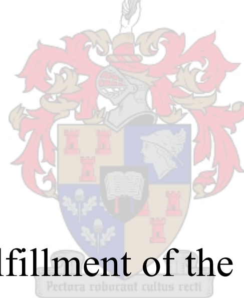
Molecular genetic analysis of preterm labour

Natalie Bruiners



Thesis presented in partial fulfillment of the requirements for the degree of Master of Science at the University of Stellenbosch

Supervisors: Drs R Hillermann-Rebello and G.S. Gebhardt

December 2007

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 part submitted it at any university for a degree.

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Abstract

The World Health Organisation (WHO) has defined preterm labour as the onset of labour before 37 completed weeks of gestation with an incidence ranging between 5-10%. Although patient care has improved, the rate of preterm birth has slowly been increasing and currently impacts significantly on maternal and fetal mortality and morbidity. The complex condition of preterm labour involves multiple etiologies and risk factors, which complicates the search for candidate markers and / or biomarkers.

The aim of this prospective study was to investigate potential genetic associations with preterm labour. The study cohort consisted of consecutive first-time booking, low-risk primigravid pregnant women from a restricted geographical region.

The study cohort comprised 421 [306 Coloured and 115 Black] pregnant women presenting at the Paarl Hospital Obstetric clinic. Subsequently, DNA was extracted from whole blood and investigated for a range of known polymorphisms in pro-inflammatory and anti-inflammatory cytokines, as well as the novel *LGALS*13 gene, for potential variants that may impact on pregnancy outcome. Screening techniques involve combinations of allele-specific PCR amplification, Multiphor SSCP/HD analysis, restriction enzyme analyses and DNA sequencing.

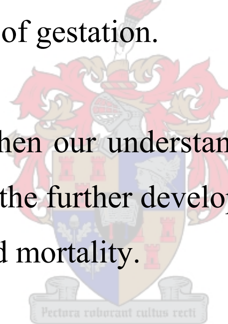
A significant association was demonstrated between the *IL*-1*RN**2-allele and adverse pregnancy outcome, mainly in the preterm labour and hypertension group. The presence *TNF*α-308 A-allele was associated with overall adverse pregnancy outcome and preterm labour. In addition to this, a novel *IL*-1*RN* allele was identified in the control group.

Mutation screening and subsequent statistical methods revealed an association between a novel *LGALS*13 exonic variant, 221delT, and preterm labour in Coloured women. Two previously-documented intronic variants (IVS2-22A/G and IVS3+72T/A) demonstrated linkage disequilibrium, signifying evolutionary conservation of exon three. Additionally, two novel intronic variants, IVS2-36 G/A and IVS2-15 G/A, demonstrated no association with adverse pregnancy outcome.

In this study we identified rare novel exonic variants; two non-synonymous variants in exon three (M44V, [N=2] and K87R, [N=1]) and a silent variant in exon four (P117P, [N=1]) - all identified in individuals from the control cohort. Within coding exon three, an interesting variant ["hotspot"] was identified, which represents six polymorphic bases within an 11bp stretch. No associations were demonstrated with these variants and pregnancy outcome.

Furthermore, a previously documented 5' "'promoter" variant, -98 A/C, was identified and demonstrated no association with adverse pregnancy outcome. However, subdivision of late-onset pre-eclamptic cases revealed a significant association with the A-allele and late-onset pre-eclampsia.

Genotype-phenotype investigation demonstrated association between the *IL*-10 -1082 A/G, *IL*-4 C/T and 221delT loci and poor pregnancy progress which manifested as (i) delivery of infants weighing <2000g, (ii) before 37 weeks of gestation.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The findings of this study will strengthen our understanding of the pathophysiology underlying pregnancy complications and facilitate the further development of effective treatment strategies to reduce maternal and fetal morbidity and mortality.

Opsomming

Die Wêreld Gesondheid Organisasie (WHO) klassifiseer voortydse kraam as kontraksie voor 37 volledige weke, met ‘n insidensie tussen 5-10%. Alhoewel pasiënte-sorg verbeter het, neem die tempo van voortydse geboorte steeds toe, wat ‘n groot impak het op moederstrefte en fetale mortaliteit en morbiditeit. Die komplekse kondisie van voortydse kraam sluit veelvoudige oorsake en risiko faktore in, wat die navorsing van kandidaat en / of biologiese merkers kompliseer.

Die doel van hierdie prospektiewe studie, was die potensiële navorsing van genetiese assosiasies met voortydse kraam. Die studie kohort bevat opeenvolgende eerste bespreking van lae risiko primigravida swanger vrouens vanaf ‘n beperkte geografiese omgewing.

Die studie kohort beslaan 421 [306 Kleurling en 115 Swart] swanger vrouens teenwoordig by die Paarl Hospitaal Verloskunde kliniek. Vervolgens was DNS geëkstraeer van bloedmonsters en geondersoek vir ‘n verskeidenheid van bekende polimorfismes in pro-inflammatoriese en anti-inflammatoriese sitokiene, insluitend die nuwe sifting van die *LGALS*13 geen potensiaal vir variante wat ‘n impak op swangerskap uitkomste sal hê. Die siftings tegnieke toegepas, sluit in ‘n kombinasie van alleel-spesifieke amplifikasie, Multiphor enkelstring konformasie polimorfisme / heterodupleks analise, restriksie ensiem verterings en volgorde bepalings tegnieke.

‘n Betekenisvolle assosiasie was gedemonstreer tussen die *IL*-1*RN**2-alleel en nadelige swangerskap, beperk tot voortydse kraam en die hipertensie groep. Die teenwoordigheid van die *TNF*α-308 A-alleel was geassosieer met algehele nadelige uitkomste en voortydse kraam. Daarby, was ‘n nuwe *IL*-1*RN* alleel geïdentifiseer in die kontrole groep.

