele. In the larger cohort of mothers, (n=120) there was one maternal sample homozygous for the t allele and none in the control group. This variant is located in the 5' UTR of the gene, and could therefore impact on the transcription of the gene and subsequently, gene expression.

In the cordblood samples, the heterozygote state (c/t) seemed to be more prevalent (0.28) than in the maternal samples (0.20), however they corresponded to the control samples (0.27) (p=0.638). The maternal samples, in the extended cohort, had a lower frequency of heterozygotes than the corresponding control samples although this was not significant (p=0.070).

The genotype distribution in each study group was found to be in Hardy-Weinberg equilibrium. No statistically significant differences were found between mothers and controls of both groups, nor between babies and controls or babies and mothers. There was however a significant difference (p=0.036) for genotypes in the dominant model (c/c vs. c/t and t/t) between mothers and controls from the extended cohort.

b) Exon 4 IVS3 -53 (g/t)

A novel intronic (g/t) transversion was identified in the genomic interval between exon 3 and 4 of the *TAC3* gene. The transversion is located at position -53 relative to the intron-exon boundary of exon 4. This variant was only identified using Multiphor SSCP/HD analysis (Figure 7) and was subsequently confirmed by automated sequencing. The variation, at this position, did not create or abolish a restriction enzyme recognition site. Genotype and allele frequencies are shown in Table 16.

This polymorphism was submitted to the EMBL database and is now accessible via reference number **AM042557** (http://www.ebi.ac.uk/submission/webin.html).

There was no evidence that the polymorphism was located within a conventional boundary or splicing site which is usually within ~60bp before the splice acceptor site (3' of the intron).

Figure 7: Variant IVS3-53 g/t conformational change identified by Multiphor SSCP/HD gel electrophoresis. The conformational change is evident in the heteroduplex component of the gel in Lane 1. The arrows indicate the double bands present in the heteroduplex region.

Table 16: Genotype and allele frequencies of the IVS3-53 g/t polymorphism in cordblood samples of pilot study together with additional 120 maternal samples.

		Pilot study			Extended cohort	
		Cordblood	**Mothers**	**controls**	**Mothers**	**Controls**
TAC3	*No of Samples*	*33*	20	*18*	*120*	*25*
Exon 4	g/g	33 (1.00)	17 (0.85)	17 (0.94)	118 (0.98)	25 (1.00)
IVS3-53 g/t	g/t	0	3 (0.15)	1 (0.06)	2 (0.02)	0
	t/t	0	0	0	0	0
	g	33 (1.00)	37 (0.93)	35 (0.97)	236 (0.99)	25 (1.00)
	t	0	3 (0.07)	1 (0.03)	2 (0.01)	0

In the original pilot study a higher rate of heterozygotes was obserevd among maternal samples (0.15), this was disproven in the extended maternal cohort which reflected a low frequency of 0.02 which was comparable with the controls. From the mother and cordblood pilot study there was no transmission of this rare variant from mother to baby.

The observed genotypes in both maternal cohorts as well as the pilot study control cohort were in Hardy-Weinberg equilibrium. Due to lack of variation in the infant or extended control cohorts, Hardy-Weinberg equilibrium could not be determined. No significant difference was identified in genotype or allele frequencies when comparing the relevant groups to one another, except for a significant (p=0.048) difference for genotypes in the dominant model (g/g vs. g/t and t/t) between cordblood and maternal samples.

c) Exon 6 (295 t/c / S99P)

Multiphor SSCP/HD gel electrophoresis revealed a conformational variation in exon 6 of *TAC3* (Figure 8a). Following automated sequencing, (Figure 8b), the variant was characterised as a novel t/c transition at nucleotide position 295. On translation of this transition, a Serine amino acid residue is replaced by a Proline residue (S99P). This results in the replacement of a hydrophilic amino acid with a hydrophobic amino acid. This SNP is

positioned 3bp into the exon and 68bp before the TAG stop codon. Genotype and allele frequencies are shown in Table 17.

This polymorphism was submitted to the EMBL database and is now accessible via reference number **AM179832** (http://www.ebi.ac.uk/submission/webin.html).

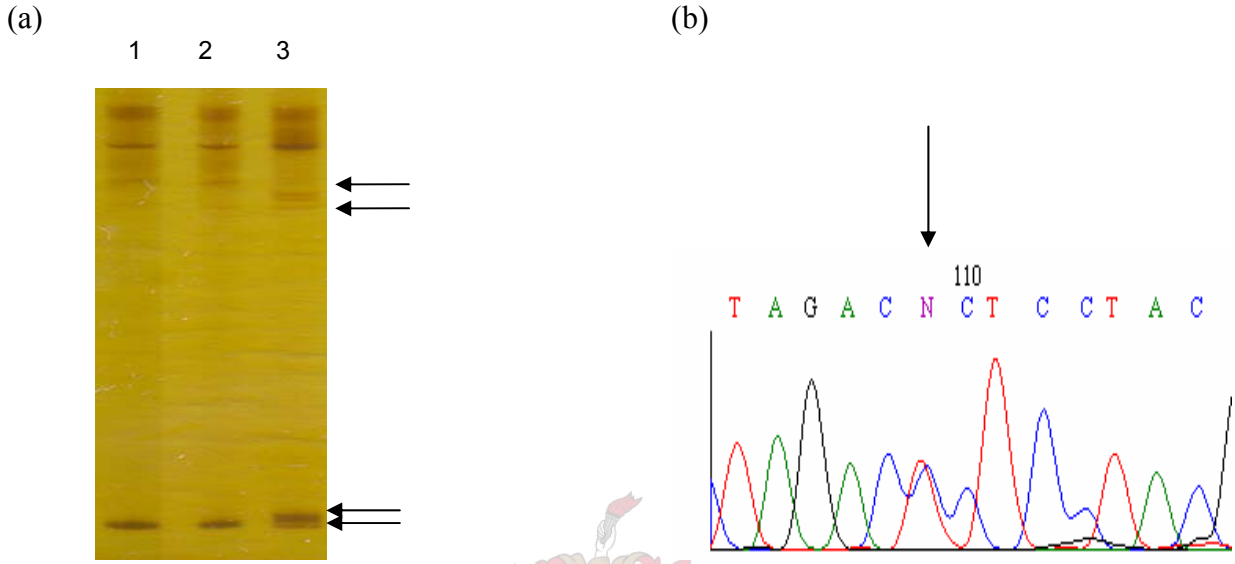


Figure 8: (a) The S99P conformational change observed by Multiphor SSCP/HD gel electrophoresis. The conformational change is evident in the SSCP as well as the heteroduplex component of the gel in lane 3 (arrowed), (b) the corresponding S99P variant confirmed by sequencing (forward orientation). A heterozygous t/c genotype is shown in the electropherogram (arrowed).

The enzyme *Hinf I* (New England Biolabs, Ma, USA) was found to recognise the wildtype t allele. This recognition site was abolished by the variant c allele. Restriction enzyme analysis was utilised to genotype the remaining samples. Figure 9 shows a restriction enzyme digest with *Hinf I*, resolved on a 3.4% polyacrylamide gel.

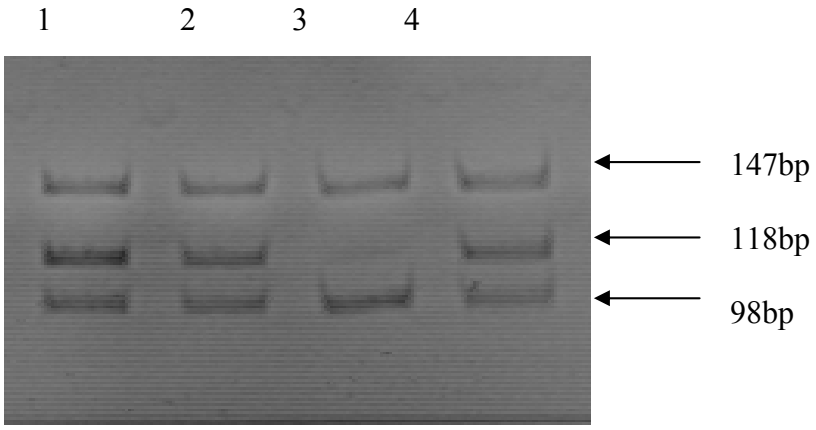


Figure 9: A photograph of the *Hinf I* restriction digest. Lane 1, 2 and 4 show heterozygote (t/c) genotypes and the remaining lane 3 represents a homozygous t/t genotype.

Table 17: Genotype and allele frequencies of the S99P polymorphism in cordblood samples of the pilot study together with the extended cohort of 120 maternal samples.

		Pilot study			Extended cohort	
		Cordblood	**Mothers**	**controls**	**Mothers**	**Controls**
TAC3	*No of alleles*	*30*	*20*	*30*	*120*	*18*
Exon 6/ S99P	t/t	22 (0.73)	19 (0.95)	26 (0.86)	101 (0.84)	15 (0.83)
	t/c	7 (0.23)	1 (0.05)	4 (0.13)	19 (0.16)	3 (0.17)
	c/c	1 (0.03)	0	0	0	0
	t	51 (0.85)	39 (0.98)	56 (0.93)	221 (0.92)	33 (0.92)
	c	9 (0.15)	1 (0.02)	4(0.07)	19 (0.08)	3 (0.08)

In the mother and cordblood pilot study, the cordblood samples showed an increased rate of heterozygosity compared to maternal samples (0.23 vs. 0.05). The only homozygous individual was an infant. Maternal and control samples of the extended cohort seemed to appear relatively similar with heterozygote frequencies of 0.16 and 0.17, respectively. The maternal heterozygous frequency was increased in the extended cohort but still remained lower than infant frequencies.

The genotype distribution in each of the sample groups was in Hardy-Weinberg equilibrium. There was a significant (p=0.038) increase in heterozygotes in the infant study group compared to the maternal study group. Further analysis revealed that the genotypes in the dominant model (t/t vs. t/c and c/c), between mothers and babies, approached the p=0.05 significance threshold (p=0.052).

d) Exon 7 3'UTR (479 t/c)

A single conformational variant was visualised in the exon 7 amplicon. A t/c transition was identified at nucleotide position 479 relative to the ATG start codon and 113bp downstream of the TAG stop codon.

This conformational variant was identified by Multiphor SSCP/HD gel electrophoresis (Figure 10), and was subsequently sequenced. No created or abolished restriction enzyme

recognition site was present at this particular position. Genotype and allele frequencies are shown in Table 18.

This polymorphism was submitted to the EMBL database and is now accessible with reference number **AM179832** (http://www.ebi.ac.uk/submission/webin.html).

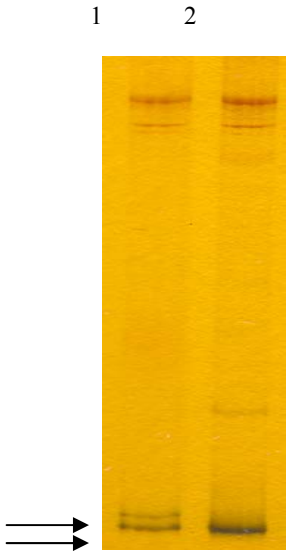


Figure 10: A photo of the conformational variant, +479 t/c, identified by Multiphor SSCP/HD gel electrophoresis. The conformational change (arrowed) was apparent in the heteroduplex component of the gel as indicated in lane 1.

Table 18: Genotype and allele frequencies of the +479 t/c polymorphism in cordblood samples of the pilot study together with the extended cohort of 120 maternal samples.

		Pilot study			Extended cohort	
		Cordblood	**Mothers**	**controls**	**Mothers**	**Controls**
TAC3	*No of alleles*	*26*	*20*	*18*	*120*	*18*
Exon 7	t/t	26 (1.00)	19 (0.95)	18 (1.0)	101 (0.84)	15 (0.83)
295 t-c	t/c	0	1 (0.005)	0	19 (0.16)	3 (0.17)
	c/c	0	0	0	0	0
	t	26 (1.00)	39 (0.98)	18 (1.0)	221 (0.92)	33 (0.92)
	c	0	1 (0.02)	0	19 (0.08)	3 (0.08)

The c allele was only identified in 1 maternal sample in the pilot study, at a frequency of 0.05, compared to none in the cordblood control samples. In the extended cohort, the maternal and control samples had similar heterozygous (t/c) frequencies and no homozygous (c/c) samples were present in either cohort.

All genotype distributions studied were in Hardy-Weinberg equilibrium. No significant differences were evident in any of the groups between genotype or allele frequencies.

3.3.2 *TACR3*

Seven amplicons were designed to cover the complete coding region of the gene, as well as flanking intron-exon boundaries. Of these seven amplicons, three presented with previously recorded variations.

a) Exon 1 -103 t/c (5' UTR)

A previously documented SNP (dbSNP: rs3733632) was identified in the first amplicon which encompasses the 5'UTR (-103 t/c). The conformational variant was first identified by Multiphor SSCP/HD gel electrophoresis (Figure 11a), and subsequently confirmed by automated sequencing (Figure 11b). Genotype and allele frequencies are shown in Table 19.

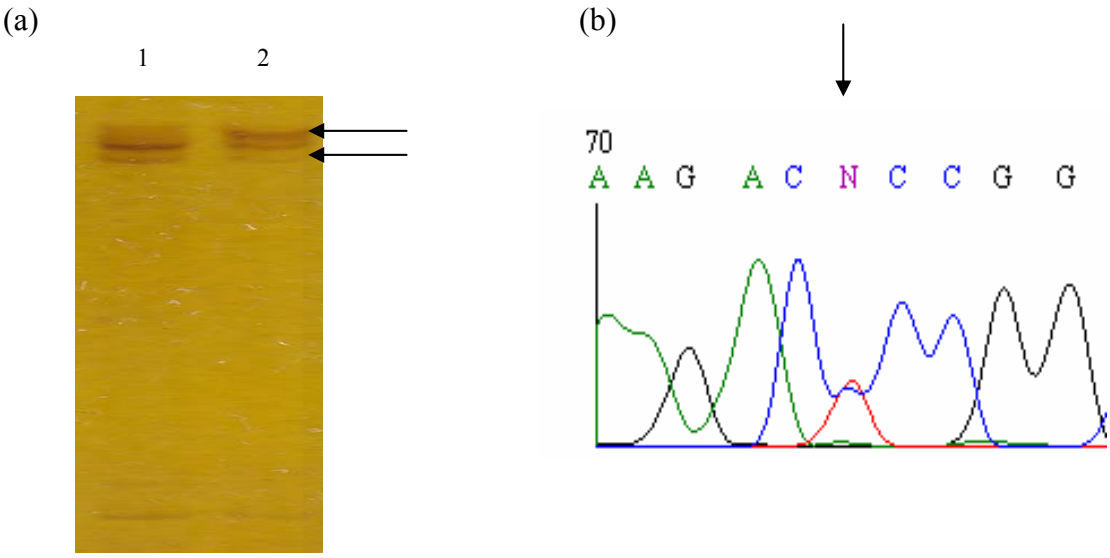


Figure 11: (a) Conformational variation observed on a Multiphor gel. The conformational change is evident in the SSCP component of the gel in lane 2 (arrowed), and (b) the corresponding variant confirmed by sequencing (forward orientation). A heterozygous t/c genotype is shown in the electropherogram (arrowed).

Using the "Restriction map" facility of Bioedit, a cutting site for the restriction enzyme *Nci I* (New England Biolabs, Ma, USA) was identified. The recognition site was created by the (t) allele and abolished by the variant (c) allele (Figure 12).

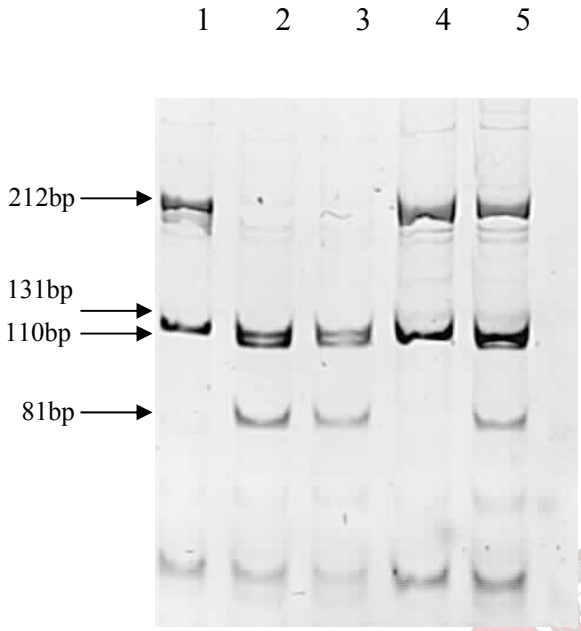


Figure 12: A photograph of the *Nci I* restriction digest resolved on a 5% polyacrylamide gel. Lanes 2 and 3 represent homozygous wildtype genotypes (t/t), lane 1 and 4 homozygote (c/c) genotypes and the remaining lane 5 a heterozygote (t/c) genotype.

Table 19: Genotype and allele frequencies of the -103 t/c polymorphism for cordblood samples of the pilot study together with the extended cohort of 120 maternal samples.

		Pilot study			**Extended cohort**	
		Cordblood	**Mothers**	**controls**	**Mothers**	**Controls**
TACR3	*No of samples*	*18*	*15*	*29*	*120*	*62*
Exon 1	t/t	0	0	2 (0.07)	6 (0.05)	11 (0.18)
-103 t/c	t/c	13 (0.72)	8 (0.53)	15 (0.52)	50 (0.42)	30 (0.48)
	c/c	5 (0.27)	7 (0.67)	12 (0.41)	64 (0.53)	21 (0.34)
	t	13 (0.36)	8 (0.23)	19 (0.33)	62 (0.26)	52 (0.42)
	c	23 (0.64)	22 (0.77)	39 (0.67)	178 (0.74)	72 (0.58)

In the pilot study cohort, the maternal samples (0.53) had similar heterozygote (t/c) frequencies as the controls (0.52). However, the cordblood samples seemed to have a higher heterozygous frequency of 0.72 but allele frequencies were not statistically different. In the extended cohort, the maternal and control samples had similar heterozygote frequencies (0.42 and 0.48), however the control samples had an increased frequency of homozygous wildtype (t/t) samples (0.18 vs.0.05) while the patients had an increased frequency of homozygous c alleles (0.53 vs. 0.34).

When comparing sample frequencies with database statistics, our maternal cohort (t = 0.26 and c = 0.74) differ from the frequencies reported in Europeans (t = 0.77 and c = 0.23) and Asians (t = 0.72 and c = 0.28), while corresponding closely to those reported in a sub-Saharan African cohort of 60 individuals (Hapmap-YRI), with frequencies of t = 0.12 and c = 0.88, possibly reflecting the "African" ancestry in our cohort. In contrast, our cohorts' heterozygous genotype frequency (t/c genotype of 0.42) resembles that of the European and Asians groups more (t/c genotype frequencies of 0.433 and 0.467, respectively), than that of the African group (t/c genotype of 0.13).

All the genotypes of the groups studied were in Hardy-Weinberg equilibrium. No significant differences were identified between genotype or allele frequencies in the pilot study samples groups. There was a significant difference evident between genotype (p=0.04) and allele (p=0.016) frequencies in the extended maternal and control study cohort. There were also significant differences in the dominant (t/t vs. t/c and c/c) and the recessive (c/c vs. t/c and t/t) models (p=0.0051 and p=0.0126), respectively.

b) Exon 3 R286K

A sequence variant in exon 3 which has been previously reported (dbSNP: rs2276973) was identified by multiphor SSCP/HD gel electrophoresis (Figure 13). Upon sequencing, the variant was identified as an a/g nucleotide substitution which resulted in the substitution of a lysine amino acid residue with a glycine residue, R286K. No restriction enzyme recognition site was created or abolished in the region of the variant. Samples were consequently genotyped according to multiphor patterns. Genotype and allele frequencies are shown in Table 20.

Using a bioinformatic programme, Conpred II, it was deduced that the variant is located within the intracellular region between the 5th and 6th transmembrane domains (Conpred II programme by Aria *et al*., 2004).

Figure 13: Conformational change shown on a Multiphor gel. The conformational change is present in the SSCP component of the gel in Lane 1 and 2 (arrowed).

Table 20: Genotype and allele frequencies of the R286K variant for cordblood samples of the pilot study together with the extended cohort of 120 maternal samples.

		Pilot study			**Increased Samples**	
		Cordblood	**Mothers**	**controls**	**Mothers**	**Controls**
TACR3	*No of alleles*	*18*	*20*	*21*	*120*	*21*
Exon3	t/t	18 (1.00)	19 (0.95)	19 (0.90)	111 (0.92)	19 (0.90)
R286K	t/c	0	1 (0.005)	2 (0.10)	9 (0.07)	2 (0.10)
	c/c	0	0	0	0	0
	t	18 (1.00)	39 (0.975)	40 (0.95)	231 (0.96)	40 (0.95)
	c	0	1 (0.005)	2 (0.05)	9 (0.04)	2 (0.05)

The cordblood and maternal pilot study samples showed very low heterozygote frequencies. In the extended cohort, these frequencies did not increase much in maternal or control samples. These low frequencies were very similar to the frequencies noted on electronic databases for European as well as African-American individuals. This confirms the conserved nature of this sequence variant.

The genotypes of all the sample groups were in Hardy-Weinberg equilibrium and no significant differences were evident when comparing any of the groups to one another.

c) Exon 5 +75 t/c

A sequence variant was identified in the second amplicon of exon 5 by multiphor SSCP/HD gel electrophoresis (Figure 14). Subsequent sequencing revealed a previously reported SNP (dbSNP: rs2765) which substitutes a t nucleotide for a c nucleotide. Genotype and allele frequencies are shown in Table 21.

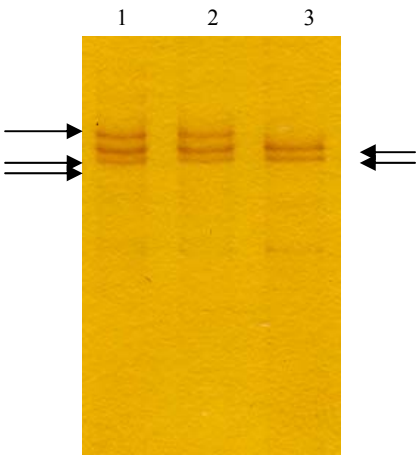


Figure 14: Conformational variant shown on a Multiphor gel. The conformational change is present in the SSCP component of the gel in lane 3 (arrowed).

The restriction enzyme recognition site for *BseN I* (Fermentas, Burlington, USA) was identified in the variant sequence (Figure 15).

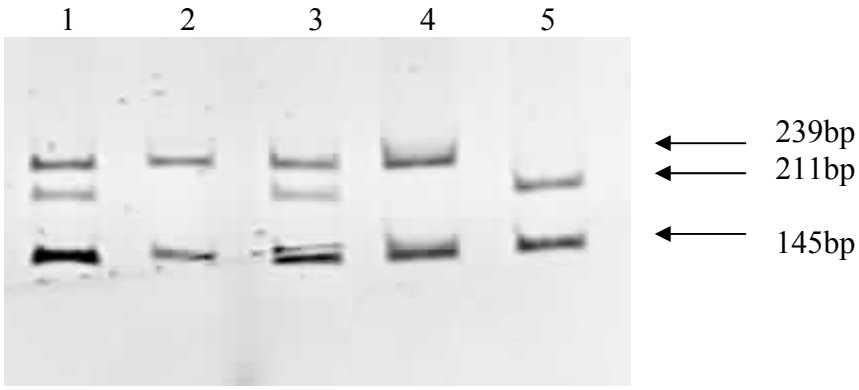


Figure 15: A photograph of the *BseN I* restriction digest. Lane 1 and 3 represent homozygous t/t genotypes, Lane 2 and 3 show heterozygote (t/c) genotypes and the remaining Lane 5 represents a homozygous (c/c) genotype.

Table 21: Genotype and allele frequencies of the +75t/c variant for cordblood samples of the pilot study together with the extended cohort of 120 maternal samples.

		Pilot study			**Increased Samples**	
		Cordblood	**Mothers**	**controls**	**Mothers**	**Controls**
TACR3	*No of alleles*	*12*	*12*	*13*	*120*	*13*
Exon 5	t/t	1 (0.08)	1 (0.08)	2 (0.15)	35 (0.29)	2 (0.15)
+75 t/c	t/c	7 (0.58)	7 (0.58)	6 (0.46)	57 (0.48)	6 (0.46)
	c/c	4 (0.33)	4 (0.33)	5 (0.38)	28 (0.23)	5 (0.38)
	t	9 (0.38)	9 (0.38)	10 (0.38)	127 (0.53)	10 (0.38)
	c	15 (0.62)	15 (0.62)	16 (0.62)	113 (0.47)	16 (0.62)

In the pilot study, the frequencies for all three genotypes were the same for mother and cordblood samples. These frequencies remained relatively constant throughout the extended cohort in maternal and control samples. In the maternal samples of the extended cohort, the homozygous

wildtype allele seemed to be present at higher frequencies (0.29) than in the control cohort (0.15) although this did not reach statistical significance.

All genotype distributions in the sample populations were in Hardy-Weinberg equilibrium. No genotype or allele frequencies were significantly different in the pilot or extended study cohorts.

3.4 In Summary:

Extension of the cohort showed differences for the genotype and allele frequencies for most of the variants. These differences did not, however, lead to new statistical findings, as most of the statistical differences were recognised between mothers and babies and these could not be disputed by the extended cohort as no infant samples were available. For the *TACR3* -103t/c variant, statistical difference was identified in the extended cohort and not the pilot study cohort. The differences in these two cohorts confirm the importance of adequate sample sizes. Increased patient and control populations reflect associations which are more likely to be true compared to smaller cohorts whose association can represent false positives. Therefore in association studies, population/sample sizes are of critical importance.

3.5 Transmission between mothers and babies

Genotypes of maternal samples were compared to those of the corresponding cordblood samples to track the transmission pattern of each *TAC3* and *TACR3* variant allele, as each parental allele has a 50% chance of being transmitted to the infant.

In the *TAC3* gene there were two mothers who were homozygous for the -25 c/t variant. One mother parented a heterozygous (c/t) infant while the other parented a homozygous (t/t) baby. The homozygous infant would have received one t allele paternally while the other infant would have only received the t allele maternally. No clear form of transmission was evident for this variant. Of the three mothers who were heterozygous for the IVS-53 t/c variant, none transmitted the c allele to their infants, resulting in all of the infants being homozygous for the wildtype t allele. It could possibly be deduced that this variant was also transmitted paternally. The S99P variant was present in seven infants who could only have received the allele paternally since none of their

mothers carried the variant. None of the mothers carrying the 295 t/c transmitted the c allele to their infants as there were no infants carrying this variant.

In the *TACR3* gene the -103 c/t variant was transmitted from all heterozygous mothers to their infants. This could be statistically significant since there is only a 50% chance of infants inheriting this allele and in our cohort there was 100% inheritance. However, it should be considered that the t allele was present in high frequencies within the patient and control cohorts. The R286K variant was only present in maternal samples and not in any infant samples and therefore not transmitted maternally. For the +75 t/c variant, it appears that all infants received the allele maternally, however, as with the -103 c/t variant the c allele was common within all the sample groups.

From the data obtained it is cannot be clearly deduced whether there is a bias in the transmission of any of the variants to infants, mainly due to small sample size. However, the IVS -53t/c, S99P, 295c/t and R286K variants could potentially be paternally inherited since none of the mothers of these infants carried these alleles. This can only be proven once fathers of these infants are screened for the relevant variants.

3.6 eNOS

The eNOS *Glu*298*Asp* variant was screened in 124 of the pre-eclamptic samples by restriction enzyme analysis (Hillermann *et al*., 2005) (Figure 16).

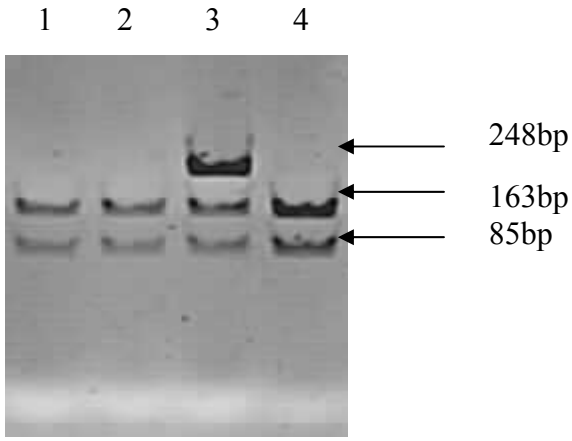


Figure 16: A photograph of the *BseN I* restriction digest analysis on a 5% polyacrylamide gel. Lane 1, 2 and 4 represent homozygous wildtype genotypes while Lane 3 represents a heterozygous genotype.

Table 22: Genotype and allele frequencies of the *Glu*298*Asp* variant for the maternal and cohorts.

		Mothers	**controls**
eNOS	*No of alleles*	*124*	*84*
	g/g	96 (0.77)	66 (0.78)
Glu298Asp	g/t	24 (0.19)	16 (0.19)
g/t	t/t	4(0.03)	2 (0.02)
	g	216 (0.87)	148 (0.88)
	t	32 (0.13)	20 (0.12)

Genotype as well as allele frequencies were very similar in maternal samples and control samples (p=0.935 and 0.762, respectively).

Genotypes of both the study cohorts were in Hardy-Weinberg equilibrium. No significant differences were evident between genotype or allele frequencies when the two cohorts were compared to one another.

In summary, it appeared that the eNOS variant *Glu298Asp* is not significantly associated with pre-eclampsia susceptibility *per se* in this particular population

3.7 Genotype: phenotype comparisons

Genotypes and allele frequencies for the *TAC3* and *TACR3* variants were determined in at least 120 samples from early-onset severe pre-eclampsia cases as well as mother-baby combinations. Comparisons were made between genotypic and clinical data to determine if there was a link between a specific genotype and clinical phenotype. Clinical data was retrieved, from questionnaires and clinical notes, by authorised personnel. Phenotypic outcomes viz, blood pressure, gestational age of complication onset and infant birthweight, were examined to determine whether they were associated with a particular genetic variant.

Significant associations were observed between the *TAC3* S99P variant and infants who were born below 1000g (<1000g, n=11 and >1000g, n=5). The S99P variant alleles showed a significant difference (p=0.043) when compared to infants below and above 1000g. Further analysis revealed that genotypes of the dominant model (homozygous t/t and heterozygous t/c vs. homozygous c/c) also differed significantly (p=0.05) between the two infant groups.

3.8 Biochemical analysis

To determine whether or not there was an association between a specific gene variant or group of variants on the DNA level and the NKB plasma levels, NKB measurements were obtained in 54 samples. Plasma samples were sent to collaborators to perform biochemical analysis using radioimmunoassay. Table 23 shows NKB levels of patient samples together with the relevant *TAC3* and *TACR3* variations they harbour.

Table 23: A composite table of 55 patient samples which were screened with their corresponding NKB levels.

NKB No	NKB value ng/L	-25 C/T	IVS3-53 G/T	S99P	479 C/T	-103 T/C	857 A/T	1471 T/C	eNOS G298A
12	162	1	1	1	1	2	1	2	1
13	60	1	1	1	1	3	2	2	1
14	112	1	1	1	1	2	2	2	1
15	80	1	1	2	1	3	1	1	1
16	86	2	1	1	1	3	1	2	1
17	50	1	1	1	1	3	1	2	1
18	62	1	1	1	2	3	1	3	1
19	88	3	1	1	1	2	1	2	2
20	94	1	1	1	1	2	1	3	1
21	58	1	1	1	1	2	1	3	1
22	52	1	1	1	1	2	1	2	1
23	52	1	1	1	2	2	1	3	1
24	92	2	1	1	1	2	1	3	1
25	48	2	1	1	1	2	1	1	n/a
26	60	1	2	1	1	2	1	1	n/a
27	100	1	1	1	1	3	1	2	n/a
28	148	1	1	1	1	1	1	3	1
29	58	1	1	2	2	3	1	2	1
30	72	2	1	1	1	n/a	1	1	n/a
31	72	1	1	1	1	3	1	2	1
32	80	2	1	1	1	3	1	1	1
33	52	2	1	1	1	3	1	2	1
34	308	1	1	1	1	3	1	1	1
35	66	1	1	1	1	2	1	2	1
36	48	1	1	1	1	3	1	2	1
37	326	1	1	1	1	3	1	2	2
38	50	1	1	1	1	3	1	2	1
39	328	1	1	1	1	2	1	3	1
40	328	2	1	1	1	1	1	2	1
41	64	1	1	n/a	1	3	1	2	1
42	320	1	1	1	2	2	1	1	1
43	52	1	1	1	1	3	1	2	1
44	64	1	1	1	1	3	1	2	2
45	332	1	1	n/a	1	n/a	1	n/a	1
46	322	1	1	1	1	3	1	n/a	1
47	270	1	1	n/a	1	3	1	1	n/a
48	332	1	1	n/a	1	3	1	n/a	n/a
49	328	1	1	n/a	1	3	1	n/a	n/a

Table 23: Continued

53	174	1	1	2	1	2	1	1	1
54	60	1	1	1	1	2	2	3	1
55	34	2	1	1	1	1	1	2	1
56	332	1	1	1	1	2	1	1	2
57	322	1	1	2	1	3	1	3	1
58	108	1	1	1	2	1	1	2	2
59	310	1	1	2	1	2	1	1	1
60	330	1	1	1	1	3	1	1	2
61	122	1	1	1	1	3	1	1	2
62	334	1	1	2	1	3	1	3	1
63	66	1	1	1	1	2	1	2	n/a
64	94	1	1	1	1	3	1	2	1
65	76	2	1	1	1	3	2	2	1
66	334	1	1	1	1	3	1	3	2
67	322	1	1	1	1	1	1	1	1
68	52	1	1	1	2	1	1	1	2
70	50	1	1	2	1	2	1	1	2

** 1=homozygous wildtype; 2= heterozygous; 3= homozygous variant; N/A=no amplification.

To identify whether any individual variant or combination of variants was directly associated with a specific range of NKB levels, the variants were considered in isolation with NKB levels and subsequently in combinations. A significant association (p=0.02) was evident, with the *TAC3* -25 c/t variant, when patients with NKB levels below 30ng/L were compared to patients with NKB levels above 30ng/L. When the other two variants were analysed, no association was identified [TACR3 -103t/c (p=0.26) and Glu298Asp g/t (p=0.39)].

3.9 NKB and eNOS

The *TAC3* -25 and *TACR3* -103 variants are located in gene regulatory regions (promoters) with the potential to significantly influence gene expression. In a recent publication by D'Anna (2004) it was speculated that eNOS activates the NK3 receptor for which NKB has the greatest affinity.

To investigate this interaction in our cohort, we studied three gene variants from these genes, in different combinations, in an attempt to identify any combinations which were more common in one cohort, if at all. These combinations were also analysed together with patient NKB levels to determine any association.

3.9.1 Genetic Association with circulating NKB levels

To simplify interpretation, genotypes at each locus were assigned a number, viz homozygous wildtype = 1, heterozygote = 2, homozygote variant = 3.

Results shown in the Table 24 reveal no combination of variants predispose to a significantly high level of circulatory NKB. The shaded rows represent the most common "haplotype" for each combination of variants. (*TACR3* -103t/c / *TAC3* -25c/t [3-1= 46%]; *TACR3* -103c/t / *eNOS* Glu298Asp g/t [3-1= 46%] and *TAC3* -25c/t / *eNOS* Glu298Asp g/t [1-1= 65%]. However, in all these cases it is evident that the NKB circulatory levels range from both extreme high to extreme low.

In the analysis of genotypes across all three loci (Table 25), of the possible combinations, 10 could be discerned. "Haplotypes" 3-1-1 and 2-1-1 were identified at frequencies of 36% and 25%, representing mean NKB levels of 84.2 and 16.05 ng/l, respectively.

Table 24: Dual-combined genotypes representing loci *TACR3* -103 / *TAC3*-25 / eNOS *Glu298Asp* determined in 52 patients with pre-eclampsia, in whom NKB levels had been measured. *Homozygous wildtype genotype = 1, heterozygote genotype = 2, homozygous variant genotype = 3.* The shaded row indicates the most common "haplotypes" identified.

Combined Genotype	**No. of individuals**	**[NKB] mean (ng/l)**	**[NKB] range (ng/l)**
***TACR3*-103t/c / *TAC3*-25c/t**			
1-1	4	69.65	21.4-202.2
1-2	1	18.2	N/A
2-1	15	64.8	9.6-211.8
2-2	2	17.7	15-20.4
2-3	1	25.2	N/A
3-1	24	91.38	16.8-267.8
3-2	5	55.48	21.2-180.4
***TACR3*-103t/c / *Glu298Asp* g/t**			
1-1	3	83.3	18.2-202.2
1-2	2	23.4	21.4-25.4
2-1	15	43.46	9.6-211.8
2-2	3	63.7	19-147
3-1	24	78.23	16.8-267.8
3-2	5	42.2	20.8-211
***TAC3*-25c/t / *Glu298Asp* g/t**			
1-1	34	77.6	9.6-267.8
1-2	9	68.3	19-198.6
2-1	8	41.37	15-180.4
3-2	1	25.2	N/A

Table 25: Combined genotypes representing loci *TACR3* -103 / *TAC3*-25 / *eNOS* Glu298Asp determined in 52 patients with pre-eclampsia, in whom NKB levels had been measured**.** *Homozygous wildtype genotype = 1, 2= heterozygote genotype, 3= homozygous variant genotype.* The shaded row indicates the most common "haplotypes" identified.

Genotypes	**No. of individuals**	**[NKB] mean (ng/l)**	**[NKB] range (ng/l)**
1-1-1	2	115.9	29.6-202.2
1-1-2	2	23.4	21.4-25.4
1-2-1	1	18.2	N/A
2-1-1	13	16.05	9.6-211.8
2-1-2	2	83	19-147
2-2-1	2	17.7	15-20.4
2-3-2	1	25.2	N/A
3-1-1	19	84.2	16.8-267.8
3-1-2	5	118.4	20.8-211
3-2-1	5	55.4	21.2-180.4

3.9.2 Allelic interaction analysis

a) Dual combinations

Based on the report by D'Anna *et al*. (2004) who speculated an interaction between NKB, NK3 and eNOS, genotypes in patients and controls were compared at three loci (*TAC3* -25c/t, *TACR3* -103t/c and *eNOS* Glu298Asp). Dual combinations (Table 26-28) as well as triplicate combinations (Table29) were analysed to identify whether a specific genotype combination showed significant interaction in patients vs. controls.

Table 26: Frequencies in patients vs. controls for the combined loci representing *TAC3* -25c/t and *TACR3* -103t/c for all possible genotype combinations. Patient frequencies are shown in upper triangle and control frequencies are shown in lower shaded triangle. Shaded blocks represent statistically significant combinations.

		***TAC3* -25c/t**		
		c/c	**c/t**	**t/t**
***TACR3* -103t/c**	**c/c**	0.47 / 0.25	0.06 / 0.06	0 / 0
	t/c	0.38 / 0.54	0.04 / 0.04	0.02 / 0
	t/t	0.03 / 0.07	0.01 / 0.02	0 / 0

When comparing dual genotypes between *TAC3* and *TACR3*, the *TAC3* -25 **c/c** and *TACR3* -103 **c/c** combination (highlighted in table) showed a statistically significant difference in patients and controls (p=0.003).

Table 27: Frequencies in patients vs. controls for the combined loci representing *TAC3* -25c/t and *eNOS* Glu298Asp g/t for all possible genotype combinations. Patient frequencies are shown in upper triangle and control frequencies are shown in lower shaded triangle.

		***TAC3* -25c/t**		
		c/c	**c/t**	**t/t**
Glu298Asp	**t/t**	0.02 / 0.04	0 / 0	0.01 / 0
	g/t	0.17 / 0.29	0.02 / 0.05	0.01 / 0
	g/g	0.68 / 0.54	0.09 / 0.07	0. / 0

Due the large amount of genotypes which were not represented in patients or controls (frequency=0), statistical analysis could not be performed on all the combinations. When comparing the combinations that were present, no statistically significant combinations were evident.

Table 28: Frequencies in patients vs. controls for the combined loci representing *TACR3* -103t/c and *eNOS* Glu298Asp g/t for all possible genotype combinations. Patient frequencies are shown in upper triangle and control frequencies are shown in lower shaded triangle.

Glu298Asp	***TACR3* -103t/c**		
	t/t	**t/c**	**c/c**
t/t	0 / 0.01	0.02 / 0.02	0.02 / 0.01
g/t	0.02 / 0.03	0.06 / 0.21	0.11 / 0.09
g/g	0.02 / 0.05	0.35 / 0.34	0.39 / 0.21

The *TACR3* -103 **t/c** and *eNOS* Glu298Asp **g/t** (highlighted in table) genotypes combination showed statistically significant when compared in patients and controls (p=0.013).

b) Triplicate combinations

Triplicate combinations were determined for the three loci by examining the *TAC3* and *TACR3* frequencies in combination with a specific *eNOS* (Glu298Asp) allele background.

Table 29: Frequencies in patients vs. controls for the combined loci representing *eNOS* Glu298Asp g/t, *TAC3* -25 c/t and *TACR3* -103t/c for all possible genotype combinations. Patient frequencies are shown in upper triangle and control frequencies are shown in lower shaded triangle.

eNOS –Glu298Asp g/g background

		***TAC3* -25c/t**		
		c/c	**c/t**	**t/t**
***TACR3* -103t/c**	**c/c**	0.17 / 0.34	0.04 / 0.04	0 / 0
	t/c	0.32 / 0.32	0.02 / 0.03	0 / 0.
	t/t	0.04 / 0.02	0 / 0.01	0 / 0

eNOS –Glu298Asp g/t background

		***TAC3* -25c/t**		
		c/c	**c/t**	**t/t**
***TACR3* -103t/c**	**c/c**	0.08 / 0.10	0.01 / 0.01	0 / 0
	t/c	0.20 / 0.04	0.02 / 0.01	0 / 0.01
	t/t	0.01 / 0.02	0.02 / 0.	0 / 0

eNOS –Glu298Asp t/t background

TACR3 -103t/c	*TAC3* -25c/t: c/c	c/t	t/t
c/c	0.02 / 0.01	0 / 0	0 / 0
t/c	0.01 / 0.02	0 / 0	0.01 / 0
t/t	0. / 0.01	0 / 0	0 / 0

When comparing the three loci for all the possible genotype combinations, statistical significance was only evident for *eNOS* Glu298Asp **g/t**, *TAC3* **c/c** and *TACR3* **t/c** (shaded in table) and showed a p value of 0.024. This combination was more prevalent in the control cohort compared to the patient cohort.

3.10 Summary

When comparing the *TAC3, TACR3* and *eNOS* loci in dual and triplicate combinations, various allelic interactions were identified. For the *TAC3* and *TACR3* genes, interaction was evident between the -25 **c/c** and -103 **c/c** genotypes. In the interaction analysis between *TACR3* and *eNOS*, -103 **t/c** and Glu298Asp **g/t** showed a significant association. In the triplicate analysis, interaction was evident between *eNOS* Glu298Asp **g/t**, *TAC3* **c/c** and *TACR3* **t/c.**

4. DISCUSSION

In this study, several polymorphisms were identified in the *TAC3* and *TACR3* genes, of which three are novel. These novel variants are located in exonic (S99P t/c), intronic (IVS3 -53g/t) and untranslated regions (+113t/c) of the *TAC3* gene. By identifying previously reported SNPs, the methodology used in this study is substantiated.

The S99P variant was identified in the coding region of exon 6. A statistically significant difference (p=0.038) was identified in the comparison of maternal and infant samples in the pilot study. Subsequently, no homozygous t genotype (t/t) could be identified in either of the maternal cohorts and the heterozygote frequency was higher in the infants when compared to the maternal samples. This t/c transition causes a substitution of a Serine with a Proline residue at amino acid position 99. This results in a hydrophilic to a hydrophobic change, which could potentially affect the nature of the gene product, and consequently, it's function. When analyzed using SIFT (Sorting Intolerant From Tolerant, http://blocks.fhcrc.org/sift/SIFT.html), this substitution is predicted to be intolerable. Exon 6 is highly conserved in humans and has >90% homology with *Macaca mulatta, Canis familiaris* and *Sus scrofa* (http://ww.ncbi.lm.nih.gov/BLAST/) indicating a possible functional role. This exonic variant also has the potential to affect the splicing signals for this transcript due to its position, 3bp from the intron/exon boundary. The variant either disrupts the recognition of the existing splicing unit at that junction (possibly leading to exon skipping and an altered transcript), or an additional recognition site is created, again leading to altered exon splicing and transcript generation. Motifs such as the GT and AG dinucleotides which are present at the 5' and 3' intron/exon boundaries determine accurate cleavage and rejoining. These are, however, not the only motifs and other consensus sequences have been derived.

An association (p=0.043) was observed between infants with birthweight below 1000g and the presence of the S99P variant (P-allele). There were no infants weighing above 1000g who carried the S99P allele in a homozygous state. This association was strengthened when the dominant model (t/t vs. t/c and c/c) for this variant was analysed (p=0.05).

Investigation of the transmission of these alleles, by analysing mother/cordblood combinations, revealed that the S99P variant was transmitted via the paternal lineage in 85% of the cases. In a study performed by Dunger *et al*, (2006) they reported that fetal genes, and

in particular paternally expressed genes, had an effect on fetal growth during pregnancy. The S99P variant allele, which is predominantly inherited paternally in this study, showed significant association with infant birthweight. It has been reported that changes in the expression of imprinted genes play a major role in the developmental programming and could affect prognosis for infants born small for gestational age (Fowden *et al*., 2006). Therefore, future studies should attempt to include maternal, paternal and infant samples in a larger cohort to investigate this phenomenon (which is currently based on "inferred" paternal genotypes).

The association demonstrated with the low birthweight could be due to the infant inducing a more severe form of complication in the mother, to which clinicians react by inducing delivery. This early delivery then reflects with a low infant birthweight as well as poor outcome.

Two previously reported variants identified in the *TAC3* and *TACR3* genes, -25c/t (dbSNP:2291855) and -103t/c (dbSNP: rs3733632), are interesting by virtue of their position. Both these polymorphisms are present in the 5'UTRs, close to the start codons and therefore could be positioned in the regulatory regions of the genes. Variation in the promoter region may exert an effect on the transcription of the gene and in turn, influence gene expression. Examples include uroporphyrinogen III synthase erythroid promoter variants which have been shown to cause erythropoeitic porphyria (Solis *et al*., 2001) and a single IL-3 promoter region SNP associated with rheumatoid arthritis (Yamada *et al*., 2001). Hoogendoom *et al*., 2003, reported that one third of promoter variants exert a functional effect on gene expression. Statistical significance was evident in our study (p=0.02) between NKB levels below 30ng/L and the *TAC3* -25c/t polymorphism.