Mutasie sifting en opeenvolgende statistiese metodes, het ‘n assosiasie getoon tussen ‘n nuwe *LGALS*13 koderende variant, 221delT, en voortydse kraam in Kleurling vrouens. Twee voorafbeskryfde introniese variante (IVS2-22 A/G en IVS3+72 T/A), het ‘n betekenisvolle bewys opgelewer dat daar koppelings-onewewig bestaan tussen hierdie variante, en toon evolusionêre

konservasie van ekson drie. Addisioneel was twee nuwe introniese variante ontdek, IVS2-36 G/A en IVS2-15 G/A, wat geen assosiasie getoon nie.

In hierdie studie het ons ‘n nuwe seldsame koderende variante geïdentifiseer in die kontrole groep, waarvan twee nie-sinonieme variante was in ekson drie (M44V, N=2 en K87R, N=1) en ‘n stil variasie in ekson vier (P117P, N=1). Geleë in die koderende area van ekson drie, was ’n interessante variant [“hotspot’] ontdek, waarvan ses basisse in ‘n 11 basis paar area polimorfies is. Geen assosiasie was getoon met hierdie variante en swangerskap uitkomste nie.

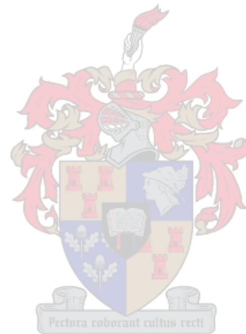
Verder was ‘n voorafbeskryfde 5' ‘promotor’ variant, -98 A/C, geïdentifiseer wat geen assosiasie getoon met nadelige swangerskap uitkomste nie. Onderverdeling van laat-aanvangs pre-eklampsie, het getoon dat die A-alleel ‘n betekenisvolle assosiasie getoon het met die ontwikkeling van laat pre-eklampsie.

Genotipe-fenotipe interaksies het ’n assosiasie getoon tussen die *IL*-10 -1082 A/G, *IL*-4 C/T en 221delT lokusse en nadelige swangerskap uitkomste, wat manifesteer as (i) kraam van suigelinge wat <2000g weeg, (ii) geboorte voor 37 weke.

Die bevindings van hierdie studie sal ons basiese kennis verbeter oor die patologie beskrywend aan swangerskap komplikasies, asook die fasilitering en ontwikkeling van effektiewe behandelings strategieë, om moederstrefte en fetale mortaliteit en morbiditeit te verminder.

NRF acknowledgement

The financial assistance of the National Research Foundation (NRF) towards this research project is hereby acknowledged. Opinions expressed and conclusions arrived at, are those of the author and not necessarily to be attributed to the NRF.



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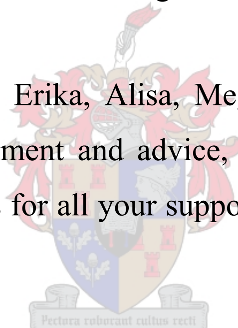

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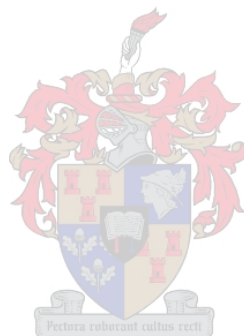

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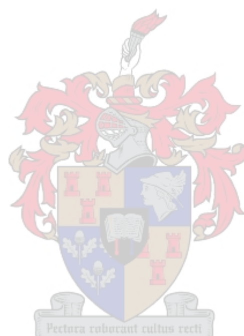

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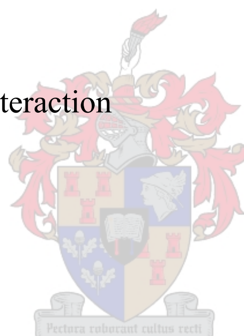

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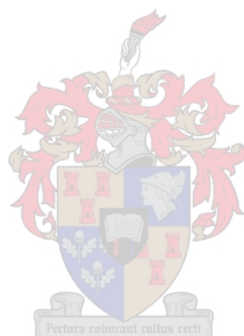

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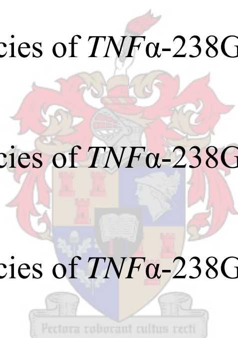

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List of Abbreviations

% - percentage
~ - approximately
@ - at
˚C - degree celcius
' - prime
< - less than
> - greater than
≥ - greater and equal to
© - control (primers)
µL - microliter
^{13}P-NMR - phosphorus-13 nuclear magnetic resonance
5'UTR - 5' untranslated region

A - adenosine
AA - arachidonic acid
AMP - adenosine monophosphate
AP - abruptio placentae
APS - ammonium persulphate
Arg - arginine
ARMS - amplified refractory system
Asp - aspartic acid
ASSP - alternative splice site predictor
ATG - start codon
ATPase - adenosine triphosphatase

BLAST - Basic Local Alignment Search Tool
bp - base pairs
BV - bacterial vaginosis

C	-	cytosine
Ca^{2+}	-	calcium ion
cAMP	-	cyclic adenosine monophosphate
cGMP	-	cyclic guanosine monophosphate
CI	-	confidence interval
Cl^{-}	-	chloride ion
COX	-	cyclooxygenase
CRD	-	carbohydrate recognition domain
CSFs	-	colony stimulating factors
CVD	-	cardiovascular disease
Cx	-	connexin
Cys	-	cystein residues
D'	-	measure of linkage disequilibrium
dbSNP: rs	-	database single nucleotide polymorphism: reference sequence
ddH_2O	-	double distilled water
dH_2O	-	distilled water
delT	-	deletion of a single thymine base
DHPLC	-	denaturing high performance liquid chromatography
DNA	-	deoxyribonucleic acid
dNTPs	-	2´-deoxy-nucleotide-5´-triphosphates
E	-	exon
EDTA	-	ethylenediaminetetraacetic acid
ELBW	-	extremely low birth weight
ELISA	-	enzyme-linked immunosorbent assay
ESE	-	exonic splice elements
EtOH	-	ethanol
F	-	forward primer
FIRS	-	fetal inflammatory response syndrome