It has been reported that NKB is present in higher levels in pre-eclamptic patients when compared to normotensive pregnant women (Page and Lowry, 2000) . These increased levels could be a result of increased gene expression, which in turn could be as a result of a regulatory error. Polymorphisms or mutations like the *TAC3* -25c/t and the *TACR3* -103t/c variants, could influence transcription factors and consequently affect transcriptional activity, thus causing alterations in circulating NKB levels. This, however, can only be proven by expression studies. An upstream region of up to 1000 bp could be included in the analysis on promoter activity and DNA footprinting, among other techniques, could be used to identify

conserved promoter sequences/motifs in this region. The idea is that evolutionary pressure keeps the regulatory patterns free of mutations, whereas the surrounding DNA without specific function are more likely to accumulate mutation (Pennacchio and Rubin, 2001).

In silico promoter analysis was attempted, however, no common motifs were identified with the various bioinformatic programs utilised. A proposed reason is that different programs search for different motifs and it is not always possible to select a specific motif in a given search. Therefore if a motif is present in a particular sequence, it may not be detected by certain programs, making it difficult to strengthen previous findings. Perhaps using these programs to identify known promoter regions with specific motifs is more trustworthy.

MC Promoter (http://www.geneatlas.org) is a program aimed at identifying the *exact* localization of eukaryotic RNA polymerase II transcription start sites, while CpG detector (http://www.ebi.ac.uk/emboss/cpgplot/) detects regions of genomic sequences that are rich in CpG dinucleotides. These patterns are important because such regions are resistant to methylation and tend to be associated with genes which are frequently switched on (Lyko, 2001). CpG islands hint at regions of generally low methylation (often hypomethylated) and therefore of open chromatin structure. They are associated with at least 50% of vertebrate promoters, but do not exist in non-vertebrate eukaryotes (Lyko, 2001). This occurrence along with other epigenetic effects could also alter the expression or regulation of a gene. Epigenetics causes changes in gene expression without a change in DNA sequence (Serman *et al*., 2006).

The association identified between the *TAC3*-25c/t variant and lower NKB level could be expected due to the position of the polymorphism relative to the start codon. This variant is predicted to be in the promoter region, and could therefore influence the efficacy of transcription, resulting in altered levels of NKB. Page and Lowry (2000a) reported increased NKB levels in pre-eclamptic pregnancies; a study that has been followed by many similar but controversial data. In this study, elevated NKB levels within the pre-eclamptic population was not evident, however, two peaks were identified in the distribution of high NKB levels.

The intronic variant identified in this study (IVS3-53 g/t) was located in intron 3. This polymorphism was only identified in three pre-eclamptic mothers and one control sample in the pilot study cohort as well as in two mothers in the extended cohort, and no infant samples.

There was a significant difference (p=0.048), for the genotypes in the dominant model (g/g vs. g/t and t/t), between infants and maternal samples in the pilot study cohort. This could potentially represent a biomarker, however, no restriction enzymes were found to recognize the specific variant and therefore Multiphor SSCP/HD was utilised to genotype this polymorphism. The individual genotypes, (g/g; g/t and t/t), at this locus could not be discerned on this gel system alone and therefore future genotyping should be performed using direct sequencing or allele specific PCR.

The nucleotide substitution was not situated within a recognized branch site of the intron-exon boundary, it could, however, still result in alterations of the mature mRNA levels and consequently contribute to disease (Padgett *et al*., 1986). Cartegni *et al,* (2002) described how polymorphisms in the 5'and 3'splice site can additionally lead to disease by disrupting a splice recognition site and preventing the removal of an intron. Krawczak *et al* (1992) estimated that 15% of all point mutations which result in human disease, cause splicing defects. Examples of human disease caused by a splice defect include neurofibromatosis type 1 and ataxia telangiectasia (Teraoka *et al*., 1999). In the study performed by Teraoka *et al*, (1999), northern blot analysis was utilized to determine whether the variant caused a splice alteration. mRNA position on the northern blot, depicted whether there was a size difference in the normal and variant samples. These intronic variant positions varied from 12bp to 112bp from the intron-exon boundaries, suggesting that intronic polymorphisms can alter splicing even if they are not situated in recognized branch sites or in close proximity to boundaries.

A bioinformatics program, Alternative Splice Site Predictor (ASSP) (http://es.embnet.org/~mwang/assp), was utilized to identify whether this variant created or abolished a splice recognition site (Wang and Marin, 2006). No splice variants were detected by this program for this particular intronic polymorphism, and consequently, no functional effect can be ascribed to this polymorphism, without further wet-bench analysis.

The +113t/c polymorphism identified in exon 7 of the *TAC3* gene was present at similar frequencies in patient (0.16) and control (0.17) cohorts. This variant could not be associated with pre-eclampsia or any related phenotype when performing genotype-phenotype analysis. The 3'UTR regions are thought to contribute to the stability of mRNA (Jackson and Standart 1990; Ross 1995) and this polymorphism should be investigated further since SSCP/HD

typing alone cannot distinguish individual genotypes at this locus and no restriction recognition site was identified at this position.

The sequence variant identified in exon 3 of the *TACR3* gene, 857a/g / R286K, occurred at low frequencies in the patient and control cohorts. This variant results in an amino acid substitution from R (glycine) to a K (lysine). When analysed with the SIFT program, this substitution was predicted to be tolerated. This could be as a result of no change in the phobicity or charge of the altered amino acid.

In the combined analysis of the *TAC3* -25c/t, *TACR3* -103t/c and *eNOS* -298g/t loci, no specific combination of genotypes showed a significant association with a specific level of NKB. Although some combinations appeared to be present more frequently than others, these genotypes represented all levels of circulating NKB (9.6 - 267.8 ng/L). However, when allelic interaction was sought and compared in patients and controls, significant interaction (p=0.024) was evident for the following allele combination: *TAC3* **c/c**, *TACR3* **t/c** and *Glu*298*Asp* **g/t**. This particular allele interaction appeared to be present at a higher frequency in controls (0.20) compared to patients (0.04). The interaction of these genotypes in this combination may therefore potentially exercise a protective effect in the control population. The stably maintained *TACR3* -103t allele could be due to a selective force, this allele may be advantageous and is therefore present in a higher frequency that the c allele.

In a study performed by Park *et al*, (2004) a polymorphism in the interleukin 3 gene (*IL-3*) was associated with decreased risk of asthma development. The association was based on a single gene variant, and the authors suggested that further analysis should be performed to elucidate the function of the variant. Although the association found in this study was between three gene variants, interaction between these genes, as well as the impact of each individual variant should be analysed in detail to reveal the influence that this genotype combination may have.

Identifying biomarkers for pre-eclampsia, poor pregnancy outcome as well as low birthweight is critical. Infants from these pregnancies require expensive neonatal care by highly trained staff. Mothers have to be educated regarding negative effects of alcohol and smoking, and the importance of fortification of foodstuffs such as mielie-meal and breads. These biomarkers should be present early in pregnancy to determine high risk pregnancies before the clinical

onset of any disease. Costs involved to test for such a marker should be kept as low as possible, as most women in this catchment area cannot even afford basic health care. Although the S99P polymorphism may present as a marker for poor pregnancy outcome and help test for susceptibility genotypes, testing *in utero* is costly and carries a risk for the unborn infant. Therefore, while this potential susceptibility marker will be communicated to clinicians, identifying a susceptibility biomarker in mothers is more appropriate.

In this study, Multiphor SSCP/HD, was utilised as the major mutation screening method. The sensitivity of this technique appeared to be good, however, amplicon sizes were restricted to 400bp. Sensitivity of this technique was increased by varying gel-running temperatures between 7^0C and 11^0C. In this study, the heteroduplex component of the gel proved useful for genotyping purposes and therefore samples were not run for longer than 110 minutes. Running samples for longer periods of time may have improved separation but would have excluded the use of heteroduplexes as they would have run off the gel. Another means of mutation screening that could be used in future studies is denaturing High Performance Liquid Chromatography (dHPLC). This technique makes use of larger amplicon sizes and can be adapted for high-throughput (Oefner and Underhill, 1995). Sequence variation can be confirmed by automated sequencing since this technique is not cost effective for screening large cohorts independent of other screening methods.

The HAPMAP (http://www.hapmap.org/) project data was utilised to compare genotype and allele frequencies from our cohorts to other populations. Mostly, similar frequencies were found for our population and the Sub-Saharan African population data reported. The few differences that were detected, could be as a result of comparing the Sub-Saharan African group with the South African coloured population. This was a novel study and therefore patient genotype frequencies at several of these loci cannot yet be compared with other studies.

Strengths of this study can be attributed to it being the largest novel study of its kind. This study was performed in a patient cohort from one particular population in which the onset of pre-eclampsia is common. The results obtained from this study can be utilised for future studies as reference for SNPs and patient genotype frequencies. A larger cohort of related maternal, paternal and infant samples would strengthen the transmission analysis. Collecting

NKB data as well as eNOS metabolite levels from all the patients in the cohort, together with the controls would allow for a better case-control study of these factors.

Many questions are directed at the impact that genetic case-control association studies have on the field of research. This method of analysis has not always led to insight into complex diseases. Better study design and use of bioinformatics tools could improve the validity of this approach (Campbell and Rudan, 2002). Whether variants showing an association with a disease truly confer susceptibility and knowledge about genetic variability should impact on the study design and improve bias. Bioinformatic tools allow us to broaden our insight and interpret results better. If studies are designed better and can be replicated, then even casual associations can be recognised as important.

While *eNOS* Glu298Asp has been identified in the Japanese population as a susceptibility marker for pre-eclampsia (Yoshimura et al., 2000; Kobashi et al., 2001) it has generated controversial results in other studies. In a follow-up study performed by Yoshimura *et* al, (2003), it was raised that although the NOS variant did show association in their earlier study, it did not increase the risk of pre-eclampsia in developing countries. In a meta-analysis performed by Yu *et al* (2006) no significant association was identified for women carrying the Asp298 allele and an increased risk for pre-eclampsia. In a study published by Hillermann *et al* (2005) eNOS was identified as a marker for *complicated* pre-eclamptic pregnancies only, in the coloured South African population. This finding was supported by this project which did not find an association between the independent eNOS variant and pre-eclampsia *per se*.

NKB levels have been measured in numerous populations and have lead to controversial observations. This study was designed as a novel study to screen the genes which encode NKB and its receptor, NK_3. An explanation for altered NKB level was sought by investigating the genes on a DNA level. Gene expression can be regulated on various levels including transcription, translation and post modification. To help identify high risk patients, a biomarker needs to be identified.

Sampling for any specific biomarker should be non-invasive, thereby causing limited risk to the mother and the foetus. NKB levels may be increased with gestation while other molecules, such as PP13 (placental protein 13), decrease during gestation. Should the biomarker represent a specific peptide or molecule with "altered expression", then it is

important to establish what causes the increase or decrease. These altering levels could be regulated on a DNA level or any of the other levels discussed. Identifying the regulation of these plasma concentrations is crucial in the understanding of their impact of the disease.

In most health care facilities around the world, a biomarker is of critical importance and could decrease the pressure that this disease places on these services. Timeous identification of high risk patients would have a large impact on maternal and foetal morbidity. Women who present with a high risk of developing pre-eclampsia could be subjected to bed rest and earlier treatment, thereby decreasing the high demand these patients require when admitted in a serious condition.

Currently there is no method of preventing pre-eclampsia (other than avoidance of pregnancy). There have been trials in which attempts to lower salt and protein intake and increase antioxidant and Vitamin C and E levels have been thought to reduce the risk of pre-eclampsia. These have recently been shown to not be as effective as previous pilot studies suggested (Romero and Garite, 2006). Low dosages of aspirin have also shown to have a preventative measure by decreasing the incidence of pre-eclampsia and foetal death by 19% and 16% respectively (Knight *et al.*, 2000).

In summary:-

- This study successfully characterised the *TAC3* and *TACR3* genes in a specific South Africa population group
- This study covered analysis of mother-infant transmission, case-control association, and allele interaction between *TAC3, TACR3* and *eNOS*
- Seven polymorphisms were identified, of which three were novel and subsequently added to public databases for use in other studies.
- Although none of these variants were directly associated with the risk of pre-eclampsia development, there was association with infant birthweight.

The most significant overall conclusion in the analysis of these genes is that none of the sequence variants identified underlie altered NKB levels measured in pre-eclamptic patients. This implies other levels of gene regulation at these loci and opens up new avenues of NKB research.

5. Future Studies

Future transmission studies should attempt to form a larger cohort of complete father, mother and infant triads. Transmission disequilibrium tests (TDT) can then be performed on the cohort and transmission can be tracked specifically via the paternal or maternal lineages (Deng and Chen, 2001). This is an important aspect of research as it is thought that paternal genes could be responsible for certain aspects of pre-eclampsia.

It has also been suggested that epigenetic mechanisms such as DNA methylation or imprinting play a role in pre-eclampsia. Analysis using various kits such as QIAGEN ™ epigenetic kits could reveal more about this predicted phenomenon. These kits make use of methylation specific PCR and sequencing,

Control and patient groups should be made as large as possible as this is imperative in case-control association studies. Patients should be well stratified (clinically) and all racial bias should be removed.

The two "promoter" variants, -25c/t and -103t/c should be characterised fully. This can be done by means of real-time RT-PCR, whereby mRNA levels can be measured based on the idea that the mRNA amplifies exponentially and therefore, samples with higher levels of mRNA before amplification will have higher concentrations at the end of the amplification process (Bustin, 2000). It can then be deduced that the samples with higher or lower concentrations, relative to the house-keeping genes, have an over or under-expression of the gene. Another means to test the transcriptional activity of a specific region is by utilising tissue culture and expression vector systems. This would reveal whether these variants have an effect on the expression of the gene, by using them to express a protein such as green fluorescent protein (GFP). Positive or negative results would reveal activity or lack thereof.

The intronic IVS-53 variant can be tested for functionality by using the minigene system (Stoss *et al*., 1999). The minigene system makes use of genomic fragments which include the alternative exon and the surrounding introns as well as the flanking constitutively spliced exons. Constructs derived by cloning are inserted into eukaryotic expression vectors and analysed for splice variations.

The S99P polymorphism should be analysed in a larger infant cohort to determine if the association identified with this variant and low birthweight persists.

To strengthen the NKB level data gathered in this project, a larger cohort of patients and NKB levels should be collected. NKB levels should also be analysed in gestation-matched controls to compare if they are increased or decreased. This cohort should be well stratified according to early and late onset pre-eclampsia as controversial reports have considered these to be two different diseases.

Although NKB gene polymorphisms have not been shown to be related to pre-eclampsia in this study, the detection assay developed, in this study, may be useful in future studies, possibly on other vasculopathies.

Understanding the aetiology of this multifactorial disease is of utmost importance. However, a lot of research remains to be done before we will completely comprehend pre-eclampsia. Research involving multidisciplinary approaches, such as combining genetic analysis with biochemical measurements and immunological assays should be sought. These approaches will strengthen findings and lead to a better understanding of this complex disease.

6. REFERENCES

Abi-Said D, Annegers JF, Combs-Cantrell D, Frankowski RF,Willmore LJ**.** Case-control study of the risk factors for eclampsia. *Am J Epidemiol* 1995; **142**: 437–441.

Anim-Nyame N, Sooranna SR, Steer PJ, Johnson MR. Longitudinal analysis of maternal leptin concentrations during normal pregnancy and pre-eclampsia. *Hum Repro* 2000; **15:** 2033-2036.

Ansel JC, Brown JR, Payan DG, Brown MA. Substance P selectively activates TNF-alpha gene expression in murine mast cells. *J Immunol*. 1993; **15;150(10):**4478-85.

Arngrímsson R, Purandare S, Connor M, Walker JJ, Bjornsson S, Soubrier F, Kotelevtsev YV, Geirsson RT, Bjornsson H. Angiotensinogen: a candidate gene involved in pre-eclampsia? *Nat Genet* 1993; **4:** 114-115.

Arngrímsson R, Connor JM, Geirsson RT, Brennecke S, Cooper DW. Is genetic susceptibility for pre-eclampsia and eclampsia associated with implantation failure and fetal demise? *Lancet* 1994; **343:** 1643-1644.

Arngrimsson R, Hayward C, Nadaud S, Baldursdottir A, Walker JJ, Liston WA, Bjarnado ttir RI, Brock DJ, Geirsson RT, Connor JM, Soubrier F. Evidence for a familial pregnancy-induced hypertension locus in the eNOS-gene region. *Am J Hum Genet* 1997; **61:**354–362.

Arngrimsson R, Sigurard ttir S, Frigge ML, Bjarnad ttir RI, Jonsson T, Stefansson H, Baldursdottir A, Einarsdottir AS, Palsson B, Snorradottir S, Lachmeijer AM, Nicolae D, Kong A, Bragason BT, Gulcher JR, Geirsson RT, Stefansson K. A genome-wide scan reveals a maternal susceptibility locus for pre-eclampsia on chromosome 2p13.*Hum Mol Genet* 1999; **8(9):**1799-805.

Ashour AM, Lieberman ES, Haug LE, Repke JT. The value of elevated second-trimester beta-human chorionic gonadotropin in predicting development of preeclampsia. *Am J Obstet Gynecol* 1997; **176(2):**438-42.

Baines MG, Duclos AJ, Antecka E & Haddad EK. Decidual infiltration and activation of macrophages leads to early embryo loss. *Am J Reprod Immunol* 1997; **37:** 471–477.

Baker PN, Kingdom JCP (eds): Pre-Eclampsia - Current Perspectives on Management. UK, 2004.

Barden, A. E., Herbison, C. E., Beilin, L. J., Michael, C. A., Walters, B. N. and Van Bockxmeer, F. M. Association between the endothelin-1 gene Lys198Asn polymorphism blood pressure and plasma endothelin-1 levels in normal and pre-eclamptic pregnancy. *J. Hypertens*. 2001; **19:** 1775–1782

Bashford MT, Hefler LA, Vertrees TW, Roa BB, Gregg AR. Angiotensinogen and endothelial nitric oxide synthase gene polymorphisms among Hispanic patients with preeclampsia. *Am J Obstet & Gynecol* 2001; **184:** 1345-1350.

Belardelli F. Role of interferons and other cytokines in the regulation of the immune response. *APMIS* 1995; **103:** 161–179.

Belo L, Gaffney D, Caslake M, Santos-Silva A, Pereira-Leite L, Quintanilha A, Rebelo I. Apolipoprotein E and cholesteryl ester transfer protein polymorphisms in normal and preeclamptic pregnancies. *Eur J Obstet Gynecol Reprod Biol.* 2004; **112(1):**9-15.

Benson G. Tandem repeats finder: a program to analyze DNA sequences. *Nuc Acids Res* 1999; **27(2):** 573-580.

Benyo Df, Miles TM, Conrad KP. Hypoxia stimulates cytoines production by the villous eolants from the human placenta. *J Clin Endocrinol Metab* 1997; **82:** 1582-1588.

Broughton Pipkin F. What is the place of genetics in the pathogenesis of pre-eclampsia? *Biol Neonate*. 1999; **76(6):**325-30.

Broughton Pipkin F. Risk factors for preclampsia. *The New Eng J for Med* 2001; **12:** 925-926.

Brown TA, Genomes. New York: John Willey, 1999.

Brownbell P, Bell NJ, Woods RJ, Lowry PJ, Page NM, Sibley CP. Neurokinin B is a paracrine vasodilator in the human fetal placental circulation. *J Clin Endocrinol Metab*. 2003; **88(5):**2164-70

Bustin, S.A., 2000. Absolute quanti.cation of mRNA using real-time reverse transcription polymerase chain reaction assays. *J. Mol. Endocrinol*. 25, 169–193.

Butt C, Zheng H, Randell E, Robb D, Parfrey P, Xie YG. Combined carrier status of prothrombin 20210A and factor XIII-A Leu34 alleles as a strong risk factor for myocardial infarction: evidence of a gene-gene interaction. *Blood*. 2003; **101(8):**3037-41.

Buttery LD, McCarthy A, Springall DR, Sullivan MHF, Elder MG, Michel T, Polak JM. Endothelial nitric oxide synthase in human placenta: Regional distribution and proposed regulatory role at the fetal-maternal interface. *Placenta* 1994; **15:**257–265.

Campbell H, Rudan I. Interpretation of genetic association studies in complex disease. *Pharmacogenomics J*. 2002;**2(6)**:349-60.

Cartegni L, Chew SL, Krainer AR. Listening to silence and understanding nonsense: Exonic mutations that affect splicing. *Nature Genetics* 2002; **3:** 285-297.
Chesley LC. History and epidemiology of preeclampsia-eclampsia. *Clin Obstet & Gynecol* 1984; **27:** 801-820.

Chen G, Wilson R, Wang SH, Zheng HZ, Walker JJ, McKillop JH. Tumour necrosis factor-alpha (TNF-alpha) gene polymorphism and expression in pre-eclampsia. *Clin and Experim Immunol* 1996; **104:** 154-159.

Chikosi AB, Moodley J, Pegoraro RJ, Lanning PA, Rom L. 5,10 methylenetetrahydrofolate reductase polymorphism in black South African women with pre-eclampsia. *Brit J Obstet and Gynaecol* 1999; **106:** 1219-1220.

Cintado CG, Pinto FM, Devillier P, Merida A, Candenas ML. Increase in neurokinin B expression and in tachykinin NK(3) receptor-mediated response and expression in the rat uterus with age.*J Pharmacol Exp Ther.* 2001; **299(3):**934-8.

Combs A, Katz MA, Kitzmiller JL, Brescia RJ. Experimental pre-eclampsia produced at chronic constriction of the lower aorta: Validation with longitudinal blood pressure measurements in conscious rhesus monkeys. *Am J Obstet Gynecol* 1993; **169:**215-223.

Cooper CA, Robinson G, Vinson GP, Cheung WT, Broughton Pipkin F. The localization and expression of the renin-angiotensin system in the human placenta throughout pregnancy. *Placenta* 1999; **20:**467–474.

Curnow KM, Pham T, August P. The L10F mutation of angiotensinogen is rare in pre-eclampsia. *J Hyperten* 2000; **18:** 173-178.

D'Anna R, Corrado F, Baviera G, Jasonni VM. Neurokinin B peptide levels in normal pregnancy. *Eur J Obstet and Gynecol and Reprod Bio* 2002; **130:** 101-102.

D'Anna R, Baviera G, Corrado F, Crisafulli A, Lentile R, Buemi M, Squadrito F. Neurokinin B and nitric oxide plasma levels in preeclampsia and isolated intrauterine growth restriction. *Inter J Gynecol and Obstet* 2004; **111:**1046-1051.

D'Orleans-Juste P, Claing A, Telemaque S, Warner TD, Regoli D. Neurokinins produce selective venoconstriction via NK-3 receptors in the rat mesenteric vascular bed. *Eur J Pharmacol* 1991; **204:** 329–334.

Daher S, Sass N, Oliveira LG, Mattar R. Cytokine genotyping in preeclampsia. *Am J Reprod Immunol*. 2006; **55(2):**130-5.

Davey DA, MacGillivray I. The classification and definition of the hypertensive disorders of Pregnancy. *Am J Obstet Gynecol* 1988; **158 (4):** 892-898.