G	-	guanosine
g	-	gram
$G_{\alpha q}$	-	heterotrimeric G protein α q subunit
Gln	-	glutamine
Glu	-	glutamic acid
Gly	-	glycine
GTPase	-	guanosine triphosphatase
HELLP	-	hemolysis, elevated liver enzymes and low platelets
HIV	-	human immuno-deficiency virus
HLA	-	human leukocyte antigen
hrs	-	hours
HWE	-	Hardy Weinberg equilibrium
IDT	-	Intergrated DNA Technologies
IFN-γ	-	interferon gamma
IgE	-	immunoglobuline E
IL-1	-	interleukin-1 gene
IL-1	-	interleukin-1 protein
IL-1ra	-	interleukin-1 receptor antagonist protein
IL-1*RN*	-	interleukin-1 receptor antagonist gene
IL-1RN*1	-	interleukin-1 receptor antagonist allele 1 [410 bp (four repeats)]
IL-1RN*2	-	interleukin-1 receptor antagonist allele 2 [240 bp (two repeats)]
IL-1RN*3	-	interleukin-1 receptor antagonist allele 3 [500 bp (five repeats)]
IL-1RN*4	-	interleukin-1 receptor antagonist allele 4 [325 bp (three repeats)]
IL-1RN*5	-	interleukin-1 receptor antagonist allele 5 [595 bp (six repeats)]
Ile	-	isoleucine
IUD	-	intrauterine death

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IUGR	-	intrauterine growth restriction
IVS	-	intervening sequence
K^+	-	potassium ion
KCl	-	potassium chloride
kDa	-	kilo Daltons
KH_2PO_4	-	potassium dihydrogen phosphate
$KHCO_3$	-	potassium hydrogen carbonate
L	-	liter
LBW	-	low birth weight
*LGALS*13	-	lectin, galactoside-binding, soluble 13
LMW	-	low maternal weight
LOD	-	logarithm of odds
LPS	-	lipopolysaccharide stimulated
M	-	molar
MALDI-TOF	-	Matrix Assisted Laser Desorption /Ionization- Time of Flight
$MgCl_2$	-	magnesium chloride
mg	-	milligram
mg/mL	-	milligram per milliliter
min	-	minute
mL	-	milliliter
MLBW	-	moderately low birth weight
MLC	-	myosin light chain
MLCK	-	myosin light chain kinase
mM	-	millimolar
MMPs	-	matrix metalloproteinases
MoM	-	multiples of gestation-specific median value
mRNA	-	messenger ribonucleic acid

N	-	total
Na^+	-	sodium ion
NaCl	-	sodium chloride
Na_2HPO_4	-	disodium hydrogen phosphate
NaOH	-	sodium hydroxide
NCBI	-	National Center for Biotechnology Information
ng	-	nanogram
ng/µL	-	nanogram per microliter
ng/mL	-	nanogram per milliliter
NH_4Cl	-	ammonium Chloride
NK-κB	-	nuclear factor kappa B
nm	-	ultra violet detection of DNA
NO	-	nitric oxide
NSAID	-	non-steriodal anti-inflammatory drug
OR	-	odds ratio
ORF	-	open reading frame
P	-	probability
PAGE	-	polyacrylamide gel electrophoresis
PAMPs	-	pathogen-associated molecular patterns
PAPP-A	-	pregnancy-associated plasma protein A
PBS	-	phosphate-buffered saline
PCR	-	polymerase chain reaction
PDA	-	piperazine diacrylamide
pg/mL	-	picogram per milliliter
PGE_2	-	prostaglandin E_2
$PGF_{2\alpha}$	-	prostaglandin $F_{2\alpha}$
pH	-	potential of hydrogen
PI	-	pulsatility index
PIH	-	pregnancy-induced hypertension

PP13	-	placental protein 13
PPROM	-	preterm premature rupture of membranes
PROM	-	premature rupture of membranes
PRRs	-	pattern recognition patterns
PTL	-	preterm labour (Tables)
R	-	reverse primer
r^2	-	measures of linkage disequilibrium (correlation coefficient)
RE	-	restriction enzyme
REA	-	restriction enzyme analysis
RFLP	-	restriction fragment length polymorphism
Rho	-	ras homologous proteins
rpm	-	revolutions per minute
RR	-	relative risk
SBP	-	systolic blood pressure
SDS	-	Sodium dodecyl sulphate
sec	-	second
SF2/ASF	-	Splicing Factor 2/Alternative Splicing Factor
SGA	-	small-for-gestational-age
SIFT	-	sorting intolerant from tolerant
SNPs	-	single nucleotide polymorphisms
SRp40	-	splicing factor, arginine/serine-rich 5
SRp55	-	splicing factor, arginine/serine-rich 6
SSCP/HD	-	single stranded conformational polymorphism and heteroduplex
Ta	-	annealing temperature
TBE	-	Tris-Borate-EDTA
TE	-	Tris-EDTA
TEMED	-	N,N,N',N'-tetramethylethylenediamine
TGA	-	stop codon

T_{H2}	-	helper T Cells
Thr	-	threonine
TLR	-	toll like-receptors gene
TLR	-	toll like-receptors protein
Tm	-	melting temperature
*TNF*1	-	tumour necrosis factor common allele
*TNF*2	-	tumour necrosis factor minor allele
*TNF*α	-	tumour necrosis factor alpha gene
TNFα	-	tumour necrosis factor alpha protein
TRIS	-	trishydroxymethylaminomethane
TRIS-HCl	-	Tris hydrochloride
Tyr	-	tyrosine
U	-	unit
V	-	volts
VEGF	-	vascular endothelial growth factor gene
VEGF	-	vascular endothelial growth factor protein
VLBW	-	very low birth weight
WHO	-	World Health Organisation
wks	-	weeks
WT	-	wildtype
X	-	times
yrs	-	years
β/γ	-	beta/gamma
β2AR	-	beta-2-adrenergic receptor
β-hCG	-	human beta-chorionic gonadotrophin

1. Introduction

1.1. Preterm labour

1.1.1. Definitions

The World Health Organisation (WHO) defined preterm labour as the onset of labour before 37 completed weeks of gestation (Steer, 2005). However, this term has since been used more specifically, viz, “very preterm”, “extremely preterm”, “moderately preterm” and “late preterm” (Reedy, 2007).

For many years, newborn infants weighing less than 2500g were regarded premature for development. In 2001, low birth weight (LBW) was sub-divided into the following categories: moderate low birth weight (MLBW) for infants weighing between 1500-2499g, very low birth weight (VLBW) weighing less than 1500g and extremely low birth weight (ELBW) weighing less than 1000g (Cano *et al*., 2001).

The discrepancies of a valid definition for preterm birth in the literature have resulted in some scientist using birth weight as a marker for preterm (Reedy, 2007). The development of more accurate methods determining gestation led to the conclusion that most of the infants that delivered at term with a low birth weight were due to intrauterine growth restriction (IUGR) (Peltier, 2003).

Classifying neonatal outcome by birth weight does not distinguish between small-for-gestational age (SGA) and preterm labour. In a cohort of SGA infants, growth restriction can also be a cause, leading to false associations. Thus studies considering gestational age are favored when investigating the cause and outcome of preterm labour (Murph, 2007).

1.1.2. Incidence and Trends

The incidence of preterm delivery ranges between 5% and 11%. In the developing world, the rate overall has been increasing gradually or remained static over the past 10-20 years, but has decreased in some developed countries (Danielian and Hall, 2005).

In 2006 Barros and Vélez evaluated a database of 1.7 million births in the period 1985-2003 in Latin America. Preterm birth was subdivided according to the presence or absence of maternal medical or obstetric complications, spontaneous labour, and preterm labour after premature rupture of membranes (PPROM), induction of labour or elective caesarean. The most common subgroup for preterm birth was spontaneous preterm labour without maternal complication, which accounted for 60% of preterm deliveries. Delivery by elective induction and elective caesarean increased from 10% in 1985-1990 to currently, 18.5% (Barros and Vélez, 2006).

The rate of singleton preterm birth in New Zealand increased from 4.3% in 1980 to 5.9% in 1999. A notable increase of 72% was observed in the high socioeconomic groups, with only a 3.5% increase in disadvantaged groups. This observation may be attributable to the effects of delayed childbearing in wealthy career women and the increased use of assisted reproductive technology (Danielian and Hall, 2005). In Sweden the rate of preterm birth decreased from 6.3% in 1984 to 5.6% in 2001, accompanied by a rise in preterm multiple births from 0.34% to 0.71% (Murph, 2007). The general proportion of preterm deliveries in Denmark increased in the period 1995 - 2004 from 5.2 % to 6.2%. Stratification into extremely preterm (22-28 weeks), very preterm (28-32 weeks) and moderately preterm (32-36 weeks) groups demonstrated an increase of 41%, 22% and 22%, respectively. In the same population group, the incidence of spontaneous preterm deliveries in primiparous women at low risk rose by 51% from 3.8% in 1995 to 5.7% in 2004 (Langhoff-Roos *et al*., 2006).

At Tygerberg Hospital in South Africa, a tertiary referral hospital, preterm delivery accounts for 20.3% of pregnancies. At least 37.5% of all neonatal deaths can be accounted by prematurity (Schoeman *et al*., 2005). In the United States, the rate of preterm birth in 2004 for African American women was 17.6%, Native Americans 13.2%, Hispanics 12%, non-Hispanic whites

11.5% and Asians 10.4%. The increased use of assisted reproductive technology could account for the increase of preterm birth throughout 2004. Across the United States, the incidence of preterm labour and preterm birth has increased from 9.7% in 1990 to over 10.8% in 2004 among singleton and first time pregnancies (Reedy, 2007).

Figure 1: Graphical representation of the incidence of preterm labour worldwide, based on above mentioned information (red dots illustrate an increase in preterm labour and green a decrease).

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1.1.3. Risk factors

Epidemiological studies have identified certain risk factors that may be associated with preterm labour but do not *predict* labour before term (Table 1). An increasing amount of evidence suggests that infection during pregnancy may be a contributing factor for preterm labour (discussed later) (Wen *et al*., 2004). One of the strongest risk factors for preterm labour is a previous history of the condition. The expected frequency of preterm labour in subsequent pregnancies after one preterm birth is 14.3% and after two preterm births, it increases to 28% (Chandraharan and Arulkumaran, 2005b). Women with a previous history of PROM have an estimated risk of 16-32% of developing recurrent PROM and a 34-44% risk of developing preterm delivery (Danielian and Hall, 2005). Other factors that increase the risk of preterm labour

are multiple gestations, polyhydramnios or other causes of distension of the uterus with subsequent pressure on the cervix (Reedy, 2007).

Table 1: Risk factors for preterm labour (Reedy, 2007).

Medical history
History of birth ≤ 37 completed gestational weeks
History of preterm premature rupture of membranes
History of threatened preterm labour
History of cervical surgery
Factors in present pregnancy
Artificial reproductive technology
Uterine anomaly
Chronic urinary tract infection
Maternal weight
Severe socioeconomic stress
Smoking > 1 pack of cigarettes per day
Substance abuse
Polyhydramnios
Multiple gestation

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Low socioeconomic status has been implicated in preterm labour. These socio-economic factors include a younger mother not able to work, single without support from family, poor nutritional status and higher incidence of substance abuse. Racial differences are also a risk factor for preterm labour, but this link is not clearly understood (Reedy, 2007). The reported rates of preterm delivery among black and white racial groups indicate a two-fold higher incidence for preterm labour or delivery among black women (Robinson *et al*., 2001). A recent large population-based (United States, N=1,577,08) cohort study performed in 2007 by Kistka *et al*., reported that recurrent preterm birth appeared more frequently in black women than in white women (adjusted OR, 4.11; 95% CI, 3.78-4.47). An extreme of maternal age is also a possible factor with an increased frequency of spontaneous preterm labour in teenage pregnancies, whereas the older gravidas experience higher rates of medically induced preterm births (Mountquin, 2003). Research on low maternal weight (LMW) found that LMW women who conceive are at increase risk of pregnancy complications such as preterm labour, IUGR and LBW, compared to women with normal body weight (Ehrenberg *et al*., 2003).