De Groot CJ, Bloemenkamp KW, Duvekot EJ, Helmerhorst FM, Bertina RM, Van Der Meer F, De Ronde H, Oei SG, Kanhai HH, Rosendaal FR. Preeclampsia and genetic risk factors for thrombosis: a case-control study. *Am J Obstet & Gynecol* 1999; **181:** 975-980.

De Maart MP, Jansen MW, Hille ET, Vos HL, Bloemenkamp KW, Buitendijk S, Helmerhorst FM, Wladimiroff JW, Bertina RM, De Groot CJ. Preeclampsia and its interaction with common variants in thrombophilia genes. *J Thromb Haemost*. 2004; **2(9):**1588-93.

Dekker GA, de Vries JIP, Doelitzsch PM, Huijgens PC, von Blomberg BME, Jakobs PM, van Geijn HP. Underlying disorders associated with severe early-onset pre-eclampsia. *Am J Obstet Gynecol* 1995; **173:** 1042-1048.

Dekker GA, Sibai BM. Etiology and pathogenesis of preeclampsia: Current concepts. *Am J Obstet & Gynecol* 1998; **179:** 1359-1375.

Dekker GA, Sibai BM. The immunology of preeclampsia. *Semin Perinatol*.1999; **23(1):**24-33.

Dellovade TL, Merchenthaler I. Estrogen regulation of neurokinin B gene expression in the mouse arcuate nucleus is mediated by estrogen receptor alpha. *Endocrinology.* 2004; **145(2):** 736-42.

Deng HW, Chen WM. The power of the transmission disequilibrium test (TDT) with both case-parent and control-parent trios. *Genet Res.* 2001; **78(3):** 289-302.

DiFederico E, Genbacev O & Fisher SJ. Preeclampsia is associated with widespread apoptosis of placental cytotrophoblasts within the uterine wall. *Am J Pathol* 1999; **155:** 293–301.

Dizon-Townson DS, Major H, Ward K. A promoter mutation in the tumour necrosis factor alpha gene is not associated with preeclampsia. *J Reprode Immunol* 1998; **38:** 55-61.

Dizon-Townson DS, Nelson LM, Easton K, Ward K. The factor V Leiden mutation may predispose women to severe preeclampsia. *Am J Obstet & Gynecol* 1996; **175:** 902-905.

Dudding, T. E. and Attia, J. The association between adverse pregnancy outcomes and maternal factor V Leiden genotype: a meta-analysis. *Thromb. Haemostasis* 2004; **91:** 700–711

Duffield JS, Erwig LP, Wei X, Liew FY, Rees AJ & Savill JS. Activated macrophages direct apoptosis and suppress mitosis of mesangial cells. *J Immunol* 2000; **164:** 2110–2119.

Dunger DB, Petry CJ, Ong KK. Genetic variations and normal fetal growth. *Horm Res.* 2006; **65(3):** 34-40.

Eskenazi B, Fenster L, Sidney S. A multivariate analysis of risk factors for pre-eclampsia. *J Am Med Assoc* 1991; **266:** 237-241.

Fioramonti, J., Gaultier, E., Toulouse, M., Sanger, G.J., Bueno, L. Intestinal anti-nociceptive behaviour of NK3 receptor antagonism in conscious rats: evidence to support a peripheral mechanism of action. *Neurogastroenterology and Motility* 2003; **15(4):** 363–369.

Folgero T, Storbakk N, Torbergsen T, Oian P. Mutations in mitochondrial transfer ribonucleic acid genes in preeclampsia. *Am J Obstet Gynecol* 1996; **174:** 1626–1630.

Fowden AL, Sibley C, Reik W, Constancia M. Imprinted genes, placental development and fetal growth.*Horm Res*. 2006; **65(3):**50-8.

Friedman JM, Halaas JL. Leptin and the regulation of body weight in mammals. *Nature* 1998; **395:** 763-770.

Garban HJ & Bonavida B. Nitric oxide sensitizes ovarian tumor cells to Fas-induced apoptosis. *Gynecol Oncol* 1999; **73:** 257–264.

Garner PR, D'Alton ME, Dudley DK, Huard P, Hardie M. Preeclampsia in diabetic pregnancies. *Am J Obstet Gynecol*. 1990; **163(2):** 505-8.

Gecse A, Kis B, Mezei Z, Telegdy G. The effect of bradykinin and substance P on the arachidonate cascade of platelets. *Immunopharmacology*. 1996; **33(1-3):**167-70.

Genbacev O, DiFederico E, McMaster M & Fisher SJ. Invasive cytotrophoblast apoptosis in pre-eclampsia. *Hum Reprod* 1999; **14 (2)**: 59–66.

Germain AM, MacDonald PC, Casey ML. Endothelin receptor mRNAs in human fetal membranes, chorionic vessels, and decidua parietalis. *Mol Cell Endocrinol*. 1997; **132(1-2):**161-8.

Goldman-Wohl DS, Ariel I, Greenfield C, Hohcner-Celnikier D, Cross J, Fisher S, Yagel S. Lack of human leukocyte antigen-G expression in extravillous trophoblasts is associated with pre-eclampsia. *Mol Hum Reprod* 2000; **6:** 88–95.

GOPEC Consortium. Disentangling fetal and maternal susceptibility for pre-eclampsia: a British multicenter candidate-gene study. *Am J Hum Genet.* 2005; **77(1):**127-31.

Greenwood TA, Kelsoe JR. Promoter and intronic variants affect the transcriptional regulation of the human dopamine transporter gene. *Genomics* 2003; **82:** 511-520

Guo G, Wilton AN, Fu Y, Qiu H, Brennecke SP, Cooper DW. Angiotensinogen gene variation in a population case-control study of preeclampsia/eclampsia in Australians and Chinese. *Electrophoresis* 1997; **18:** 1646-1649.

Guo G, Lade JA, Wilton AN, Moses EK, Grehan M, Fu Y, Qiu H, Cooper DW, Brennecke SP. Genetic susceptibility to pre-eclampsia and chromosome 7q36. *Hum Genet* 1999; **105:** 641-647.

Haddad EK, Duclos AJ & Baines MG. Early embryo loss is associated with local production of nitric oxide by decidual mononuclear cells. *J Exp Med* 1995; **182:** 1143–1151.

Hager J, Clement K, Francke S, Dina C, Raison J, Lahlou N, Rich N, Pelloux V, Basdevant A, Guy-Grand B, North M, Froguel P. A polymorphism in the 5'untranslated region of the human ob gene is associated with low leptin levels. *Internat J of Obes* 1998; **22:** 200-205.

Hall D, Grove´ D, Carstens E. Early pre-eclampsia: What proportion of women qualify for expectant management and if not, why not*? Eur J Obstet Gynecol Reprod Biol* 2006 in press.

Hamai Y, Fujii T, Yamashita T, Nishina H, Kozuma S, Mikami Y & Taketani Y. Evidence for an elevation in serum interleukin-2 and tumor necrosis factor-alpha levels before the clinical manifestations of preeclampsia. *Am J Reprod Immunol* 1997; **38:** 89–93.

Hamasaki T, Masuzaki H, Miyamura T, Yoshimura S, Hamaguchi N, Ishimaru T. High concentrations of serum inhibin in pre-eclampsia*. Int J Gynaecol Obstet* 2000; **71(1):**7-11.

Hambartsoumian E, Srivastava RK, Seibel MM. Differential expression and regulation of inducible nitric oxide synthase (iNOS) mRNA in human trophoblasts in vitro. *Am J Reprod Immunol*. 2001; **45(2):**78-85.

Harrison GA, Humphrey KE, Jones N, Badenhop R, Guo G, Elakis G, Kaye JA, Turner RJ, Grehan M, Wilton AN, Brennecke SP, Cooper DW. A genomewide linkage study of preeclampsia/eclampsia reveals evidence for a candidate region on 4q. *Am J Hum Genet.* 1997; **60(5):**1158-67.

Hefler LA, Tempfer CB, Gregg AR. Polymorphisms within the interleukin-1 beta gene cluster and preeclampsia. *Obstet & Gynecol* 2001a; **97:** 664-668.

Higgins JR & Brennecke SP Pre-eclampsia – still a disease of theories*? Current Opinions in Obstet and Gynecol* 1998; **10:** 129–133.

Higgins JR, Kaiser T, Moses EK, North R, Brennecke SP. Prothrombin G20210A mutation: is it associated with preeclampsia? *Gynecologic and Obstetric Investigation* 2000; **50:** 254-257.

Hillermann R**,** Gebhardt GS, Isaacs R, Steyn DW, Odendaal HJ. Mutations in prothrombin and factor V genes do not contribute significantly to placental vasculopathy in a high-risk patient cohort in South Africa. *SA Med J* 2002; **92:** 526-527.

Hillermann R, Carelse K, Gebhardt GS. The Glu298Asp variant of the endothelial nitric oxide synthase gene is associated with an increased risk for abruptio placentae in pre-eclampsia. *J Hum Genet*. 2005; **50(8):**415-9.

Hoogendoom B, Coleman SL, Guy CA, Smith K, Bowen T, Buckland PR, O'Donovan MC Functional analysis of human promoter polymorphisms. *Hum Mol Genet* 2003; **(12)18**: 2249-2254.

Hubel CA, Roberts JM, Ferrel RE. Association of preeclampsia with common coding sequence variations in the lipoprotein lipase gene. *Clin Genet* 1999; **56:** 289–296.

Humphrey KE, Harrison GA, Cooper DW, Wilton AN, Brennecke SP, Trudinger BJ. HLA-G deletion polymorphism and preeclampsia/eclampsia. *British Journal of Obstetrics and Gynaecology* 1995; **102:** 707-710.

Hunt JS, Orr HT. HLA and maternal-fetal recognition. *FASEB J* 1992; **6:**2344–2348.

Irwin DE, Savitz DA, Hertz-Picciotto I, St Andre KA. The risk of pregnancy-induced hypertension: black and white differences in a military population. *Am J Pub Health* 1994; **84:** 1508-1510.

Jackson RJ, Standart N (1990) Do the poly (A) tail and 3' untranslated region control mRNA translation? Review. *Cell* **(62) 1:** 15-24.

Jansen I, Alafaci C, McCulloch J, Uddman R, Edvinsson L. Tachykinins (substance P, neurokinin A, neuropeptide K, and neurokinin B) in the cerebral circulation: vasomotor responses in vitro and in situ. *J Cereb Blood Flow Metab*. 1991; **11(4):**567-75.

Kaiser T, Brennecke SP, Moses EK. C677T methylenetetrahydrofolate reductase polymorphism is not a risk factor for preeclampsia/eclampsia among Australian women. *Hum Hered* 2001; **51:** 20-22.

Kaiser T, Brennecke SP, Moses EK. C677T methylenetetrahydrofolate reductase polymorphism is not a risk factor for preeclampsia/eclampsia among Australian women. *Hum Hered* 2001; **51:** 20-22.

Keen J, Lester D, Inglehearn C, Curtis A, Bhattacharya S. Rapid detection of single base mismatches as heteroduplexes on Hydrolink gels. *Trends in Genetics* 1991; **7:** 5.

Keller R, Geiges M & Keist R. Arginine-dependent reactive nitrogen intermediates as mediators of tumor cell killing by activated macrophages. *Cancer Res* 1990; **50:** 1421–1425.

Kim YJ, Williamson RA, Chen K, Smith JL, Murray JC, Merrill DC. Lipoprotein lipase gene mutations and the genetic susceptibility of preeclampsia. *Hypertension* 2001a; **38:** 992-996.

Knight M, Duley L, Henderson-Smart DJ, King JF. Antiplatelet agents for preventing and treating pre-eclampsia.*Cochrane Database Syst Rev*. 2000; **2.**

Knuist M, Bonsel GJ, Zondervan HA, Treffers PE. Risk factors for pre-eclampsia in nulliparous women in distinct ethnic groups: a prospective cohort study. *Obstet Gynecol* 1998; **92(2):** 174-178.

Kobari M, Tomita M, Tanahashi N, Yokoyama M, Takao M, Fukuuchi Y. Intravascular substance P dilates cerebral parenchymal vessels through a specific tachykinin NK1 receptor in cats. *Eur J Pharmacol.* 1996; **317(2-3):**269-74.

Kobashi G, Hata A, Shido K, Kato EH, Yamada H, Fujimoto S, *et al.* Association of a variant of the angiotensinogen gene with pure type of hypertension in pregnancy in the Japanese: implication of a racial difference and significance of an age factor. *American Journal of Medical Genetics* 1999; **86:** 232-236.

Kobashi G, Yamada H, Asano T, Nagano S, Hata A, Kishi R *et al.* Absence of association between a common mutation in the methylenetetrahydrofolate reductase gene and preeclampsia in Japanese women. *Am J Med Genet* 2000; **93:** 122-125.

Kobashi G, Yamada H, Ohta K, Kato E, Ebina Y, Fujimoto S. Endothelial nitric oxide synthase gene (NOS3) variant and hypertension in pregnancy. *Am J Med Genet*. 2001; **103**: 241–4.

Koga K, Osuga Y, Yoshino O, et al. Elevated serum soluble vascular endothelial growth factor receptor 1 (sVEGFR-1) levels in women with preeclampsia. J *Clin Endocrinol Metab* 2003; **88:** 2348-51.

Kosmas IP., Tatsioni A. Ioannidis JPA. Association of Leiden mutation in Factor V gene with hypertension in pregnancy and pre-eclampsia: a meta-analysis. *J. Hypertens*. 2003; **21**: 1221–1228.

Kosmas, I. P., Tatsioni, A. and Ioannidis, J. P.Association of C677T polymorphism in the methylenetetrahydrofolate reductase gene with hypertension in pregnancy and pre-eclampsia: a meta-analysis. *J. Hypertens*. 2004; **22:** 1655–1662.

Krauss T, Augustin HG, Osmers R, Meden H, Unterhalt M, Kuhn W. Activated protein C resistance and factor V Leiden in patients with hemolysis, elevated liver enzymes, low platelets syndrome. *Obstet & Gynecol* 1998; **92:** 457-460.

Kovats S, Main EK, Librach C, Stubblebine M, Fisher SJ, DeMars R. A class I antigen, HLA G, expressed in human trophoblasts. *Science* 1990; **248:** 220–223.

Krawczak M, Reiss J, Cooper D. The mutation spectrum of single base-pair substitutions in the mRNA splice juctions of human genes: causes and consequences. *Hum Genet* 1992; **90:** 41-54.

Kupferminc MJ, Eldor A, Steinman N, Many A, Bar-Am A, Jaffa A, Fait G, Lessing JB. Increased frequency of genetic thrombophilia in women with complications of pregnancy. *N Engl J Med* 1999; **340:** 9–13.

Lachmeijer AM, Armgrímsson R, Bastiaans EJ, Frigge ML, Pals G, Sigurdardóttir S, *et al.* A genome-wide scan for preeclampsia in the Netherlands. *Eur J Hum Genet* 2001a; **9:** 758-764.

Lachmeijer AM, Armgrímsson R, Bastiaans EJ, Pals G, Ten Kate LP, de Vries, *et al.* Mutations in the gene for methylenetetrahydrofolate reductase, homocysteine levels, and vitamin status in women with a history of preeclampsia. *Am J Obstet & Gynecol* 2001b; **184:** 394-402.

Lachmeijer AM, Crusius JB, Pals G, Dekker GA, Anrgrímsson R, ten Kate LP. Polymorphisms in the tumor necrosis factor and lymphotoxin-alpha gene region and preeclampsia. *Obstet & Gynecol* 2001c; **98:** 612-619.

Lachmeijer AMA, Dekker GA, Pals G, Aarnoudse JG, ten Kate LP, Arngrímsson R. Searching for preecalampsia genes: the current position. *Eur J Obstet & Gyneco and Repro Bio* 2002a; **105:** 94-113

Lade JA, Moses Ek, Guo G, Wilton AN, Grehan M, Cooper DW, *et al.* The *eNOS* gene: a candidate for the preeclampsia susceptibility locus? *Hypertens Pregnancy* 1999; **18:** 81-93.

Laivuori H, Kaaja R, Koistinen H *et al.* Leptin during and after preeclamptic or normal pregnancy: Its relation to serum insulin and insulin sensitivity. *Metabolism* 2000a; **49:** 259-263.

Laskowska M, Laskowska K, Vinson G, Leszczynska-Gorzelak B, Oleszczuk J. Evaluation of placental angiotensin type 1 receptors in women with hypertension during pregnancy. *J Matern Fetal Neonatal Med*. 2004; **16(4):**223-9.

Lecci, A., Maggi, C.A. Peripheral tachykinin receptors as potential therapeutic targets in visceral diseases. *Expert Opinion in Therapeutic Targets* 2003; **7(3):** 343–362.

Lee LC, Sheu BC, Shau WY, Liu DM, Lai TJ, Lee YH, Huang SC. Mid-trimester beta-hCG levels incorporated in a multifactorial model for the prediction of severe pre-eclampsia. *Prenat Diagn*. 2000; **20(9)**:738-43.

Levesque S, Moutquin JM, Lindsay C, Roy MC, Rousseau F. Implication of an AGT haplotype in a multigene association study with pregnancy hypertension. *Hypertension.* 2004; **43(1):**71-8.

Lewis I, Lachmeijer G, Downing S, Dekker G, Glazebrook C, Clayton D. Failure to detect linkage of preeclampsia to the region of the NOS3 locus on chromosome 7q. *Am J Hum Genet* 1999; **64:** 310-313.

Lie RT, Rasmussen S, Brunborg H, Gjessing K, Lie-Nielsen, Irgens LM. Fetal and maternal contributions to the risk of pre-eclampsia: Population-based study. *Br Med J* 1998; **316:**1343– 1347.

Liechti-Gallati S, Schneider V, Neeser D, Kraemer R. Two buffer PAGE system-based SSCP/HD analysis: a general protocol for rapid and sensitive mutation screening in cystic fibrosis and any other human genetic disease. *Eur J Hum Genet* 1999; **7:** 590-598.

Lim KH, Zhou Y, Janatpour M, McMaster M, Bass K, Chun SH & Fisher SJ. Human cytotrophoblast differentiation/invasion is abnormal in pre-eclampsia. *Am J Pathol* 1997;**151:** 1809–1818.

Linden DR, Chell MJ, El-Fakahany EE, Seybold VS. Neurokinin (3) receptors couple to the activation of neuronal nitric-oxide synthase in stably transfected Chinese hamster ovary cells. *J Pharmacol Exp Ther* 2000; **98**: 97−104.

Lindqvist PG, Svensson PJ, Dahlback B, Marsal K. Factor V Q506 mutation (activated protein C resistance) associated with reduced interpartum blood loss -- a possible evolutionary selection mechanism. *Thromb Haemost* 1998; **79:** 69-73.

Livingston JC, Barton JR, Park V, Haddad B, Phillips O, Sibai BM. Maternal and fetal inherited thrombophilias are not related to the development of severe preeclampsia. *Am J Obstet & Gynecol* 2001a; **185:** 153-157.

Livingston JC, Park V, Barton JR, Elfering S, Haddad B, Mabie WC, *et al.* Lack of association of severe preeclampsia with maternal and fetal mutant alleles for tumor necrosis factor alpha and lymphotoxin alpha genes and plasma tumor necrosis factor alpha levels. *Am J Obstet & Gynecol* 2001b; **184:** 1273-1277.

Lorentzen B, Endresen MJ, Clausen T, Henriksen T. Fasting serum free fatty acids and triglycerides are increased before 20 weeks of gestation in women who later develop preeclampsia. *Hypertens Pregnancy* 1994; **13:**103–109.

Lorentzen B, Henriksen T. Plasma lipids and vascular dysfunction in preeclampsia. *Semin Reprod Endocrinol* 1998; **16:** 33– 39.

Lunell NO, Nylund LE, Lewander R, Sarby B. Uteroplacental blood flow in pre-eclampsia measurements with indium-113m and a computer-linked gamma camera. *Clin Exp Hypertens B.* 1982; **1(1):**105-17.

Lyko F. DNA methylation learns to fly. *Trends Genet* 2001; **17:**169–172.

Maggi CA, Patacchini R, Meini S, Giuliani S. Nitric oxide is the mediator of tachykinin NK3 receptor-induced relaxation in the circular muscle of the guinea-pig ileum. *Eur J Pharmacol.* 1993; **240(1):**45-50.

Maggi, C.A. Principles of tachykininergic co-transmission in the peripheral and enteric nervous system. *Regulatory Peptides* 2000; **93 (1–3):** 53–64.

Margarit L, Griffiths A, Tsapanos V, Decavalas G, Gumenos D. Second trimester amniotic fluid endothelin concentration. A possible predictor for pre-eclampsia. *J Obstet Gynaecol*. 2005; **25(1):**18-20.

Markovic, N., Harger, G. Ness, R. B. Association between allelic variants in cytokine genes and preeclampsia. *Am. J. Obstet. Gynecol*. 2005; **193:** 209–215.

Martinez-Abundis E, Gonzalez-Ortiz M, Pascoe-Gonzalez S. Serum leptin levels and the severity of preeclampsia. *Arch Gynecol Obstet.* 2000; **264(2):**71-3.

Maruyama A, Nakayama T, Furuya K, Mizutani Y, Yamamoto, T. Association study between the human Renin gene and preeclampsia. Hypertens Pregnancy 2005; **24**: 39–48

Massi, M., Panocka, I., de Caro, G.The psychopharmacology of tachykinin NK-3 receptors in laboratory animals. *Peptides* 2000; **21 (11):** 1597–1609.

Mastrangelo D, Mathison R, Huggel HJ, Dion S, D'Orleans-Juste P, Rhaleb NE, Drapeau G, Rovero P, Regoli D. The rat isolated portal vein: a preparation sensitive to neurokinins, particularly neurokinin B. *Eur J Pharmacol* 1987; **134:** 321–326.

Maynard SE, Min JY, Merchan J, et al. Excess placental soluble fms-like tyrosine kinase 1 (sFlt1) may contribute to endothelial dysfunction, hypertension, and proteinuria in preeclampsia. *J Clin Invest* 2003; **111:** 649-58.

Meekins JW, Pijnenborg R, Hanssens M, McFadyen IR, van Assche A. A study of placental bed spiral arteries in normal and severe pre-eclamptic pregnancies. *Placenta* 1994; **15:** 511-524.

Melen E, Umerkajeff S, Nyberg F, Zucchelli M, Lindstedt A, Gullsten H, Wickman M, Pershagen G, Kere J. Interaction between variants in the interleukin-4 receptor alpha and interleukin-9 receptor genes in childhood wheezing: evidence from a birth cohort study. *Clin Exp Allergy*. 2006; **36(11)**:1391-8.

Mimuro S, Lahoud R, Beutler L, Trudinger B. Changes of resistance to activated protein C in the course of pregnancy and the prevalence of factor V mutation. *Aust NZ J Obstet Gynaecol* 1998; **38:** 200-204.

Mizuta A, Takano Y, Honda K, Saito R, Matsumoto T, Kamiya H. Nitric oxide is a mediator of tachykinin NK3 receptor-induced relaxation in rat mesenteric artery. *Br J Pharmacol*. 1995; **116(7):**2919-22.

S A Miller, D D Dykes, and H F Polesky. A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Res* 1988; February 11; **16(3):** 1215.

Morgan L, Baker P, Pipken FB, Kalsheker N. Pre-eclampsia and the angiotensinogen gene. *Br J Obstet and Gynaecol* 1995; **102:** 489-490.

Morgan T, Craven C, Ward K. Human spiral artery rennin angiotensin system. *Hypertension* 1998; 32: 683–687.