The abuse of tobacco, alcohol and recreational drugs has not been established as an underlying cause of preterm labour or preterm birth, but the use of these drugs has been implicated in preterm labour due to dangerous effects these substances have, which could place the fetus at risk of prematurity. Fetal risks associated with smoking during pregnancy include IUGR and oligohydramnios (Reedy, 2007). Odendaal *et al.* found that smoking during pregnancy increased the risk of preterm labour and abruption placentae, which are the two main causes of perinatal deaths at Tygerberg Hospital (Odendaal *et al*., 2001). Alcohol usage of more than nine drinks per week places women at risk of preterm labour, but a lower intake in the third trimester lowers the incidence of preterm birth possibly due to the use of alcohol as a tocolysis (Reedy, 2007). The use of street drugs such as cocaine increases the risk of preterm labour to 17-19% compared to the general population risk of 5-11% (Fajemirokun-Odudeyi and Lindow, 2004).

1.2. The onset of labour

The myometrium and the cervix of the human uterus function to maintain pregnancy and deliver the fetus at term. Throughout gestation, the myometrium remains relatively inactive regardless of progressive cellular growth and stretch needed to accommodate the growing fetus. During this time, the cervix is closed, functioning as a protective barrier. Towards the end of gestation, the cervix softens and the myometrium switches from inactive to an active organ required to expel the fetus and the placenta during labour (Hertelend and Zakar, 2004).

1.2.1. The myometrium

The myometrium consist of a distinctive muscular layer involved in contraction during labour. The myometrium mainly consists of smooth muscle cells but also contains fibroblasts, blood and lymphatic vessels, immune cells and connective tissue. The connective tissue serves as a supportive environment for the bundles of smooth muscle and provides a framework that expands as the uterus distends during gestation (Rehman *et al*., 2003). Surrounding the myometrial smooth muscle cells are extracellular substances composed of collagen fibers, fibroblast and bone marrow derived cells. As pregnancy progresses, the myometrium expands by means of hypertrophy and division of smooth muscle cells.

The myometrial cells interact with each other through intercellular channels known as gap junctions that link the interiors of two cells (Thomson and Norman, 2005). These channels are sites of communication, which allow rapid conduction of electrical impulses and chemical signals from one cell to another. This electrical coupling assists with organisation of each individual cell's contraction, necessary to propagate the activity of the organ (Fuchs and Fuchs, 1996). The structure of the channels is based on proteins known as connexins, which extend through the plasma membranes to form pores. Two connexin proteins are abundant in the pregnant myometrium, viz, connexin-43 (Cx-43) and connexin-26 (Cx-26), with sequential different patterns of expression. The expression of Cx-43 is low during most of gestation, increasing significantly before the start of labour, while Cx-26 expression is higher during late pregnancy and low during labour. Cx-43 is believed to regulate electrical coupling of the myometrium during labour, whereas Cx-26's involvement in myometrial contractility remains to be elucidated (Thomson and Norman, 2005).

The myometrium consist of thin, intermediate and thick filaments as well as dense bodies, which allow the attachment of contractile filaments to α-actinin (Fuchs and Fuchs, 1996). The myometrial smooth muscle filaments are not arranged in fibres and fibrils, like striated muscle, but rather as random bundles throughout the myocyte. Myometrial contraction occurs from the sliding activity of actin and myosin with no change in length of both filaments, much the same as with skeletal muscle (Thomson and Norman, 2005).

1.2.2. The cervix

The cervix is structured to keep the cervical canal closed and sustain the integrity of the intrauterine space for development of the growing fetus (Hertelendy and Zakar, 2004). The cervix is composed of fibrous connective tissue surrounded by an extracellular matrix consisting of type I and type III collagen fibers. The other fibrillar structures include elastin, proteoglycans, and a cellular portion consisting of smooth muscle, fibroblast, epithelium and blood vessels (Maul *et al*., 2006). The covalent bindings of proteoglycans with numerous glucoseaminoglycan chains such as dermatan, eparan and chondroitin sulphates, hyaluronic acid and aggrecan determine the stability of the cervix. These compounds are negatively charged to bind large amounts of water,

leading to an increased volume in contrast to weight. Remodeling of the cervical structure takes place throughout pregnancy and involves the orientation and integrity of collagen fibres. In addition to this, water content, proteoglycans and hyaluronic acid is also regulated (Facchinetti *et al*., 2005).

The fibroblasts found within the cervix, synthesise collagenase, which is also released by infiltrating neutrophils during labour, facilitated by increased prostaglandin production and interleukin-8 under the control of nuclear factor-kappa B (NK-κB). The degradation of collagenase and increased amounts of dermatan sulphate proteoglycans partially explain the pregnancy-related softening of the cervix (Shennan and Jones, 2004).

1.2.3. Myometrial contractility

The structural basis for uterine contractility is the binding of actin and myosin controlled by the enzyme myosin light chain kinase (MLCK), activated by a rise in intracellular calcium (Bernal, 2003). Receptors for β-sympathomimetics on the cell-surface membrane of the myometrial cell assist with maintaining relaxation, promoting the opening of potassium channels and keeping the inside of the myocyte relatively electronegative, reducing the likelihood of depolarisation and contraction (Smith, 2007).