Morgan L, Craven C, Laouel JM, Ward K. Angiotensinogen Thr235 variant is associated with abnormal physiologic change of the uterine spiral arteries in the first-trimester deciduas. *Am J Obstet & Gynecol* 1999a; **180:** 95-102.

Morgan L, Crawshaw S, Baker PN, Broughton PF, Kalsheker N. Maternal and Fetal Angiotensinogen gene allele sharing in pre-eclampsia. *Br J Obstet and Gynaecol* 1999b; **106:** 244-251.

Morrison ER., Miedzybrodzka ZH, Campbell DM. Prothrombotic genotypes are not associated with pre-eclampsia and gestational hypertension: results from a large population-based study and systematic review. *Thromb. Haemostasis* 2002; **87:** 779–785

Moses EK, Lade JA, Guo G, Wilton AN, Grehan M, Freed K, *et al.* A genome scan in families from Australia and New Zealand confirms the presence of a maternal susceptibility locus for pre-eclampsia, on chromosome 2. *Am J Hum Genet* **67:** 1581-1585.

Mussap, C.J., Geraghty, D.P., Burcher, E. Tachykinin receptors: a radioligand binding perspective. *J Neurochem* 1993; **60 (6):** 1987–2009.

Myers RM, Lumelsky N, Lerman LS, Maniatis T. Detection of single base substitutions in total genomic DNA. *Nature* 1985; **313:** 495-498.

Nagy B, Toth T, Rigo JJ, Karadi I, Romics L, Papp Z. Detection of factor V Leiden mutation in severe pre-eclamptic Hungarian women. *Clin Genet* 1998; **53:** 478-481

Neela J, Raman L. Seasonal trends in the occurrence of eclampsia*. Natl Med J India* 1993; **6(1):**17-8.

Nielsen AH, Schauser KH, and Poulsen K. Current topic: the uteroplacental renin-angiotensin system. *Placenta* 2000; **21:** 468–477.

Norris LA, Higgins JR, Darling MR, Walshe JJ, Bonnar J. Nitric oxide in the uteroplacental, fetoplacental, and peripheral circulations in preeclampsia. *Obstet Gynecol* 1999; **93:** 958– 963.

O'Brien M, McCarthy T, Jenkins D, Paul P, Dausset J, Carosella ED, *et al*. Altered HLA-G transcription in pre-eclampsia is associated with allele specific inheritance: possible role of the HLA-G gene in susceptibility to the disease. *Cell Mol Life Sci* 2001; **58:** 1943-1949.

Oefner PJ, Underhill PA. Comparative DNA sequencing by denaturing high performance liquid chromatography (dHPLC). *Am J Hum Genet* 1995; **57:** (suppl:A266).

Orita M, Suzyki Y, Sekiya T, Hayashi K. Rapid and sensitive detection of point mutations and DNA polymorphisms using the polymerase chain reaction. *Genomics* 1989; **5:** 874-879.

O'Shaughnessy KM, Fu B, Ferraro F, Lewis I, Downing S, Morris NH. Factor V Leiden and thermolabile methylenetetrahydrofolate reductase gene variants in an East Anglian preeclampsia cohort. *Hypertension* 1999; **33:** 1338-1241.

Ozcan T, Rinder HM, Murphy J, Kohn C, Copel JA, Magriples U. Genetic thrombophilia and hypertensive complications of pregnancy. *Obstet & Gynecol* 97:S40 (2001).

Padgett R, Grabowski P, Konarska M, Seiler S, Sharp P. Splicing of messenger RNS precursors. *Annu Rev Biochem* 1986; **55:** 1119-1150.

Page NM, Lowry PJ. Is preeclampsia simply a response to the side effects of a placental tachykinin. *J Endocrinol* 2000a; **167:** 355-361.

Page NM, Woods RJ, Gardiner SM, Lomthaisong K, Gladwell RT, Butlin DJ, Manonda IT, Lowry PJ. Excessive placental secretion of Neurokinin B during the third trimester causes pre-eclampsia. *Nature* 2000b; **405:** 797-800.

Page NM, Woods RJ, Lowry PJ. A regulatory role for neurokinin B in placental physiology and preeclmpsia. *Regul Peptides* 2001; **98:** 97-104.

Page, N.M., Bell, N.J. The human tachykinin NK1 (short form) and tachykinin NK4 receptor: a reappraisal. *Eur J Pharmacol* 2002; **437 (1–2):** 27–30.

Palma Gamiz JL . Arterial hypertension and pregnancy: diagnostic criteria and therapeutic approach. *Revista Espanola de Cardiologica* 1998; **51:** 50–58.

Park BL, Kim LH, Choi YH, Lee JH, Rhim T, Lee YM, Uh ST, Park HS, Choi BW, Hong SJ, Park CS, Shin HD. Interleukin 3 (IL3) polymorphisms associated with decreased risk of asthma and atopy. *J Hum Genet*. 2004; **49(10):**517-27.

Patak EN, Pennefather JN, Story ME. Effects of tachykinins on uterine smooth muscle. *Clin Exp Pharmacol Physiol.* 2000; **27(11):**922-7.

Patak, E., Candenas, M.L., Pennefather, J.N., Ziccone, S., Lilley, A., Martı´n, J.D., Flores, C., Manteco´n, A.G., Pinto,F.M. Tachykinins and tachykinin receptors in human uterus. *Br J Pharmacol* 2003; **139 (3):** 523–532.

Pegoraro RJ, Chikosi A, Rom L, Roberts C. Moodley,J. Methylenetetrahydrofolate reductase gene polymorphisms in black South Africans and the association with preeclampsia. *Acta Obstet. Gynecol. Scand.* 2004; **83**: 449–454

Pennefather JN, Lecci A, Candenas ML, Patak E, Pinto FM, Maggi CA. Tachykinins and tachykinin receptors: a growing family. *Life Sci.* 2004; **74(12):**1445-63.

Pijnenborg R, Bland JM, Robertson WB, Brosens I. Utero-placental arterial changes related to interstitial trophoblast migration in early human pregnancy. *Placenta* 1983; **4:**397-414.

Pijnenborg R, Anthony J, Davey DA, Rees A, Tiltman A, Vercruyse L, Van Asssche FA. Placental bed spiral arteries in the hypertensive disorders of pregnancy. *Br J Obstet Gynaecol* 1998; **98:** 648-655.

Pennacchio LA, Rubin EM. Genomic strategies to identify mammalian regulatory sequences. *Nat Rev Genet* 2001; **2:**100–109.

Perianin A, Snyderman R, Malfroy B. Substance P primes human neutrophil activation: a mechanism for neurological regulation of inflammation. *Biochem Biophys Res Commun.* 1989; **161(2):**520-4.

Petraglia F, Sawchenko P, Lim AT, Rivier J, Vale W. Localization, secretion, and action of inhibin in human placenta. *Science*.1987; **237(4811):**187-9.

Plummer S, Tower C, Alonso P, Morgan L, Baker P, Broughton-Pipkin F, Kalsheker N. Haplotypes of the angiotensin II receptor genes AGTR1 and AGTR2 in women with normotensive pregnancy and women with preeclampsia. *Hum Mutat*. 2004; **24(1):**14-20.

Polliotti BM, Fry AG, Saller DN, Mooney RA, Cox C, Miller RK. Second-trimester maternal serum placental growth factor and vascular endothelial growth factor for predicting severe, early-onset preeclampsia.*Obstet Gynecol*. 2003;**101(6)**:1266-74.

Postovit LM, Adams MA, Graham CH. Does nitric oxide play a role in the aetiology of pre-eclampsia? *Placenta.* 2001 ;**22(A):**S51-5.

Powers RW, Minich LA, Lykins DL, Ness RB, Crombleholme WR, Roberts JM. Methylenetetrahydrofolate reductase polymorphism, folate, and susceptibility to preeclampsia. *J Soc Gynecol Invest* 1999; **6:** 74-79.

Raijmakers MT, Zusterzeel PL, Steegers EA, Peters WH. Hyperhomocysteinaemia: a risk factor for preeclampsia? *Eur J Obstet & Gynecol and Reprod Bio* 2001; **95:** 226-228.

Rajkovic A, Mahomed K, Rozen R, Malinow MR, King IB, Williams MA. Methylenetetrahydrofolate reductase. *Mol Genet Metab* 2000; **69:** 33-39.

Redman CW, Roberts JM. Management of pre-eclampsia. *Lancet* 1993; **341(8858):**1451-1454.

Redman C.W, Sargent IL. Current topic: pre-eclampsia and the placenta, *Placenta* 1991; **12:**301–308.

Redman CW, Sargent IL. Latest advances in unerstanding preeclampsia. *Science* 2005; **308:**1592-1594.

Regoli, D., Boudon, A., Fauchere, J.L. Receptors and antagonists for substance P and related peptides. *Pharmacol Rev* 1994; **46 (4):** 551–599.

Reister F, Frank HG, Heyl W, Kosanke G, Huppertz B, Schroder W, Kaufmann P & Rath W. The distribution of macrophages in spiral arteries of the placental bed in pre-eclampsia differs from that in healthy patients. *Placenta* 1999; **20:** 229–233.

Rigo Jr. J, Nagy B, Fintor L, Tanyi J, Beke A, Karadi I. Maternal and neonatal outcome of preeclamptic pregnancies: the potential roles of factor V Leiden mutation and 5,10 methylenetetrahydrofolate reductase. *Hypertens Pregnancy* 2000; **19:** 163-172.

Roberts JM, Taylor RN, Musci TJ, Rodgers GM, Hubel CA, McLaughin MK. Preeclampsia: an endothelial cell disorder. *Am J Obstet and Gynecol* 1989; **161:** 1200–1204.

Roberts JM. Endothelial dysfunction in preeclampsia. *SeminReprod Endocrinol* 1998; **16:** 5–15.

Roberts CB, Rom L, Moodley J, Pegoraro RJ. Hypertension-related gene polymorphisms in pre-eclampsia, eclampsia and gestational hypertension in Black South African women. *J Hypertens*. 2004; **22(5):**945-8.

Robillard PY, Hulsey TC, Alexander GR, Keenan A, de Caunes F, Papiernik E. Paternity patterns and risk of preeclampsia in the last pregnancy in multiparae. *J Reprod Immunol* 1993; **24:** 1-12.

Robillard PY, Hulsey TC, Perianin J, Janky E, Miri EH, Papiernik E. Association of pregnancy-induced hypertension with duration of sexual cohabitation before conception. *Lancet* 1994; **344:** 973-975.

Romero R, Garite TJ. Unexpected results of an important trial of vitamins C and E administration to prevent preeclampsia. *Am J Obstet Gynecol*. 2006; **194(5):**1213-4.

Ross J. mRNA stability in mammalian cells. *Microbiol Rev* 1995; **3(59)**: 423-50.

Saito S, Umekage H, Sakamoto Y, Sakai M, Tanebe K, Sasaki Y & Morikawa H. Increased T-helper-1-type immunity and decreased T-helper-2-type immunity in patients with preeclampsia. *Am J Reprod Immunol* 1999; **41:** 297–306.

Samadi AR, Mayberry RM, Reed JW. Preeclampsia associated with chronic hypertensionamong African American women and white women. *Ehn Dis* 2001; **11(2):** 192-200.

Saving Mothers (2002-2004) – Report on Confidential Enquiries into maternal Deaths in South Africa – Department of Health. Editor: Bob Patterson

Savitz DA, Zhang J. Pregnancy-induced hypertension in North Carolina, 1988 and 1989. *Am J Pub Health* 1992; **82:** 675-679.

Savvidou MD, Vallance PJ, Nicolaides KH, Hingorani AD. Endothelial nitric oxide synthase gene polymorphism and maternal vascular adaptation to pregnancy. *Hypertension* 2001; **38:** 1289-1293.

Scott JS. Pregnancy toxaemia associated with hydrops foetalis, hydatidiform mole and hydramnios. *J Obstet and Gynaecol Br Em* 1958; **65:** 689–701.

Serman A, Vlahovic M, Serman L, Bulic-Jakus F. DNA methylation as a regulatory mechanism for gene expression in mammals. *Coll Antropol.* 2006; **30(3):**665-71.

Shah DM. Role of the renin-angiotensin system in the pathogenesis of preeclampsia. *Am J Physiol Renal Physiol* 2005; **288(4):** F614-25.

Slatineanu S. [Pregnancy induced hypertension and renin-angiotensin system: how far is the truth?] *Rev Med Chir Soc Med Nat Iasi*. 2005;**109(4):**825-30.

Shaarawy M, Abdel-Magid AM. Plasma endothelin-1 and mean arterial pressure in the prediction of pre-eclampsia. *Int J Gynaecol Obstet*. 2000; **68(2):**105-11.

Sohda S, Arinami T, Hamada H, Yamada, N, Hamaguchi H, Kubo T. Methylenetetrahydrofolate reductase polymorphism and preeclampsia. *J Med Genet* 1997; **34:** 525-526.

Solis C, Aizencang GI, Astrin KH, Bishop DF, Desnick RJ. Uroporphyrinigen III synthase erythroid promoter mutations in adjacent GATA1 and CP2 elements cause congenital erythyropoeitic porphyria. *J Clin Inves* 2001; **107**: 753-762.

Stoss O, Stoilov P, Hartmann AM, Nayler O, Stamm S. The in vivo minigene approach to analyze tissue-specific splicing. *Brain Res Brain Res Protoc*. 1999; **4(3)**:383-94.

Strachan T abd Read AP. *Human molecular genetics 3rd edition*. UK 2004 p417

Suzuki Y, Tanemura M, Suzuki Y, Murakami I, Suzumori K. Is angiotensinogen gene polymorphism associated with hypertension in pregnancy? *Hypertens Pregnancy* 1999; **18:** 261-27.

Tàlosi G, Endreffy E, Tùri S, Nemeth I. Molecular and genetic aspects of preeclampsia: State of the art. *Mol Genet and Metab* 2000; **71:** 565-572.

Takimoto E, Ishida J, Sugiyama F, Horiguchi H, Murakami K, Fukamizu A. Hypertension induced in pregnant mice by placental rennin and angiotensiongen. *Science* 1996; **274:** 995-998.

Taylor RN, de Groot CJM, Cho YK & Lim K-H. Circulating factors as markers and mediators of endothelial cell dysfunction in preeclampsia. *Semin Reprod Endocrinol* 1998;**16:** 17–31.

Tempfer CB, Dorman K, Deter RL, O'Brien WE, Gregg AR. An endothelial nitric oxide synthase gene polymorphism is associated with preeclampsia. *Hypertens Pregnancy* 2001; **20:** 107-118

Teraoka SN, Telatar M, Becker-Catania S, Liang T, Onengut S, Tolun A, Chessa L, Sanal O, Bernatowska E, Gatti RA, Concannon P. Splicing defects in the ataxia-telangiectasia gene, ATM: underlying mutations and consequences. *Am J Hum Genet.* 1999; **64(6)**:1617-31.

Thornburg KL, Jacobson SL, Giraud GD, Morton MJ. Hemodynamic changes in pregnancy. *Semin Perinatol*. 2000; **24(1):**11-4.

Torbergsen T, Oian P, Mathiesen E, Borud O. Pre-eclampsia— A mitochondrial disease? *Acta Obstet Gynecol Scand* 1989; **68:** 145–148.

Trupin LS, Simon LP, Eskenazi B. Change of paternity: A risk factor for preeclampsia in multiparas. *Epidemiology* 1996; **7:**240–244.

Tsatsaris V, Goffin F, Munaut C. Overexpression of the soluble vascular endothelial growth factor in preeclamptic patients: pathophysiological consequences. *J Clin Endocrinol Metab* 2003; **88:** 5555-63.

Tsuchida K, Shigemoto R, Yokota Y, Nakanishi S. Tissue distribution and quantitation of the mRNAs for three rat tachykinin receptors. *Eur J Biochem* 1990; 193 **(3):** 751–757.

Vaillant P, David E, Constant I, Athmani B, Devulder G, Fievet P, Gondry J, Boulanger JC, Fardelone P, Fournier A. Validity in nulliparas of increased beta-human chorionic gonadotrophin at mid-term for predicting pregnancy-induced hypertension complicated with proteinuria and intrauterine growth retardation. *Nephron.* 1996; **72(4):**557-63.

Van Pampus MG, Dekker GA, Wolf H, Huijgens PC, Koopman MM, von BB *et al.,* High prevalence of haemostatic abnormalities in women with a history of severe preeclampsia. *Am J Obstet & Gynecol* 1999; **180:** 1146-1150.

Van Wijk MJ, Kublickiene K, Boer K & Van Bavel E. Vascular function in preeclampsia. *Cardio Res* 2000; **47:** 38–48.

Vitoratos N, Chrystodoulacos G, Kouskouni E, Salamalekis E, Creatsas. Alterations of maternal and fetal leptin concentrations in hypertensive disorders of pregnancy. *Eur JObstet & Gynecol and Reprod Biol* 2001; **96:** 59-62.

Watanabe H, Hamada H, Yamada N, Sohda S, Yamakawa-Kobayashi K, Yoshikawa H, Arinami T. Association analysis of nine missense polymorphisms in the coagulation factor V gene with severe preeclampsia in pregnant Japanese women. *J Hum Genet* 2002; **47:** 131-135.

Wang M. and Marín A. Characterization and Prediction of Alternative Splice Sites. *Gene* 2006; **366:** 219-227.

Ward K, Hata A, Jeunemaitre X, Helin C, Nelson L, Namikawa C *et al.* A molecular variant of angiotensinogen associated with preeclampsia. *Nat Genet* 1993; **4:** 59-61.

Wasserman WW, Fickett JW. Identification of regulatory regions which confer muscle-specific gene expression. *J Mol Biol* 1998; **278:**167–181.

Wichukchinda N, Nakayama EE, Rojanawiwat A, Pathipvanich P, Auwanit W, Vongsheree S, Ariyoshi K, Sawanpanyalert P, Shioda T. Protective effects of IL4-589T and RANTES-28G on HIV-1 disease progression in infected Thai females. *AIDS*. 2006; **20(2):**189-96.

Yallampalli C, Garfield RE. Inhibition of nitric oxide synthesis in rats during pregnancy produces signs similar to those of preeclampsia. *Am J Obstet Gynecol.* 1993; **169(5):**1316-20.

Yamada R, Tanaka T, Unoki M, Nagai T, Sawada T, Ohnishi Y, Tsunoda T, Yukioka M, Maeda A, Suzuki K, Tateishi H, Ochi T, Nakamura Y, Yamamoto K. Association between a single-nucleotide polymorphism in the promoter of the human interleukin-3 gene and rheumatoid arthritis in Japanese patients, and maximum-likelihood estimation of combinatorial effect that two genetic loci have on susceptibility to the disease. Am *J Hum Genet*. 2001; **68(3):**674-85.

Yanagisawa M, Kurihara H, Kimura S, Goto K, Masaki T. A novel peptide vasoconstrictor, endothelin, is produced by vascular endothelium and modulates smooth muscle Ca2+ channels. *J Hypertens Suppl* 1988; **6(4):** S188-91.

Yoshimura T, Yoshimura M, Tabata A, Shimasaki Y, Nakayama M, Miyamoto Y, Saito Y, Nakao K, Yasue H, Okamura H. Association of the missense Glu298Asp variant of the endothelial nitric oxide synthase gene with severe preeclampsia. *J Soc Gynecol Investig* 2000; **7(4):** 238-41.

Yoshimura T, Chowdhury FA, Yoshimura M, Okamura H. Genetic and environmental contributions to severe preeclampsia: lack of association with the endothelial nitric oxide synthase Glu298Asp variant in a developing country. *Gynecol Obstet Invest* 2003; **56(1):**10-3.

Yu CK, Casas JP, Savvidou MD, Sahemey MK, Nicolaides KH, Hingorani AD. Endothelial nitric oxide synthase gene polymorphism (Glu298Asp) and development of pre-eclampsia: a case-control study and a meta-analysis. *BMC Pregnancy Childbirth*. 2006; **16(6):**7.

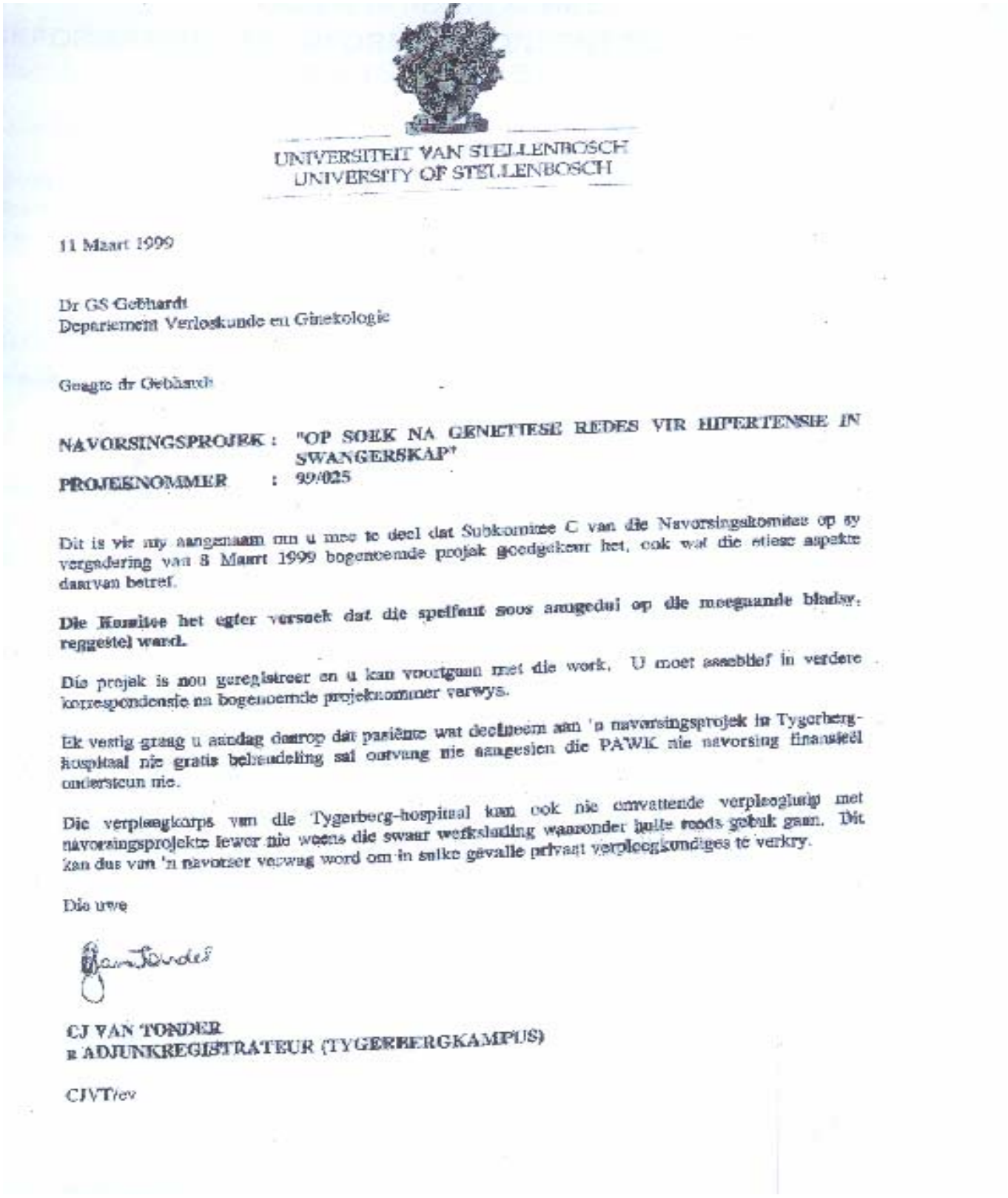
Zaahl MG, Winter TA, Warnich L, Kotze MJ. The -237C-->T promoter polymorphism of the SLC11A1 gene is associated with a protective effect in relation to inflammatory bowel disease in the South African population. *Int J Colorectal Dis*. 2006; 21(5):402-8.