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In the myometrium, at the time of labour, the electrochemical gradient is reduced by the binding of prostaglandins to the E- and F-prostaglandin receptors, which permit the opening of ligand-regulated calcium channels. The binding of oxytocin activates the $G_{\alpha q}$ protein linked to phospholipase C. Activation of phospholipase C activates the protein kinase C pathway, and most likely activates the MLCK. The release of inositol triphosphate via the binding of oxytocin facilitates the release of intracellular calcium in the stored sarcoplasmic reticulum. As the levels of calcium increase in the cell, the electro-negativity drops and activates the opening of more voltage-regulated calcium channels leading to an increase intracellular concentration of calcium resulting in depolarisation (Smith, 2007). As calcium enters the cell, it binds to calmodulin and forms an active complex activating the MLCK, which in turns phosphorylates the myosin light chain (MLC) (Bernal, 2003). The phosphorylation of the MLC causes the production of ATPase

activity leading to contraction caused the sliding of the myosin over the actin filaments. This process is reversible promoting relaxation regulated by the adenylate cyclase pathway, increasing intracellular cyclic adenosine monophosphate (cAMP) and activating the protein kinase A, which inhibits MLCK (Smith, 2007).

Uterine contractions can also be improved by a calcium-independent pathway relating to the activation of small guanosine triphosphatase (GTPase) of the Rho family. The activation of Rho A, a monomeric G protein, mediates the phosphorylation of the MLC, stimulating contraction (Bernal and TambyRaja, 2000 and Bernal, 2003).

1.2.4. Contraction and relaxation

The cells in the myometrium are electrically volatile and maintain the ionic gradients across the plasma membrane. The regulation of the transmembrane gradient of calcium (Ca^{2+}), sodium (Na^{+}), chloride (Cl^{-}) and potassium (K^{+}) is sustained by active transport of ions across membranes controlled by the actions of pumps and co-transporters (Blanks *et al*., 2007).

The basis for contraction and relaxation is the rise and fall of intracellular calcium. A complex interaction of membrane potential, voltage and receptor operating channels and Ca^{2+} pumps regulate circulating levels of calcium inside the myocyte. The resting intracellular calcium is lower than the intracellular calcium threshold for contraction. The rapid decrease of intracellular calcium are coordinated by the uptake of calcium into the sarcoplastic reticulum, the release of calcium outside the cell via plasma membrane Ca^{2+}/ATPase, and by lesser degree via the Na^{2+}/Ca^{2+} exchange. The myometrial state of relaxation entails the activation of calcium-activated large conductance K^{+} channels, resulting in membrane hyperpolarisation (Bernal and TambyRaja, 2000).

1.2.5. Infection and myometrial contractility

Bacterial species producing endo- and exotoxins have an elevated effect on cytokine and prostaglandin production in the amnion, chorion and decidua. As the levels of toxins or cytokines increase, myometrial function is impaired and abnormal labour is evident (Newton, 2005). These cytokines enhance the regulation of cyclo-oxygenase (COX)-2 in the amnion, decidua and myometrium leading to increase production of uterine prostaglandin and consequently the start of labour (Rauk and Chiao, 2000).

In two studies evaluating the role of urogenital infections on the contractility of isolated human myometrium, it was found that mycoplasma infection decreased while Chlamydia or mixed infection of both increased the myometrial contraction induced by oxytocin or prostaglandin $F_{2\alpha}$ (Zefirova *et al*., 2002a and Zefirova *et al*., 2002b).

1.3. Infection as a cause of preterm labour

Intrauterine infection is found to be a common and important mechanism in the ethiology of preterm birth. Evidence supporting the casual relation between infection and spontaneous preterm labour include (Romero *et al*., 2006a):

1. Systemic administration of microbial products to pregnant animals results in spontaneous preterm labour and preterm birth.
2. Preterm births have been associated with several extrauterine maternal infections, such as malaria, pyelonephritis, pneumonia and periodontal disease.
3. Preterm labour and preterm birth have been associated with sub-clinical intrauterine infections.
4. Mid-trimester pregnant women with intra-amniotic infection or intrauterine inflammation (defined as an increased concentration of cytokines in the amniotic fluid and matrix degrading enzymes) are at increased risk of subsequent preterm birth.
5. Preterm birth can be prevented in experimental models of chorioamnionitis, by treating the intrauterine infection with antibiotics.

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6. Treating asymptomatic bacteriuria may prevent preterm birth.

1.3.1. Pathways of infection

There are various routes in which microorganisms can gain access to the amniotic cavity and fetus: (1) ascending from the vagina and cervix; (2) haematogenous dissemination through the placenta; (3) retrograde seeding from the peritoneal cavity through the fallopian tube; and (4) accidental introduction at the time of invasive procedures, such as amniocentesis, percutaneous fetal blood sampling, chronic villus sampling, or shunting (Romero *et al*., 2006a).

The most common pathway of intrauterine infection is the ascending route, which is characterised by four stages (Figure 1). During the first stage, there's a change in the vaginal and cervical microbial flora or the presence of pathological organisms in the cervix. Bacterial vaginosis (BV) may be a first indication of stage I infection. In stage II, as the microorganisms gain access to the intrauterine cavity, they inhabit the decidua and cause an inflammatory response leading to deciduitis. As infection spreads they invade the fetal vessels or the amnion in the amniotic cavity leading to choriovasculitis or amnionitis, respectively. This leads to microbial invasion of the amniotic cavity or an intra-amniotic infection known as stage III. As bacteria enter the amniotic cavity they gain entry to the fetus (stage IV) by targeting amniotic fluid leading to congenital pneumonia. Infection of the amniotic fluid can cause otitis, conjunctivitis and omphalitis in the fetus, and via these sites of infection, gain access to fetal circulation causing bacteremia and sepsis (Romero et al, 2003).

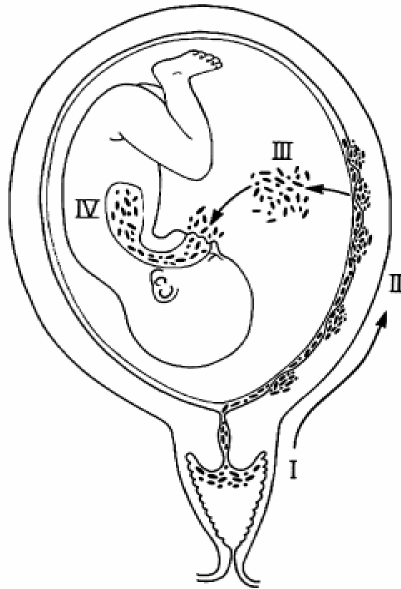


Figure 2: The ascending route of intrauterine infection. (I) Vaginal infection, (II) inflammation of fetal membranes, (III) microbial invasion of the amniotic cavity, (IV) and fetal inflammation (Romero *et al*., 2006a).

1.3.2. Microbiology of uterine infection

The most frequent microorganisms isolated from the amniotic cavity are genital *Mycoplasma* species, particularly *Ureaplasma urealyticum*, implicated in women with preterm labour and intact membranes. Additional microorganisms encountered in the amniotic cavity include *Streptococcus agalactiae*, *Escherichia coli*, *Fusobacterium* species and *Gardnerella vaginalis* (Romero *et al*., 2006a).

1.3.3. Infection of the genital tract

1.3.3.1. Fetal infection

Fetal infection (stage IV) is the most advanced and severe stage of ascending intrauterine infection (Figure 2). In general, the mortality rate of neonates with congenital neonatal sepsis ranges between 25% and 90% (Gonçalves *et al*., 2002). Inflammatory invasion of the umbilical cord vessels (funisitis) reflects a more accurate indication of fetal infection in contrast to the inflammation of the membranes, which are largely of maternal origin. Funisitis have been

associated with amniotic fluid infection, congenital neonatal sepsis and the fetal inflammatory response syndrome (Tasci *et al*., 2006).

The Fetal Inflammatory Response Syndrome (FIRS) is defined as an increase of pro-inflammatory cytokines in the fetal circulation, with an IL-6 concentration of > 11ng/mL in the fetal blood. It is present in most cases of preterm labour or PPROM and is related to an *in utero* multi-organ dysfunction that can cause septic shock and fetal death (Di Naro *et al*., 2006). Fetal organs targeted during FIRS include the haematopoietic system, adrenals, heart, brain, lungs and skin (Figure 3) (Romero *et al*., 2006b).

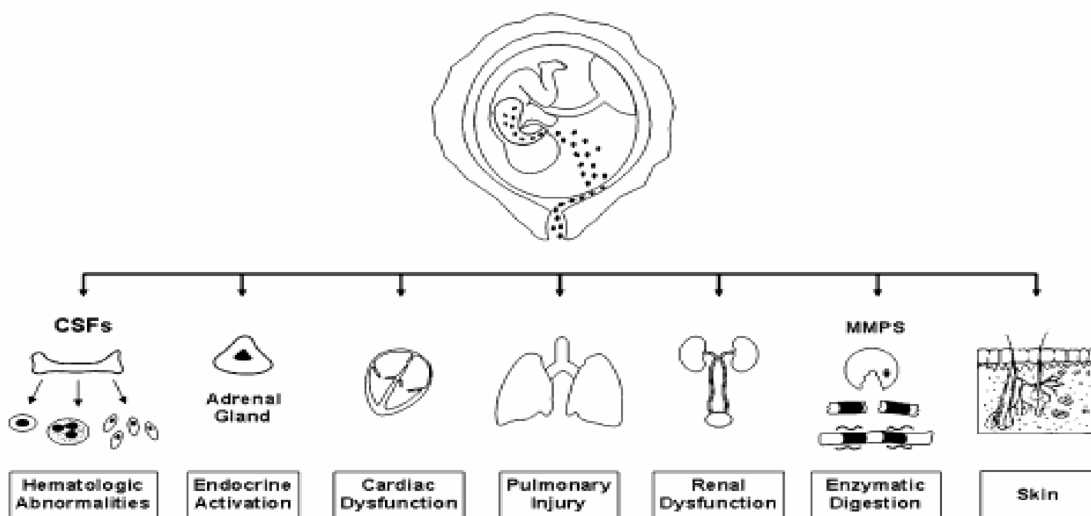


Figure 3: Fetal organs proposed to be target organs during the fetal inflammatory response syndrome (FIRS) are the haematopoietic system, adrenals, heart, brain, lungs and skin. (CSFs, colony stimulating factors; MMPs, matrix metalloproteinases) (Romero *et al*., 2006b).

Fetuses affected by FIRS have an increased rate of severe neonatal morbidities such as respiratory distress syndrome, suspected or proven neonatal sepsis, pneumonia, bronchopulmonary dysplasia, intraventricular haemorrhage, periventricular leukomalacia and necrotising enterocolitis (Romero *et al*., 2006b).

Suspected cases of FIRS can be diagnosed based on the levels of pro-inflammatory cytokines measured in cord blood samples collected at delivery. Pathological assessment of the umbilical cord is another approach to establish whether fetal inflammation was present before birth, since funisitis and chorionic vasculitis are histopathological features of FIRS. Additional approaches

include determining (i) C-reactive protein concentration in the umbilical cord blood, found to be elevated in patients with amniotic fluid infection, funisitis, and congenital neonatal sepsis, as well as (ii) neutrophils in the amniotic fluid. The white blood cell count in the amniotic fluid can also be applied as an indirect manifestation of fetal inflammation (Romero *et al*., 2006a).