Zhou Y, McMaster M, Woo K, et al. Vascular endothelial growth factor ligands and receptors that regulate human cytotrophoblast survival are dysregulated in severe preeclampsia and hemolysis, elevated liver enzymes, and low platelets syndrome. *Am J Pathol* 2002; **160:** 1405-23.

Zunker P, Happe S, Louwen F, Evers S, Ringelstein EB. Peripartal temporal course of endothelin 1, angiotensin II, and atrial natriuretic peptide in pre-eclampsia and normotensive pregnancy. *Fetal Diagn Ther*. 1998; **13(5):**309-14.

7. APPENDICES

Appendix 1: Study cohort ethical approval: C99/025



UNIVERSITEIT VAN STELLENBOSCH
UNIVERSITY OF STELLENBOSCH

11 Maart 1999

Dr GS Gebhardt
Departement Verloskunde en Ginekologie

Geagte dr Gebhardt

NAVORSINGSPROJEK : "OP SOEK NA GENETIESE REDES VIR HIPERTENSIE IN SWANGERSKAP"
PROJEKNOMMER : 99/025

Dit is vir my aangenaam om u mee te deel dat Subkomitee C van die Navorsingskomitee op sy vergadering van 8 Maart 1999 bogenoemde projek goedgekeur het, ook wat die etiese aspekte daarvan betref.

Die Komitee het egter versoek dat die spelfout soos aangedui op die meegaande bladsy, reggestel word.

Die projek is nou geregistreer en u kan voortgaan met die werk. U moet asseblief in verdere korrespondensie na bogenoemde projeknommer verwys.

Ek vestig graag u aandag daarop dat pasiënte wat deelneem aan 'n navorsingsprojek in Tygerberg-hospitaal nie gratis behandeling sal ontvang nie aangesien die PAWK nie navorsing finansieel ondersteun nie.

Die verpleegkorps van die Tygerberg-hospitaal kan ook nie omvattende verpleegsorg met navorsingsprojekte bewerkstellig weens die swaar werkslading waaronder hulle reeds gebuk gaan. Dit kan dus van 'n navorser verwag word om in sulke gevalle privaat verpleegkundiges te verkry.

Die uwe

CJ VAN TONDER
ADJUNKREGISTRATEUR (TYGERBERGKAMPUS)

CJVT/ev

Appendix 2: Patient consent form template

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

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.

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 ..... on .....20.....
(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 ..... on .....20.....
(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 ..... on .....20.....
(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

Appendix 3: Patient questionnaire template

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: |_____|

KREAT: |_____|

AST: |_____|

ALT: |_____|

LDH: |_____|

WCC: |_____|

HB: |_____|

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 4: Control cohort ethical approval: C050/2001

UNIVERSITEIT VAN STELLENBOSCH
UNIVERSITY OF STELLENBOSCH

25 April 2001

Dr GS Gebhardt
Departement Verloskunde en Ginekologie

Geagte dr Gebhardt

NAVORSINGSPROJEK: "DEVELOPMENT OF A DNA BANK TO SERVE AS A CONTROL GROUP FOR ONGOING AND FUTURE MOLECULAR STUDIES IN SEVERAL DISEASE-CAUSING MUTATIONS IN TANDEM WITH CAPACITY DEVELOPMENT OF A PROSPECTIVE CANDIDATE IN HEALTH SCIENCES"

PROJEKNOMMER : 2001/C050

Dit is vir my aangenaam om u mee te deel dat Subkomitee C van die Navorsingskomitee bogenoemde projek goedgekeur het op 26 April 2001, ook wat die etiese aspekte daarvan betref.

Die projek is nou geregistreer en u kan voortgaan met die werk. U moet asseblief in verdere korrespondensie na bogenoemde projeknommer verwys.

Ek vestig graag u aandag daarop dat pasiënte wat deelneem aan 'n navorsingsprojek in Tygerberg-hospitaal nie gratis behandeling sal ontvang nie aangesien die FAWK nie navorsing finansieel ondersteun nie.

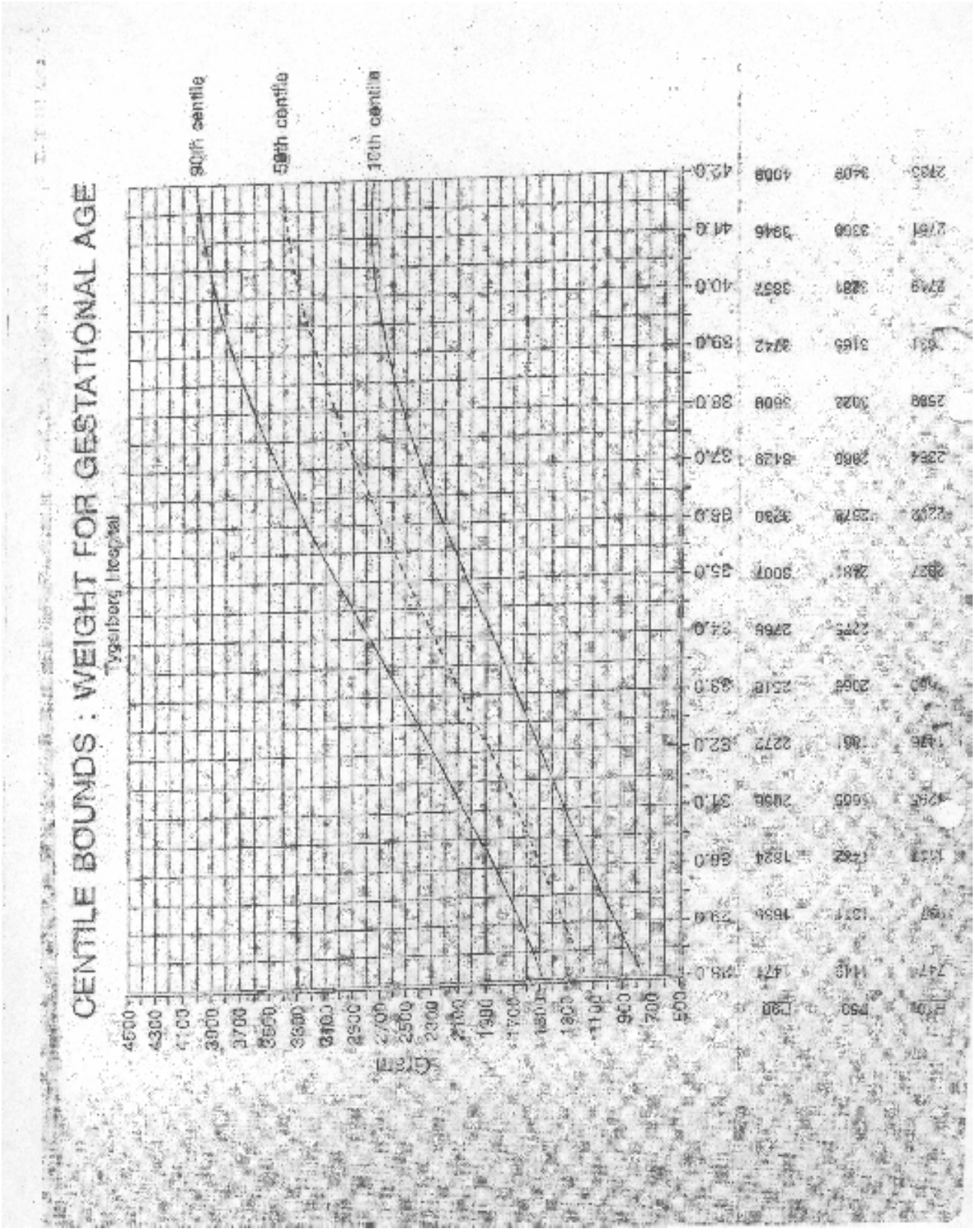
Die verpleegkorps van die Tygerberg-hospitaal kan ook nie omvattende verpleegbulp met navorsingsprojekte lewer nie weens die swaar werkslading waaronder hulle reeds gebuk gaan. Dit kan dus van 'n navorser verwag word om in sulke gevalle privaat verpleegkundiges te verkry.

Met vriendelike groete

CJ VAN TONDER
NAVORSINGSONTWIKKELING EN -STEUN (TYGERBERG)

CJVT/ev

Appendix 4: Fetal growth chart



Appendix 6: NCBI *TAC3* gene sequence annotation (Updated 1/10/2006)

Annotated sequence file:

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sequence.
ACCESSION   NC_000012 REGION: complement(55690051..55696592)
VERSION     NC_000012.10  GI:89161190
PROJECT     GenomeProject:168
KEYWORDS    HTG.
SOURCE      Homo sapiens (human)
  ORGANISM  Homo sapiens
            Eukaryota; Metazoa; Chordata; Craniata; Vertebrata;
Euteleostomi;
            Mammalia; Eutheria; Euarchontoglires; Primates; Haplorrhini;
            Catarrhini; Hominidae; Homo.
REFERENCE   1  (bases 1 to 6542)
  AUTHORS   Scherer,S.E., Muzny,D.M., Buhay,C.J., Chen,R., Cree,A.,
Ding,Y.,
            Dugan-Rocha,S., Gill,R., Gunaratne,P., Harris,R.A., Hawes,A.C.,
            Hernandez,J., Hodgson,A.V., Hume,J., Jackson,A., Khan,Z.M.,
            Kovar-Smith,C., Lewis,L.R., Lozado,R.J., Metzker,M.L.,
            Milosavljevic,A., Miner,G.R., Montgomery,K.T., Morgan,M.B.,
            Nazareth,L.V., Scott,G., Sodergren,E., Song,X.Z., Steffen,D.,
            Lovering,R.C., Wheeler,D.A., Worley,K.C., Yuan,Y., Zhang,Z.,
            Adams,C.Q., Ansari-Lari,M.A., Ayele,M., Brown,M.J., Chen,G.,
            Chen,Z., Clerc-Blankenburg,K.P., Davis,C., Delgado,O.,
Dinh,H.H.,
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            Jacob,L.S., Kelly,S.H., Li,L., Li,Z., Liu,J., Liu,W., Lu,J.,
            Maheshwari,M., Nguyen,B.V., Okwuonu,G.O., Pasternak,S.,
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            Waldron,L., Wang,Q., Williams,G.A., Zhang,J., Zhou,J.,
Allen,C.C.,
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David,R.,
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D'Souza,L.,
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            Nwaokelemeh,O.O., Nwokenkwo,S., Obregon,M., Oguh,M.,
```

```
Oragunye,N.,
            Oviedo,R.J., Parish,B.J., Parker,D.N., Parrish,J., Parks,K.L.,
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            White,F., Williamson,A.L., Wleczyk,R., Wooden,H.S.,
Wooden,S.H.,
            Yen,J., Yoon,L., Yoon,V., Zorrilla,S.E., Nelson,D.,
            Kucherlapati,R., Weinstock,G. and Gibbs,R.A.
  TITLE     The finished DNA sequence of human chromosome 12
  JOURNAL   Nature 440 (7082), 346-351 (2006)
   PUBMED   16541075
REFERENCE   2  (bases 1 to 6542)
  AUTHORS   International Human Genome Sequencing Consortium.
  TITLE     Finishing the euchromatic sequence of the human genome
  JOURNAL   Nature 431 (7011), 931-945 (2004)
   PUBMED   15496913
COMMENT     GENOME ANNOTATION REFSEQ:  Features on this sequence have been
            produced for build 36 version 2 of the NCBI's genome annotation
            [see documentation].
            On Mar 3, 2006 this sequence version replaced gi:51511728.
            The DNA sequence is part of the fourth release of the finished
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consultation
            with NCBI staff.
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using
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Origin

1-9000 Continuous sequence

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 9121 tttggcatcc tcaaaaacca aagagattag gtaatgtagt gtagaagaga gcagagcttt
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10021 CCGGCAGCAC CAGTGTGTGA GGGGAGCAGG CAGCGGTCCT AGCCAGTTCC TTGATCCTGC
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TAC3-Exon2 (10702-10820 -> 119bp)                              TOP
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      start
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10801 CTGGCGGGGG CCGCAGCAAG gtaagtctcc cctggcagag tactggggac atcacgggaa
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TAC3-Exon3 (12833-12926 -> 94bp)
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12961 ggcaggattc tgaagataag aggcctgg>ga gat<cctttca gatgggagag agatggggga
13021 tagcttagtg aatcggtgag ggttgtgatc tgaaccccgc tctcatcact ^ttccaacttc
(13071)(branch site - YNYYRAY)
TAC3-Exon4 (13116-13145 -> 30bp)
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13561 gacccatgtc tggggagatg cttcactttc tcatcatcac ^tgctgacaat gttggccctt
(13601)(branch site - YNYYRAY)
TAC3-Exon5 (13629-13682 -> 54bp) TOP
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13681 AGgtaggagt gtgtggaggt acagtggaag ggcttagggt actggcagag tatgacagaa
13741 gtcacgtgcc tcatatttgt cacca<gaggg aaagacagga cctttcttac cttcagtgag
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stop
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16141 cagtgggact tttttacagg atataaga^tg tgatttcagt g>tttttttt gttttgtttt
(16169)(branch site - YRYRAY)

TAC3-Exon7 (16216-16502 -> 287bp) TOP

16201 gttttttgtc ctcagTACTC CACTTCCGGA CTCCTGGAC^T ^GCATTAGGAA GACCTCTTTC
(16240-16377)(STS:D12S1906), (16241-16429)(STS:A004P28)
16261 CCTGTCCCAA TCCCCAGGTG CGCACGCTCC TGTTACCCTT TCTCTTCCCT GTTCTTGTAA
16321 CATTCTTGTG CTTTGACTCC TTCTCCATCT TTTCTACCTG ACCCTGGTGT GGAAACTGCA
16381 TAGTGAATAT CCCCAACCCC AATGGGCATT GACTGTAGAA TACCCTAGAG TTCCTGTAGT
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17461 tcaccgttaa ctggctgcct tagttattat aaagggaaaa catttatctc ccactcctct
17521 ctaaagtgcc tgttaccagc tcctgcagct ctgacttaac agtccccaga atgtgtaagg

17521-19921 Continuous sequence

Appendix 7: NCBI *TACR3* gene sequence annotation (updated 1/10/2006)

Annotated sequence file:

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LOCUS       NC_000004 130349 bp DNA linear   CON 30-AUG-2006
DEFINITION  Homo sapiens chromosome 4, reference assembly, complete
sequence.
ACCESSION   NC_000004 REGION: complement(104730074..104860422)
VERSION     NC_000004.10  GI:89161207
PROJECT     GenomeProject:168
KEYWORDS    HTG.
SOURCE      Homo sapiens (human)
  ORGANISM  Homo sapiens
            Eukaryota; Metazoa; Chordata; Craniata; Vertebrata;
Euteleostomi;
            Mammalia; Eutheria; Euarchontoglires; Primates; Haplorrhini;
            Catarrhini; Hominidae; Homo.
REFERENCE   1  (bases 1 to 130349)
  AUTHORS   International Human Genome Sequencing Consortium.
  TITLE     Finishing the euchromatic sequence of the human genome
  JOURNAL   Nature 431 (7011), 931-945 (2004)
   PUBMED   15496913
COMMENT     GENOME ANNOTATION REFSEQ:  Features on this sequence have been
            produced for build 36 version 2 of the NCBI's genome annotation
            [see documentation].
            On Mar 3, 2006 this sequence version replaced gi:51511464.
            The DNA sequence is part of the fourth release of the finished
            human reference genome. It was assembled from individual clone
            sequences by the Human Genome Sequencing Consortium in
consultation
            with NCBI staff.
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using
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evidence
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using
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evidence
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Origin

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(9986)(branch site - YNYYRAY)
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11641-70380 Continuous sequence

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(71036)(T/C)(dbSNP:7682099)
 71041 ccatggaaaa aaattatctt ccataaaacc gatccttggt gccaaaaggc tgggcaccgc
 71101 tgacttacat ctgttgatcc tcactttacc ccaagagatg ggtgttctta tttcaatttt
 71161 aaaaataata aagctgaggt tcaaaaagtt taaacaagtg tcagaataga atttgaacct
 71221 agatcttcct gattcctaga ctatgccatg attaccattc tacgctgcct ctactcaagc
 71281 agtccttatc aactggttt^c ccaaaagagc aatgactgta actattgaaa cgcctttgaa
       (71300)(C/A)(dbSNP:10516505)
 71341 ataccttga aactcc>attt caaactctgt tataaagaaa accaatgaaa tggttttgtt
```

TACR3-Exon2 (71414-71602 -> 189bp)

```
 71401 tgtttttata tagGTATATG GCTATTATTG ATCCCTTGAA ACCCAGACTG TCTGCTACAG
 71461 CAACCAAGAT TGTCATTGGA AGTATTTGGA TTCTAGCATT TCTACTTGCC TTCCCTCAGT
 71521 GTCTTTATTC CAAAACCAAA GTCATGCCAG GCCGTACTCT CTGCTTTGTG CAATGGCCAG
 71581 AAGGTCCCAA ACAACATTTC ACgtaagtta attctctatt atggttttca attcagttta
 71641 tcaaacattt a<ggaaactac agattaggag caacaattaa ataagactga catttccccc
 71701 agggttcata cattatttgg aaaaggaaat atataaaaat actgtaatat ggtatgattt
 71761 gtgtgtggtc aataagttgt gtaagttact aaggtttta attggaaata tgaggatgtc
 71821 tatgtgggat ggcactggag agtttcctga aggaagtgat aaaagataaa agcaatgtgc
 71881 aaagaaaagc attgtgttat tgattgagaa atgagtgaga aggaagaaat caaagcccaa
 71941 atgtgttgat tgctctttta ggaagtgtag ctgaagggaa ggagcaccct agtaggttat
 72001 ctaattttat tttggtttga tttggtttgt gttcttttgc ttgtctctag acaagcaaaa
 72061 agcattaaaa aacctgaaca ctagattgaa aaggaaatac aaatgctata atatatggta
 72121 aaatttggtt catattttat acccacatta aatagagcaa gaaggaataa taatgtaatt
 72181 gtgtaattta ttgcatttga tttttaaatt ataatcttaa attttatttt aataatgtac
 72241 ttatttttct attttactca ccaatttttc cctttaaaaa taacaaatgg tgcaaa^taat
       (72297)(T/G)(dbSNP:3822290)
 72301 ggttgccatt taaagcagtc attttatcta cctttgctct acccaaagtt cagatttttg
 72361 aacaaaaata ttaggaggca cttatttaag gaaaatcaga cagctcatat taaactgagt
 72421 accctattca tattattaat acatcttaat tgtatgataa tgttatcatc ttcaaattat
 72481 ttgttgactg aattatttcc tcattgactt taaatcaata cttagtaatc tactgagaaa
 72541 tggaaaggca aggatattta agattcactt tacaaaaaac aaaacaaaa^c aaaaaaa^aca
       (72590)(A/-)(dbSNP:11445973), (72598)(C/-)(dbSNP:3839188)
 72601 ctggtatgga gaagaccgtt ctaacaaact ttttaattac tttaaagctt tccaagacag
```

72601-73020 Continuous sequence

```
73021 tttatgtatg tctgatgttg cagcaataga gaagacagtc ctatatagga gaaaacatat
 73081 gccggttgat tcatttattc tatcccttcc cttttcctcc aatgatacat ttaattggtt
 73141 tagctcaaca attaccaagc agagtacata gcagtggaaa gtcgttcctc ctcaacctct
 73201 aggcaaggag aacttatgtt taactgagcc cttcaaccca gctatagttt tcatatacca
 73261 agtaaagatt gacaacatca actggcagca tttgaaacat aaatataagt gacaatagca
 73321 tgatattttg atggaatttt gagatgaatg tatagagata atgtagaaat aactcttggt
 73381 agttttaatt ^atgattaaa gctgagccaa ttcagt^cc>cc aatatttcat tccaataagg
       (73391)(G/A)(dbSNP:17033945), (73417)(branch site - YNYYRAY)
```

TACR3-Exon3 (73473-73623 -> 151bp)

73441 tcttcagcat gtgtttttct tatttttcat agTTACCATA TTATCGTCAT TATACTGGTG
73501 TACTGTTTCC CATTGCTCAT CATGGGTATT ACATACACCA TTGTTGGAAT TACTCTCTGG
73561 GGAGGAGAAA TCCCAGGAGA TACCTGTGAC A^AGTATCATG AGCAGCTAAA GGCCAAAAGA
(73592)(G/A)(dbSNP:2276973)
73621 AAGgtactgg tccatgttgt ttacctagca tttgtatagg ttatggtata tcagaaagaa
73681 aa<gcaatcta gtcatggcat gttaatacaa tatgatctga tgctctactc ctgtttttaa
73741 tgtctttgtt ttaatttttt ttcttttctt ttggttttgt ataagctgct gtccacatac
73801 tgtaatcagt ctgttatata atagttaatt cattacataa ggacttctta aaaattgcct
73861 tttgtaaagt attcttaatt actctgctga aattgctaaa atttcactat tccagttgat
73921 tgtatttttg caa^ctctatc tttctttgtc atttattgaa aagaatgcct ctgatgtatt
(73934)(G/C)(dbSNP:17033943)
73981 ctataatctg ctcaaggctt cttttatccc aggaatgaca tctcttctag aaagtgcaat
74041 aattttctaa tatacatcta tctttcctgt atcccctcgt ctcttttttat ttctctctca
74101 ctttctctgt gtgttctttc ccctgataag acattttata tgtatgtatg attaaaggat
74161 gaggattgta ttaatactat tctataattt attgagtgac attaaaaagg ctgtttctaa

74160- 137460 Continuous sequence

137461 tcataaaagc taatgcataa aacagttgca taaaccaata atctatttgc aaatctcaag
137521 ttagtatttt cctcacacta acgtgaatat ttacacataa tcactatttg gggaaatctt
137581 ccccaatatc agattataag agaaaccaaa ttaatattca taaggttacg tagtgagcag
137641 gctattaaag aaatctaaat agtcttaagc tatagtcagg ctatagaaga gtactgactt
137701 gaacaaaccc agccctagca gcctagtaaa cctccatagt gacttaacag ttgatttcct
137761 ggagataata tctccactat cgtacaatgc aggctacaca gccacatcct agttcctatc
137821 tctctctgtt ccaggctctc tgggtttttg taaacactga ttttcttcag gccaatcaaa
137881 atatatttgt ccgtgtttga gttagcacta cccattcact tagataggta gaaatgaata
137941 gtgacatagc ataatttctt gagacttacc ttaggcaact gtccgtatat tgcttcacca
138001 ttttttaata tattgactat tccctaggca tgatgtgtat ttaactgtat tggcagaaaa
138061 gcatgatttg c>aataagcta ttggaactat tatgagtatt atatttgtca aaaatgactt
TACR3-Exon4 (138134-138330 -> 197bp)
138121 tttttcttta tagGTTGTCA AAATGATGAT TATTGTTGTC ATGACATTTG CTATCTGCTG
138181 GCTGCCCTAT CATATTTACT TCATTCTCAC TGCAATCTAT CAACAACTAA ATAGATGGAA
138241 ATACATCCAG CAGGTCTACC TGGCTAGCTT TTGGCTGGCA ATGAGCTCAA CCATGTACAA
138301 TCCCATCATC TACTGCTGTC TGAATAAAAG gtaaaaacaa aacta^cgaaa tgcaagttgc
(138346)(T/C)(dbSNP:6822961)
138361 ttgtcacacc <caccttcact ggaaacatac aatgttgttc cattttcttg gttcaaattc
138421 aaaatgcaga aggaagggaa aatttaagaa ttcactgaga gaggcacagg ctttatcttt
138481 caagaccccc atacacactt tataaagcat cccacattaa ta^attagatg ccccaaataa
(138523)(G/A)(dbSNP:6847994)
138541 tgatcttggg gttctaactg gatgattctt ctatgggcca acttcttcct ccaaaatctc
138601 agaattttac ctgccaaaga aaaagaagtg ccctcatgat taaaggagaa ttgaaaatat
138661 gctcaaatcc tcaatgaaat gaagttactt cctagaaatt cttgaataat ttctataaga
138721 tggagagatg atggcatatg tgagcggact ttattaaaaa ggatatgttg taaataaata
138781 aactttgaag tagaaggttt tgtaaaaaca atgggaactc caatcctcct tcctgcattg

138782 – 138782 Continuous sequence

139381 gagactttaa tttataaatg tgtaatcatt tctgaaagaa actattttga aatatatcat
139441 ttttaagtgc cttaactggc cagcctaatt caattatatt tatgtaaatg tatacaatta
139501 ttttagaaaa atgtactgtt tctttaacat attgattacc tgatgctatg cattcgctat
139561 tgatgacttc tcactgatgg gaatgagatg cttttcattg ctgggaatca atgcttctca
139621 ttaatgggaa attaagattc aattcaaaca agaactaaac gactaacaac agtactgtga
139681 cataaattct aagagtctgg ctaaaaacca aactcaaaaa ctgagttgtt ataactgcct
139741 taggtagaat tttctgtggc agc>tgaaaga catatctgct tgaaaaaata acttttttctt
TACR3-Exon5 (139823-140349 -> 527bp)
139801 tctgtggcct gcttttcctc agATTTCGAG CTGGCTTCAA GAGAGCATTT CGCTGGTGTC
139861 CTTTCATCAA AGTTTCCAGC TATGATGAGC TAGAGCTCAA GACCACCAGG TTTCATCCAA
139921 ACCGGCAAAG CAGTATGTAC ACCGTGACCA GAATGGAGTC CATGACAGTC GTGTTTGACC
139981 CCAACGATGC AGACACCACC AGGTCCAGTC GGAAGAAAAG AGCAACGCCA AGAGACCCAA
140041 GTTTCAATGG CTGC>TCT<CGC AGGAATTCCA AATCTGCCTC C^GCCACTTCA AGTTTCATAA

(140082)(G/A)(dbSNP:17033889)
140101 GCTCACCCTA TACCTCTGTG GATGAATATT CT^**TAA**TTCCA TTTCCTGAGG TAAAAGATTA
stop
140161 ^GGTGTGAGACC ATCATGGT^GC CAGTCTAGGA CCCCATTCTC CTATTTA^CCA GTCCTGTCCT
(140161-140273)(STS:WI-9174), (140179-140328)(STS:WI-18877),
(140208)(T/C)(dbSNP:2765)
140221 ATATACCCTC TAGAAACAGA AAGCAATTTT TAGGCAGCTA TGGTCAAATT GAGAAAGGTA
140281 GTGTATAAAT GTGACAAAGA CACTAATAAC ATGTTAGCCT CCACCCAAAA TAAAATGGGC
140341 TTTAAATTTa ttctttgaaa actctaaatt attatatgca atgaacaaaa atat<gtcagg
140401 aaaatacttg taaactcgcc agtctatctc atttacaaat tgcaatatac atttgtgaca
140461 ttaaaatgat atatagtttt tccaagagat taaagaatct ttaaaacata atattgtaag
140521 tgaagggaaa caaaatctgt ^ataaaatctg tataaaatag gctttttgc ctagagatat
(140541)(G/A)(dbSNP:13148229)
140581 aaaatgggaa aaaaagttaa atgattcatt ttcccactag agtaatggaa aattaacctc
140641 aagaagtagg aactgaaaat ctttgctcaa gaaaactcat tttgtagaat gtaaagattt
140701 aaagatttaa agatttcttc catggcattt aactgaaaag gaaatctagt taaatcatta
140761 ggcaaatgta tatttcattc tcttgatttt ttttctgaga aagtgaaatt ttaaaaaatt
140821 atatactttt tgaccattac ttatttttga ataccaagct aaaaagtagt cagttaagac
140881 cctaaaaata tgaagaaaat tcctatcatt ttttatttt cacagatttt ttaaatagct
140941 agattataaa acagtaaagg tacattccaa taaatagagg aagaatacta tttatgttct
141001 cttcctctcc cctcactgaa aatagaataa ggactaggag aaaatatctt caaaggcaac
141061 tccttaggga aaaaaagtca caagtgtgac aaaag^actat atggttgaaa aataccaata
(141096)(C/A)(dbSNP:7438251)
141121 tatgttacat atgagtaaac taggcaaggt gtagacaagt gaattacctc ttagaaaacg

Appendix 8: Rapid DNA Extraction protocol (Kit)

Rapid DNA Isolation from 300µl whole blood:

Puregene® DNA Isolation Kit (Gentra Systems™ Minneapolis, USA)

Cell Lysis

- Add 300µl whole blood to 900µl RBC lysis solution and incubate for 1 min at room temperature; invert gently 10 times during incubation.
- Centrifuge for 20s at 13 000-16 000g. Remove supernantent (leave ~10µl liquid) and vortex to rsuspend the pellet.
- Add 300µl Cell Lysis Solution and pipette up and down.

Protein Precipitation

- Add 100µl Protein Precipitation Solution and vortex at high speed for 20s. Centrifuge for 1 min at 13 000-16 000g.

DNA Precipitation

- Transfer supernatant to clean Eppendorf tube containing 300µl 100% isopropanol and mix by inverting samples 50 times
- Centrifuge at 13 000-16 000 g for 30s
- Pour of supernatant and drain tube on clean absorbent paper. Add 300 µl 70% ethanol and invert tube several times to wash pellet
- Centrifuge at 13 000-16 000g for 60s. Carefuly pour off ethanol and invert and dain tube for 30min on clean paper towl.

DNA Hyration

- Add 20µl DNA Hydration Solution and dissolve for 30 min. Store at 4^{o}C

Appendix 9: DNA Extraction protocol

Extractions Buffers and solutions

Nuclear lysis buffer (1L, pH 8.2 set with 1M NaOH, store at 4°C)

1.211g	Tris-Cl	0.01M
23.4g	NaCl	0.4M
0.6g	EDTA	0.002M

Cell lysis Buffer (1L, pH 7.4 set with concentrated HCl, store at 4°C)

8.3g	NH_4Cl	0.155M
1.1g	$KHCO_3$	0.01M
0.03g	EDTA	0.0001M

Phosphate buffered saline (PBS) (1L, store at 4°C)

2g	KCl	0.027M
8g	NaCl	0.137M
1.14g	Na_2HPO_4	0.008M
0.2g	KH_2PO_4	0.0015M

10% SDS (100mL, Store at room temperature to prevent precipitation)

10g	SDS

Work in vapour hood

Proteïenase K (store at -20°C)

10mg/mL diluted in dH_2O, aliquot in 1.5mL eppendorf tubes

6M NaCl (500mL, saturated solution)

175.32g	NaCl

Genomic DNA Extractions from Blood:

- Place ~10ml blood in a 50ml Falcon tube
- Add 3ml cold Lysis Buffer
- Place on ice for 15 min, shaking each 5 min interval
- Centrifuge @ 1500rpm for 10min
- Pour off supernatant , keeping pellet
- Add 10ml PBS and mix
- Centrifuge @ 1500rpm for 10min
- Pour off supernatant, keeping pellet
- Dissolve pellet in : 3ml Cell – Lysis buffer

  30µl Proteinase K (10mg/ml)

  300µl 10% SDS
- Mix well and incubate overnight in 55°C waterbath

Day 2:

- Add 1ml 6M NaCl and shake for 1min
- Centrifuge @ 3500rpm for 30 min
- Transfer supernatant to new tube and shake for 15s
- Centrifuge @ 2500rpm for 15 min
- Transfer supernatant to new tube without foam or pellet
- Add 2 volumes ice cold 100% ethanol to precipitate DNA
- Scoop out DNA and place in eppi with 500µl 70% ethanol
- Centrifuge @ 14 000rpm for 10 min @ 4°C

Dissolve pellet in 200-800µl ddH_2O

Appendix 10: Multiphor SSCP/HD gel electrophoresis

Multiphor solutions

40% acrylamide PDA solution 1L

396g acylamide
4g piperazine diacrylamide
Bring to volume with dH_2O

Keep wrapped in foil in fridge

0.75M TRIS-Formate Buffer 1L

90.8 g TRIS
600ml dH_2O (dissolve)
pH=9.0 with formic acid
Bring to volume with dH_2O

41% Glycerol 100ml

41ml glycerol
59ml dH_2O

TRIS-Borate Buffer 1L

125.9g TRIS
17.3g Boric acid
700ml dH_2O (dissolve)
50µl Bromophenol Blue (4% solution)
Bring to volume with dH_2O

Set pH=9.0

SSCP loading dye 50ml

47.5ml 95% Formamide
0.16g 100mM NaOH
0.125g 0.25% Bromophenol Blue
0.125g 0.25% Xylene cyanol
Bring to volume with dH_2O

10% APS 1.5ml

0.15g Ammonium persulphate
Bring to volume with dH_2O

Gel mix (12% non-denaturing) x10

53ml 40% acylamide-PDA
85ml TRIS-Formate buffer
30ml 41% Glycerol
Keep wrapped in foil in fridge

Per 1 gel

15ml gel mix
150µl 10% APS
15µl TEMED

Plate Glue

50µl 0.25% plate glue (3-Trimethoxysilyl)propyl methacrylate
Dilute in 1% ethanol

Silver Staining Solution

Solution 1 1L

0.1% silver nitrate
Bring to volume with dH_2O

Keep in dark bottle

Solution 2 1L

15g NaOH
Bring to volume with dH_2O
3ml 40% Formaldehyde

Keep in dark bottle

Multiphor protocol

Gel Plates preparation:

- Wash plates 4X with ethanol
- Every 4 runs clean plate with blocks with gelslick especially over blocks
- Put 50µL plateglue and 6µL 10% acetic acid on plate without blocks
- Wipe well until resistance is felt and wash a few times with ethanol
- Clean spacers and place them on plates
- Camp well on both short sides and long area close to blocks and then place upside down on bench
- Prepare gel mix and pour quickly using a syringe
- Allow to set at room temp for 30min (can stand for 3 days –don't wrap! It shrinks)
- Separate plates (gel should be on one without blocks)
- Put water on surface of multiphor
- Don't get water on plates when placing on multiphor apparatus
- Slide plate facing upwards over water (no bubbles)
- Use 2 buffer strips/gel-side/2h run soaked in TRIS-borate buffer
- Place strips on both sides of gel close to the wells
- Wash electrodes of multiphor gently each time before use

PCR Products:

- Take 3-5µL PCR product and 3-5µL SSCP loading dye
- Denature @ 95°C for 5min in PCR thermocycler
- Place on ice immediately for 3 min
- Load 3µL (skipping ends of gel) onto gel and run @ 9°C first (if resolution not good, run at 11°C)
- 2h 45min at 355V

Visualization of bands:

- remove gel from apparatus
- rinse in dH_2O (remove buffer because it interferes with the stain)
- incubate for 10min @room temp in solution I (silver nitrate intercalates with DNA)
- rinse X2 with dH_2O
- incubate for 10min @room temp in solution II
- rinse X2 with dH_2O
- blot dry with paper towel
- cover with filter paper
- leave overnight, then put paper off glass and label paper with dried gel

Appendix 11: DNA purification protocols

BioLine SureClean® (Bioline, UK)

- Add equal volume of BioLine SureClean® to PCR sample and incubate at room temperature for 10 min
- Centrifuge for 10 min at 13 000 rpm and remove supernatant
- Add 100µl of 70% ethanol and vortex for 30 seconds
- Centrifuge for 10 min at 13 000 rpm, remove supernatant and air-dry
- Resuspend in nuclease-free water (starting volume)

Appendix 12: Polyacrylamide gel electrophoresis protocol

5%C, 4% stock 100ml

38g Acrylamide
2g Bis-acrylamide
Bring to volume with dH_2O

10% APS 100ml

0.15g Ammonium persulphate
Bring to volume with dH_2O

Mix for 2 gels

- 7ml gel mix
- 4ml 5X TBE
- 9ml dWater
- 200µl APS
- 80µl Temed

Gel Plates preparation

- Wash plates well with ethanol
- Clean spacers with ethanol
- Place spacers in the correct place and close plates
- Clamp plates down in Gel Caster

Preparing Gel Mix

- Pour quickly using a syringe
- Put comb into place
- Allow to set at room temerature
- Once set remove comb
- Clean wells with 1X TBE and syringe needle
- Open cast at bottom
- Place in Gel aparatus
- Fill up with 1X TBE

Digest products

- Add loading dye to 20µl of digest product
- Load 25µl into the wells
- Run @ room temperature for 60-90 minutes @ 120V

Visualisation of bands

- Remove top plate from gel
- Do ethidium bromide staining for 10 minutes
- De-stain in distilled water for 3 minutes
- Remove gel from plate and visualise under UV light

Appendix 13: Congress outputs

- Presented on poster at Internation Society for the Study of Hypertension in Pregnancy (ISSHP), July 2006, Lisbon, Portugal.

Neurokinin B (NKB) and endothelial nitric oxide synthase (eNOS) gene variants do not interact to increase susceptibility to pre-eclampsia

Megan Stolk[1], Kashefa Carelse Tofa[2], George Rebello[3], Stefan Gebhardt[2], Verena Geissbuehler[4], Renė Moser[5] and Renate Hillermann[1]

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Background: In normal pregnancy, physiological cardiovascular adaptation includes an increase in total blood volume and a decreased peripheral vascular resistance in most tissues, including the uterine arteries and placental vessels. These changes are accompanied by increased endogenous nitric oxide (NO) production, under the control of the enzyme NO synthase. The activation of NO synthase is modulated, among other factors, by the circulating factor, neurokinin B (NKB)-specific NK_3 receptor. On the basis of the interaction between NKB, NK_3 and nitric oxide and a previous observation that the *eNOS* Glu298Asp variant impacts on the risk of complicated pre-eclampsia in our population, we investigated whether potentially functional sequence variants in the *TAC3* and *TACR3* genes and the *eNOS* polymorphism Glu298Asp influence the balance in this relationship.

Methods: Circulating plasma NKB levels were determined in 50 plasma samples using a standard radioimmunoassay protocol. In genomic DNA samples from the corresponding patients, specific *TAC3*-25c/t, *TACR3*-103t/c and *eNOS* Glu298Asp polymorphisms were amplified by PCR and genotyped by restriction enzyme analysis. Genotypes at single loci and in combinations were examined and compared with circulating NKB levels.

Results:
A marginal association (p=0.055) was demonstrated between the *eNOS* Glu298Asp variant and pre-eclampsia. No significant association could be demonstrated between the promoter region *TAC3* and *TACR3* and the *eNOS* polymorphism, susceptibility to pre-eclampsia, or the level of circulating NKB, whether the loci were considered independently, or in combination.

Conclusions: While NKB and its receptor could be involved in maternal haemodynamic adaptation via nitric oxide production in healthy pregnancy, there is no evidence that genetic variation at the DNA level perturbs that balance in pregnancies complicated by pre-eclampsia.

-Presented on poster at Internation Society for the Study of Hypertension in Pregnancy (ISSHP), July 2006, Lisbon, Portugal.

The binding region of the human galectin/placental protein -13 gene, *LGALS13*, is enriched with nucleotide sequence variation

Megan Stolk[1], George Rebello[3], Stefan Gebhardt[2], Kashefa Carelse Tofa[2], Berthold Huppertz[4], Sinuhe Hahn[5] and Renate Hillermann[1]

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Background: The observation that placental protein 13 (PP13) levels are differentially expressed early in pregnancies subsequently affected by pre-eclampsia has raised the possibility that PP13 may serve as an early detection biomarker for pre-eclampsia. We performed mutation analysis of the *LGALS13* gene which encodes PP13, to determine whether there are any sequence variants or polymorphisms which may impact on PP13 expression.

Methods: This pilot study cohort comprised 60 samples representing: 20 women with early onset severe pre-eclampsia together with 20 infants resulting from these pathological pregnancies and a control group of 20 healthy, matched individuals. Genomic DNA was amplified by PCR and the entire coding region of the *LGALS13* gene screened by Multiphor SSCP/heteroduplex analysis. Conformational variants were further characterized by automated sequencing and subsequently restriction enzyme analysis, where appropriate.

Results: In the 5' untranslated region of the gene, a previously documented C/A substitution [rs3764843] was identified at position -98 relative to the ATG transcription start site. Two intronic variants were identified in intron 2 – viz, previously documented IVS2-22 (A/G) [rs2233706] and novel IVS2-36 (A/G). In exon 3 (211bp), a complex mutation involving 6 different substitutions within a 11bp region was identified in three pre-eclamptic women, who transmitted it to their infants. This variation was not observed in any of the 20 control samples. An additional two pre-eclampsia samples revealed a deletion T (222delT / L74W), which causes truncation of the PP13 protein by 37 amino acids, excluding a portion of the protein considered to be critical for its function. The deletion was transmitted to their infants, and a paternally-derived variant (inferred) was also identified in one other infant. The deletion was not identified in any of the 20 control samples analysed.

Conclusions: The identification of different forms of genetic mutations in a critical portion of the PP13 encoding gene, *LGALS13*, provides novel evidence of regulation of PP13 expression at the DNA level. Further analysis such as a genotype/phenotype study in a substantial patient cohort and a study to investigate a possible correlation between circulating PP13 levels and *LGALS13* genotype is warranted.

Appendix 14: Submitted Manuscript

Novel sequence variants in the *TAC3* and *TACR3* genes are not significantly associated with susceptibility to pre-eclampsia.

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The novel nucleotide sequence data reported here are available in the Genbank database under the accession numbers AM042557 and AM179832.

Abstract

The placental dysfunction that underlies pre-eclampsia is associated with several factors. These include among others, failure of the cytotrophoblast to invade maternal spiral arteries and the release of circulatory molecules. Neurokinin B (NKB) has been considered such a circulating factor, but its role in pre-eclampsia has been controversial. This study is the first to examine the genes encoding NKB (*TAC3*) and its receptor (*TACR3*) in patients with pre-eclampsia.

Seven sequence variants were identified in the *TAC3* and *TACR3* genes, of which three are novel. These include a *TAC3* intronic transversion (IVS3-53G→T), an exon 6 substitution (g.295T→C), and a transition in the 3' untranslated exon 7 (c.635T→C). Of the four previously-documented sequence variants, two are located in close proximity to the transcriptional start sites and are predicted to have functional consequences.

From this study, it would appear that *TAC3* and *TACR3* DNA polymorphisms do not underlie susceptibility to pre-eclampsia, and that other phenomena such as post transcriptional modification should be considered.

Keywords: neurokinin B/ pre-eclampsia/ polymorphism/ *TAC3/ TACR3.*

Introduction

Pre-eclampsia only occurs when placental tissue is present; consequently the placenta is thought to play a pivotal role (Redman 1991; Kenny and Baker, 1999). A current hypothesis proposes that a variety of underlying conditions (genetic and/or immunological) together with environmental factors and a susceptible phenotype contribute to defective first trimester uterine invasion (Cross 2003; Redman and Sargent, 2005). The resulting transient placental ischaemia may contribute to poor fetal growth (Lunell *et al*., 1984; Zhou *et al*., 1997) and the release of an unknown substance(s) in the maternal circulation.

A plausible candidate for such a circulatory factor is neurokinin B (NKB). Page and colleagues were the first to report that plasma concentrations of NKB were significantly elevated in pre-eclampsia and that NKB is expressed, among other sites, in the outer syncytiotrophoblast of the placenta (Page *et al*., 2000; Pinto *et al*., 2004).

NKB is a member of the tachykinin family, a group of structurally related peptides with the capacity to contract smooth muscle. The three best documented mammalian peptides of this family are Substance P (SP) and Neurokinin A and B. The receptors for each of these peptide are termed NK_1, NK_2 and NK_3, respectively (Maggi 1995; Patak *et al*., 2003). These receptors mediate processes such as activation of the immune system (Ansel *et al*., 1993), vasodilation (Brownbell *et al.,* 2003), vascular reactivity (D'Orleans-Juste *et al*., 1991) and smooth muscle contraction (Patak *et al*., 2000).

While placental NKB production increases gradually throughout normal pregnancy, reaching its highest levels at term (D'Anna *et al*., 2002; Sakamoto *et al*., 2003), its role in pre-eclampsia, measured quantitatively, has been controversial. Page *et al*. (2000) reported that excessive placental secretion of NKB during the third trimester of pregnancy was associated with pre-eclampsia, although the trend in increased secretion of NKB could not be substantiated in a study by Schlembach *et al.* (2003). In an *in vitro* study, Wareing *et al*. (2003) found no evidence that NKB (on its own) could alter the vascular reactivity in vessels. D'Anna and colleagues reported high levels of NKB in pre-eclampsia as well as in pregnancies with intra-uterine growth restriction (IUGR) and suggested that NKB could be involved in maternal haemodynamic adaptation via nitric oxide production (D'Anna *et al*., 2004).

Since the quantitative measurement of NKB levels may be influenced by factors such as gestation and possible interaction with other vasoactive substances (Schlembach *et al*., 2003), an approach was adopted to investigate the role of NKB in pre-eclampsia on a genetic level. DNA genotypes are invariant and can be correlated with both biochemical and clinical data.

Human NKB mRNA (NM_013251) is encoded by seven exons and spans a genomic region of 5.4 kb. Exons 1 and 7 correspond to the 5' and 3' untranslated regions of the transcript, respectively, while exons 2 to 6 encode the precursor and exon 5, the sequence of NKB (Page *et al*., 2001). The *TACR3* gene (NM_001059) which encodes the neurokinin B receptor (NK3), is localised to human chromosome 4, contains 5 exons and is expressed widely (Pennefather *et al*., 2004). To date, no mutation detection screening of these genes, has been published.

In this novel study, the genes encoding NKB (*TAC3*) and the NKB receptor (*TACR3*) were screened for sequence variations and results correlated with the clinical phenotype.

This broad approach was adopted to challenge the candidacy of NKB as a significant risk factor for the development of pre-eclampsia and its potential use as a predictive marker for the disease.

MATERIALS AND METHODS

Institutional and ethical approval for the study was granted and informed consent was obtained from each participant. To minimise genetic heterogeneity, participants were largely restricted to the South African Coloured population of the Western Cape (Botha 1972).

Patients and Samples

Mutation screening

The mutation screening cohort (n=120) consisted of mainly primigravidae with severe pre-eclampsia. Women with a history of hypertensive disease, recurrent miscarriage or congenital abnormalities were excluded from the study. A control panel (n=93), representing women with healthy, term deliveries was available for comparative analysis. Their babies weighed >10th centile according to gestational age for this specific population group, thereby reducing the chances of including cases of possible undiagnosed placental vasculopathy.

Diagnosis of pre-eclampsia

A diagnosis of pre-eclampsia was determined by the criteria established by the International Society for the study of hypertension in pregnancy (Davey and MacGillivray, 1988). This is a diastolic blood pressure measurement of ≥90 mmHg (using Korotkoff V) on two occasions at least four hours apart, accompanied by significant proteinuria (>300 mg/24 hours urine collection or persistent 2+ on a diagnostic strip) arising after 20 weeks of gestation (Dekker *et al*., 1995).

Methodology

Genetic analysis

DNA was extracted from whole blood samples using the GENTRA™ PureGene® genomic DNA purification kit (Minneapolis, USA). The *TAC3* and *TACR3* genes were annotated using Locuslink at the NCBI locus link (http://www.ncbi.nlm.nih.gov/). Primers (Table 1) were designed for each exon-flanking intronic region using Primer3 (Rozen and Skaletsky, 2000). Primer specificity was verified by BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) (Altschul *et al*., 1990).

PCR amplification

Each amplicon was generated in a total reaction volume of 50 µl comprising 10x reaction buffer, 15 pmol each forward and reverse primer (Inqaba Biotech, Pretoria, South Africa), 200 µM dNTPs, 25 mM $MgCl_2$, 0.5 U Taq polymerase (Bioline Biotaq, Promega, Madison, USA) and ~50 ng genomic DNA as template.

The PCR thermal cycling (GeneAmp®PCR System 9700) for the *TAC3* gene was initiated at 95 ºC for 2 minutes, followed by 30 cycles of : denaturation at 94 ºC for 30 seconds, annealing at 52 ºC (exon 1), 55 ºC (exons 2, 4, 5, 6, 7) 57 ºC (exon 3) for 15 seconds and extension at 72 ºC for 1 minute. A final extension step at 72 ºC for 5 minutes was included.

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

Multiphor SSCP/heteroduplex analysis

Approximately 3 µl of PCR amplicon was added to an equal volume of loading dye prior to heat denaturation for 5 minutes. The denatured products were subsequently resolved on Multiphor gel electrophoresis gels, as described by Liechti-Gallati *et al*. (1999).

Automated sequencing

PCR products for sequencing were purified with the Wizard® PCR Preps DNA Purification System (Promega, Madison, USA). The ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster Ca) was used to generate bi-directional sequences.

Restriction enzyme analysis

Where sequence variants were shown to create or abolish a particular restriction enzyme recognition site, genotyping was performed following restriction enzyme analysis according to manufacturer's recommendations.

Genotyping

Genotypes and allele frequencies were determined in 120 samples from severe pre-eclamptic cases. In 98 of these cases, genotypes could be assigned across all 7 loci, which faciliated the investigation into allelic interaction.

Statistical analysis

Genotype and allele frequencies were established by direct genotype and allele counting. The genotype frequencies were compared by chi-squared analysis using Statistica® version 7. A threshold value ($p<0.05$) was considered as statistically significant.

RESULTS

Patient demographics

The patient demographic details are summarised in Table 2.

Genetic analysis

Fifteen amplicons, encompassing the coding and partial 5' and 3' untranslated regions of the *TAC3* and *TACR3* genes, were screened by a combination of Multiphor SSCP/HA (single stranded conformational polymorphism/heteroduplex analysis), direct sequencing and restriction enzyme analysis (Table 1).

Seven polymorphisms were identified, of which three were novel. Allele and genotype frequencies are shown in Table 3. All genotype frequencies determined were in Hardy Weinberg equilibrium.

Three novel sequence variants in the *TAC3* gene

A novel intronic (G→T) transversion [AM042557] within 53 bp of the exon 4 boundary (IVS3-53G→T) was identified in two patients (0.008), of whom one also carried the *TAC3* exon 7 c.635 T→C variant. No homozygous T/T genotype was discerned. This low frequency polymorphism was not identified in any control samples. There was no statistically significant difference demonstrated in the comparison of patient and control groups (p=0.516).

The sequence variant is not located within theRNA splicing branch site positioned ~60 bp before the splice acceptor site (3' of the intron). The polymorphism does not create or abolish a known restriction enzyme recognition site.

A novel T→C transition [AM179832] was identified at nucleotide position 295 (g.295 T→C), 3 bp into exon 6, and 111 bp before the TAA translation stop codon. The restriction enzyme *Hinf* I (New England Biolabs, Ipswich, USA) was used to distinguish between alleles, with the C allele abolishing the restriction recognition site (g↓antc) for this enzyme. The variant

was only identified in heterozygous form, in 19 pre-eclamptic women (0.079). It occurred at a similar frequency (0.08) in a small panel of 18 control samples and no significant association was demonstrated (p=0.928).

This nucleotide change, located immediately adjacent to an intron-exon boundary splicing consensus sequence, causes the substitution of a hydrophilic serine residue by a hydrophobic proline residue (S99P).

Further downstream, in non-coding exon 7, a novel base pair substitution [AM179832] was identified (c.635 T→C) which is located 110 bp after the TAA translation STOP codon. The variant (in heterozygous form) was identified in 10 pre-eclamptic women (0.042) and not in any of the control samples tested. There was no statistical difference in the allele or genotype frequencies when comparing patient and control groups at this low frequency locus (p=0.203).

Of the previously-documented sequence variants identified in this study, three (*TAC3* -25 C→T [dbSNP:rs2291855], *TACR3* -103 T→C [dbSNP: rs3733632] and *TACR3* 1471 A→G [dbSNP: rs2765] are high-frequency polymorphisms, while one, *TACR3* 857 A→G [dbSNP: 2276973] occurs at a frequency of <5%.

The first two polymorphisms are located in the 5' untranslated region of their respective genes, in close proximity to the start codons. Variation in those regions may impact on transcription, and consequently, gene expression. *TAC3* sequence variant g.-25C→T was identified in patients at a frequency of 0.075. The T allele abolishes a *Msp*I (New England Biolabs, Ipswich, USA) restriction enzyme recognition site (cc↓gg). In this study, only one patient was homozygous T/T for the variant (0.008) and of the 16 heterozygotes (0.13) identified, none carried any other *TAC3* variant reported in this study.

The g.-25C→T variant had already been documented in the SNP database (dbSNP: 2291855) in an undefined population, at a frequency of 0.10, and more recently, in a Sub-Saharan African cohort at a frequency of 0.04. In our control samples, the variant was found at a frequency of 0.14 and no homozygous T/T genotypes were discerned. There was no statistical

difference in the allele or genotype frequencies when comparing patients and controls at this locus (p=0.923 and p=0.391, respectively).

The other 5' UTR sequence variant, *TACR3* (g.-103T→C) polymorphism (dbSNP:3733632) was characterised by restriction enzyme analysis with *Nci*I (New England Biolabs, Ipswich, USA) enzyme, with the C allele creating the restriction enzyme recognition site (cc↓ngg). It was found at a frequency of 0.740 in pre-eclamptic women, of whom 0.416 and 0.533 had heterozygous (T/C) and homozygous (C/C) genotypes, respectively. These frequencies were very similar to those in the control panel (0.484 and 0.339, respectively) of this study (p= 0.509).

Interestingly, our cohort allele frequencies at this locus (T = 0.26 and C = 0.74) differ from those reported in Europeans (T = 0.77 and C = 0.23) and Asians (T = 0.72 and C = 0.28), while corresponding more closely to those reported in a sub-Saharan African cohort of 30 Nigerian mother-father-child trios (Hapmap-YRI), with frequencies of T = 0.12 and C = 0.88. This possibly reflects the "African" ancestry in our cohort.

The previously reported sequence variant, dbSNP: 2276973, located in *TACR3* gene exon 3, was identified in 9 patients (0.040). This polymorphism results in the substitution of a lysine with a glycine residue, R286K. The variant is located within the intracellular region between the 5th and 6th transmembrane domains (according to the Conpred II programme of Arai *et al*., 2004). R286K was identified in control samples at a frequency of 5%. There was no statistical difference in the allele or genotype frequencies when comparing patient and control groups (p=0.511).

In the SNP database, the allele and genotype frequencies reported in European and African-American individuals, were very similar to those we obtained in this study, confirming the low-frequency/conserved nature of this sequence variant.

Finally, a *TACR3* 3'UTR sequence variant (g.1471 T→C) was identified (dbSNP:2765, described as a G→A polymorphism, on the + strand, that translates to a C→T change on the –

strand, on which the *TACR3* gene resides). The dbSNP allele frequencies at this locus are reported as 0.642 and 0.358, for the T and C alleles, respectively. In this study, the polymorphism was genotyped by restriction enzyme analysis using *BseN*I (Fermentas, Burlington, USA), the recognition site (actg↓g) being created by the C allele. The frequencies determined in this study were largely similar within the different groups examined and were in agreement with the published frequencies for these alleles.

No sequence variants were detected in the amplicons representing *TAC3* exons 2, 3 and 5 and *TACR3* exons 2 and 4, despite the introduction of varying experimental conditions such as temperature and gel matrix.

Genotype combinations

In the analysis of 98 complete genotypes for the 7 polymorphisms reported in this study, there was no significant allelic association or trend, apart from the observation that *TACR3* -103C and 1471C alleles (dbSNP:rs3733632, and dbSNP:rs2765 respectively) frequently occurred together, regardless of status at any other polymorphism in *TAC3* or *TACR3*. This is not unexpected, since they each occur commonly within this particular population.

DISCUSSION

Of the seven sequence variants identified in the *TAC3* and *TACR3* genes by mutation screening, three *TAC3* variants were novel. These are localised to the distal portion of the gene: IVS3-53G→T is located in the exon 3/exon 4 intervening interval, g.295T→C in the coding region of exon 6 and c.635 T→C in the 3'-untranslated region.

The IVS3-53G→T genotype was identified exclusively in two pre-eclamptic women, of whom one also carried an additional *TAC3* sequence variant. Despite the variant being localised beyond the consensus intron-exon splice boundary, and not within a recognised splice branch site, it could still cause splicing defects. This could result in an alteration in the level of the mature mRNA generated, and consequently contribute to the disease (Padgett *et al*., 1986). This is reported to be the case for 15% of all intronic polymorphisms (Krawczak *et al*., 1992).

The novel polymorphism, c.635 T→C, located in the 3'-untranslated region of *TAC3* exon 7, is rare (0.042). This genomic region is thought to contribute to mRNA stability (Jackson and Standart 1990; Ross 1995).

Of the two exonic variants, identified in this study, one was novel. The third base pair of *TAC3* coding exon 6 contains a novel T→C polymorphism (g.295T→C) which is adjacent to a consensus splice site boundary. The effect of this base pair substitution (identified equally in patients and controls) is a change from a hydrophilic serine to a hydrophobic proline amino acid residue. It is interesting to note that there are at least two isoforms of *TAC3* (tv1-NM_001006667 and tv2_NM013251): isoform 1 contains no exon 6, whereas isoform 2 contains exon 6 and the region appears to be well conserved. It could be speculated that tissues harbouring isoform 2 with a "mutated" exon 6 may be more prone to the consequences of this variant. Application of the ESEfinder program (http://exon.cshl.edu/ESE) predicted that in the presence of the variant C allele, a SC35 and a SRp40 binding site is abolished, while a novel one is created.

The other exonic variant identified in this study is *TACR3*g.857A→G / R286K, which was very rare (up to 0.050), but present in both patient and control groups. The polymorphism occurs in exon 3 and represents a change in amino acid from K (lysine) to a R (glycine).

Application of the ESEfinder program predicted that in the presence of the variant G allele, single SF2/ASF and SC35 sites are abolished, and a novel SRp55 site is created. Collectively these alterations could lead to aberrant mRNA species that either encode a defective protein isoform, or demonstrate instability.

In this study, various types of polymorphisms were identified. While polymorphisms are often considered as "inert" variations with no significant effect on gene product or resulting protein, there is growing evidence of the involvement of the "low frequency" polymorphisms, in the clinical expression of complex diseases (Cargill *et al*., 1999; Yang *et al*., 2001). Furthermore, the effect of so-called "silent mutations" should not be ignored. In contrast to being considered as "neutral polymorphisms", they can still have an effect on mRNA levels and consequently, the translated gene product (Cartegni *et al*., 2002).

The previously published *TAC3* and *TACR3* variants -25C→T (dbSNP:2291855), and -103T→C (dbSNP: rs3733632) respectively, identified in this study, are interesting by virtue of their position. Each is located in close proximity to the start codon and most likely lies within the promoter region of the gene. While variation within this region is not likely to affect the process of translation, transcription of the gene may be influenced, which in turn could lead to altered gene expression. Examples of this phenomenon include the uroporphyrinogen III synthase erythroid promoter variants, which have been shown to cause erythropoeitic porphyria (Solis *et al*., 2001) and a single IL-3 promoter region SNP associated with rheumatoid arthritis (Yamada *et al*., 2001). According to Hoogendoom *et al*., 2003, one third of promoter variants exert a functional effect on gene expression.

Functional NKB is thought to establish the early trophoblast by dilating the uterine spiral arteries. The resulting vascular changes most likely increase maternal blood pressure and direct blood to the uterus and placenta, where it is in demand. In early-onset, severe pre-eclampsia, the absence of appropriate trophoblast invasion and incomplete spiral artery remodelling may (independent of *TAC3* and *TACR3* gene mutation status) trigger the further secretion of NKB into the maternal circulation, culminating in maternal disease. Alternatively, the expression of the genes could be influenced by a factor or factors (possibly even related to the poor invasion and remodelling process) that result in elevated levels (moderately or severely) of NKB in most pregnancies complicated by pre-eclampsia.

To follow on this study, which represents the largest on NKB to date, future studies should include the full characterisation of the reported variants. Intronic variants can be investigated further by using the RNA-based minigene system to evaluate splicing efficiency (Cartegni *et al*., 2002), and the effect of exonic or coding variants on gene expression can be investigated by transfection studies such as those utilising the Luciferase reporter assay system. In these studies, particular alleles can be challenged *in vitro* with potential "influencing factors" such as hormones, etc. The genetic mutation screening could also be extended to include more of the 5' regulatory region further upstream of the genes' translation start codons and so facilitate full characterisation of the relevant promoters.

Gene expression regulation occurs at several levels. From this study, it would appear that *TAC3* and *TACR3* DNA polymorphisms do not underlie susceptibility to pre-eclampsia, and that other phenomena such as post transcriptional modification should be considered.

Antagonists for all three NKB receptors have been developed and are currently being tested in phase I and II trials (Mesnage *et al*., 2004). The first specific NK_3 receptor antagonist (*(S)-(N)-(1-(3(1-Benzoyl-3-(3,4-dichlorophenyl)piperidin-3-yl)propyl)-4-phenylpiperidien-4-yl)-N-methylacetamide*) and other derivates may reduce the vasoconstrictive effects of neurokinin and be important for clinical trials in the management of pre-eclampsia. In relation to the study presented here, it may be worthwhile investigating whether patient responses to these compounds differ, depending on their mutation status at the *TAC3* and *TACR3* gene loci.

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REFERENCES

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J Mol Biol 215 3, 403-410.

Ansel JC, Brown JR, Payan DG and Brown MA (1993) Substance P selectively activates TNF-alpha gene expression in murine mast cells. J Immunol 150 (10), 4478-4485.

Arai M, Mitsuke H, Ikeda M, Xia J.-X, Kikuchi T, Satake M and Shimizu T (2004) ConPred II: a consensus prediction method for obtaining transmembrane topology models with high reliability. Nucleic Acids Res, W390-W393.

Botha MC (1972) Blood group gene frequencies. An indication of the genetic constitution of population samples in Cape Town. Am J Roentgenol Radium Ther Mucl Med 115, 1-27.

Brownbell P, Bell NJ, Woods RJ, Lowry PJ, Page NM and Sibley CP (2003) Neurokinin B is a paracrine vasodilator in the human fetal placental circulation. J Clin Endocrinol & Metab 88, 2164-2170.

Cargill M, Altshuler D, Ireland J, Slkar P, Ardilie K, Patil N, Shaw N, Lane CR, Lim EP, Kalyanaraman N, Nemesh J, Ziagaura L, Friedland L, Rolfe A, Warrington J, Lipschutz R, Daley GQ, Lander ES (1999) Characterisation of single-nucleotide polymorphisms in coding regions of human genes. Nat Genet 22, 231-238.

Cartegni L, Chew SL, Krainer AR (2002) Listening to silence and nonsense mutations: exonic mutations that affect splicing. Nat Rev 3, 285-198.

Cross JC (2003) The genetics of pre-eclampsia: a feto-placental or maternal problem? Clin Genet 64, 96-103.

Davey DA, MacGillivray I (1988) The classification and definition of the hypertensive disorders of pregnancy. Am J Obstet Gynecol 158 4, 892-898.

D'Anna R, Corrado F, Baviera G, Jasonni VM (2002) Neurokinin B peptide levels in normal pregnancy. Eur J Obstet Gynecol Reprod Biol 103, 101-102.

D'Anna R, Baviera G, Corrado F, Crisafulli A, Lentile R, Buemi M, Squadrito F (2004) Neurokinin B and nitric oxide plasma levels in pre-eclampsia and isolated intra-uterine growth restriction. BJOG 111, 1046.

D'Orleans-Juste P, Claing A, Telemaque S, Warner TD, Regoli D (1991) Neurokinin B produces selective venoconstriction via NK3 receptors in the rat mesenteric vascular bed. Eur J Pharmacol 204, 329-334.

Dekker GA, de Vries JIP, Doelitzsch PM, Huijgens PC, von Blomberg BME, Jakobs PM, van Geijn HP (1995) Underlying disorders associated with severe early-onset pre-eclampsia. Am J Obstet Gynecol 173, 1042-1048.

Hoogendoom B, Coleman SL, Guy CA, Smith K, Bowen T, Buckland PR, O'Donovan MC (2003) Functional analysis of human promoter polymorphisms. Hum Mol Genet (12) 18, 2249-2254.

Jackson RJ, Standart N (1990) Do the poly (A) tail and 3' untranslated region control mRNA translation? Review. Cell (62) 1, 15-24.

Kenny L, Baker PN (1999) Maternal pathophysiology in pre-eclampsia. Baillieres Best Pract Res Clin Obstet Gynaecol 13, 59-75.

Krawczak M, Reiss J, Cooper DN (1992) The mutational spectrum of single base-pair substitutions in mRNA splice junctions of human genes: causes and consequences. Hum Genet 90, 41-54.

Liechti-Gallati S, Schneider V, Neeser D, Kraemer R (1999) Two buffer PAGE system-based SSCP/HD analysis: a general protocol for rapid and sensitive mutation screening in cystic fibrosis and any other human genetic disease. Eur J Hum Genet 7, 590-598.

Lunell NO, Lewander R, Momoun I, Nylund L, Sarby S, Thromstrom S (1984) Uteroplacental bloodflow in pregnancy induced by hypertension. Scand J Clin Lab Invest Suppl 169, 28-35.

Maggi CA (1995) The mammalian tachykinin receptors. Gen Pharmacol 26, 911-944.

Mesnage V, Houeto JL, Bonnet AM, Clavier I, Arnulf I, Cattelin F, Le Fur G, Damier P, Welter M and Agid Y (2004) Neurokinin B, Neurotensin, and Cannabinoid Receptor Antagonists and Parkinson Disease. Clin Neuropharmacol 27(3), 108-110.

Padgett R, Grabowski P, Konarska M, Seiler S, Sharp P (1986) Splicing of messenger RNA precursors. Ann Rev Biochem 55, 1119-1150.

Page NM, Lowry PJ (2000) Is pre-eclampsia simply a response to the side effects of a placental tachykinin? J Endocrin 167, 355-361.

Page NM, Woods RJ, Gardiner S M, Lomthaisong K, Gladwell RT, Butlin DJ, Manyonda IT and Lowry PJ (2000) Excessive placental secretion of neurokinin B during the third trimester causes pre-eclampsia. Nature 405, 797-800.

Page NM, Woods RJ and Lowry PJ (2001) A regulatory role for neurokinin B in placental physiology and pre-eclampsia. Regulatory Peptides 98, 97-104.

Patak EN, Ziccone S, Story ME, Fleming AJ, Lilley A, Pennefather JN (2000) Activation of neurokinin NK2 receptors by tachykinin peptides causes contraction of uterus in pregnant women near term. Mol Hum Reprod 6, 549-554.

Patak E, Candenas ML, Penefather JN, Ziccone S, Lilley A, Matrin JD, Flores AG, Mantecon AG, Story ME, Pinto FM (2003) Tachykinins and tachykinin receptors in human uterus. Br J Pharmacol 139, 523-532.

Pennefather JN, Lecci A, Candenas ML, Patak E, Pinto FM, Maggi CA (2004) Tachykinins and tachykinin receptors: a growing family. Life Sciences 74, 1445-1463.

Pinto FM, Almeida TA, Hernandez M, Devillier P, Advenier C, Candenas ML (2004) mRNA expression of tachykinins and techykinin receptors in different human tissue. Eur J Pharmacol 494, 233-239.

Redman CW (1991) Pre-eclampsia and the placenta. Placenta 12, 301-308.

Redman CW and Sargent IL (2005) Latest advances in understanding pre-eclampsia. Science 10 (308) 5728, 1592-1594.

Ross J (1995) mRNA stability in mammalian cells. Review. Microbiol Rev (59) 3, 423-50.

Rozen S, Skaletsky H (2000) Primer3 on the WWW for general users and for biologist programmers. Methods Mol Biol 132, 365-386.

Sakamoto R, Osada H, Litsuka Y, Masuda K, Kaku K, Seki K and Sekiya S (2003) Profile of neurokinin concentrations in maternal and cord blood in normal pregnancy. Clin Endocrinol 58, 597-600.

Schlembach D, Scalera F, Fischer T, Marx SG, Beinder E, Garfield RF (2003) Neurokinin B peptide levels are higher in normotensive pregnant women than in pre-eclamptic pregnant women. Am J Obs Gynecol 3 (198), 1418-1432.

Solis C, Aizencang GI, Astrin KH, Bishop DF, Desnick RJ (2001) Uroporphyrinigen III synthase erythroid promoter mutations in adjacent GATA1 and CP2 elements cause congenital erythyropoeitic porphyria. J Clin Inves 107, 753-762.

Wareing M, Bhatti H, O'Hara M, Kenny L, Warren AY, Taggart MJ, Baker PN (2003) Vasoactive effects of neurokinin B on human blood vessels. Am J Obstet Gynecol 188, 196-202.

Yamada R, Tanaka T, Unoki M, Nagai T, Sawada T, Ohnishi Y, Tsunoda T, Yukioka M, Maeda A, Suzuki K, Tateishi H, Ochi T, Nakamure Y, Yamamoto K (2001) Association between a single nucleotide polymorphism in the promoter of human IL-3 gene and rheumatoid arthritis in Japanese patients, and maximum-likelihood estimation of a

combinatorial effect that two genetic loci have on susceptibility to the disease. Am J Hum Genet 68, 674-684.

Yang Z, Zhu T, Ma G, Yin H, Qian W, Zhang F, Cao K, Ma W (2001) Apolipoprotein E polymorphism in the early-onset of coronary heart disease. Chin Med J 114, 983-985.

Zhou Y, Fisher SJ, Janatpour M, Genbacev O, Dejana E, Wheelock M, Damsky CH (1997) Human cytotrophoblasts adopt a vascular phenotype as they differentiate: a strategy for successful endovascular invasion? J Clin Invest (9) 99, 2139-2151.