1.3.3.2. Vaginal infection

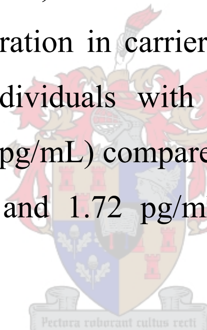
Infection of the vaginal tract leads to a condition known as bacterial vaginosis (BV). Commonly, the vaginal flora consists of both aerobic and anaerobic bacteria. The majority of microorganisms in the vaginal flora are *Lactobacillus spp.* representing 95% of all bacteria. These organisms provide a defense mechanism against infection by keeping the pH of the vagina acidic. Additionally, individuals with normal lactobacilli inhibit the colonisation of BV-associated organisms [usually lacking free radical scavengers] by producing high levels of hydrogen peroxide (Yudin, 2005).

BV is a clinical condition characterised by a change of vaginal flora, resulting in a decreased production of the normal, protective *Lactobacillus* species (Nelson *et al*., 2007) and an increased growth of pathogenic bacteria such as *Gardnerella vaginalis*, *Bacteriod spp*., *Mobiluncus spp.* and *Prevotella bivia* (Pretorius *et al*., 2007). The diagnosis of BV is often asymptomatic but may result in vaginal discharge that is grey in colour with a characteristic 'fishy odour'. The clinical diagnosis of BV is confirmed by three of the four following criteria (McDonald *et al*., 2007):

1. Vaginal pH > 4.7
2. The presence of clue cells (vaginal epithelium cells spotted with bacteria) on a Gram stain or wet mount of the vaginal discharge
3. The presence of thin homogenous discharge
4. The release of a fishy odour when potassium is added to a sample of discharge

The risk factors associated with BV include black ethnicity, low socioeconomic status, increased sexual activity and vaginal douching. These factors are mostly found in cases of symptomatic BV, with an uncertainty whether these can be applied to asymptomatic BV (Nelson *et al*., 2007).

BV has frequently been addressed as a risk factor for adverse obstetric outcomes such as preterm labour and delivery, PPROM, spontaneous abortion, chorioamnionitis and postpartum endometritis (O'Brien, 2005 and Yudin, 2007). Substances produced by BV-related organisms include endotoxins and enzymes such as sialidase, collagenase, protease, elastase, phospholipase and mucinase. The release of these substances has been shown to cause tissue damage, promoting the release of pro-inflammatory cytokines and prostaglandins. Pro-inflammatory cytokines such as interleukin (IL)-1, IL-6, IL-8, tumour necrosis factor alpha (TNFα), and prostaglandins E_2 and $F_{2\alpha}$ are found to be increased in the amniotic fluid of women with BV delivering preterm (Pretorius *et al*., 2007). A case-control study by Macones *et al*., investigated the role of the *TNF*α -308G/A genotype in women presenting with PPROM and preterm labour in combination with symptomatic BV. They found that maternal carriers of the *TNF*-308A allele were at increased risk of developing spontaneous preterm birth (OR 2.7, 95% CI 1.7-4.5). This association was further strengthen by the presence of BV (OR 6.1, 95% CI 1.9-21.0) (Macones *et al*., 2004). Genc *et al*., determined the vaginal *TNF*α concentration in carriers of the *TNF*-308A allele with abnormal vaginal flora. They showed that individuals with the *TNF*-308A allele with BV had a significantly higher *TNF*α level (10.94 pg/mL) compared to individuals with the *TNF*-308A allele without BV (1.77 pg/mL) (P=0.02) and 1.72 pg/mL in *TNF*-308G homozygotes with BV (P=0.01) (Genc *et al*., 2006).



1.3.3.3. Infection of the fetal membranes

The fetal membranes consist of the amnion and chorion, functioning as a defense barrier and helping to maintain normal pregnancy (Menon and Fortunato, 2007). Acute inflammation of the amnion and chorion membranes results in chorioamnionitis and is the primary cause of maternal and fetal complications, including preterm birth and neonatal infection (Lau *et al*., 2005). The result of chorioamnionitis, mostly subclinical, is primarily due to infection via the ascending route of the lower genital tract (Lahra *et al*., 2007) that resides in the choriodecidual space (Kidokoro *et al*., 2006). In other cases, infection crosses the intact chorioamniotic membranes into the amniotic fluid where it may infect the fetus.

Postpartum examination of the placenta revealed that the incidence of histological chorioamnionitis was 18% in women with delivery at term and 32% in women delivering preterm (Hung *et al*., 2005). The prevalence of *sub-clinical* histological chorioamnionitis is greater in earlier gestation: 50% at 24 to 28 weeks, 30% at 28 to 32 weeks, 20% at 33 to 36 weeks and 10% at more than 37 weeks. The likelihood that chorioamnioiotic cultures are positive for infection following delivery of smaller fetuses at caesarean section with intact membranes is 80% at less than 1000g, 60% at 1000 to 1499g, 35% at 1500 to 2499g and 30% at more than 2500g (Newton, 2005). Severe acute morbidity, including sepsis, necrotising enterocolitis and lung disease place the preterm infants at risk of developing intraventricular hemorrhage and periventricular leukomalacia, which is a strong predictor of mental retardation and cerebral palsy (Hagberg *et al*., 2002).

Microorganisms ascending from the vaginal cavity produce phospholipase A2 and C which cleave arachidonic acid in the fetal membranes, releasing free arachidonic acid available for production of prostaglandin, which in turn, could trigger preterm labour. Activated macrophages and lymphocytes in response to choriamnionitis induce an inflammatory cascade of effects, which play important role in the initiation of preterm labour (Saji *et al.*, 2000). Patients presenting with choriamnionitis have elevated levels of pro-inflammatory cytokines such as IL-1β, IL-6, TNFα and chemokine IL-8 in the amniotic fluid, cervical secretion, placenta and other sections of the placental-maternal unit. In a study performed by Willi *et al*., they evaluated the concentrations of IL-6 and IL-8 in patients with choriamnionitis compared to those without. They found that the levels of IL-6 (61.5pg/mL vs. 19.4 pg/mL, $P<0.01$) and IL-8 (162.3 pg/mL vs. 13.4 pg/mL, $P>0.001$) were significantly higher in patients with choriamnionitis (Willi *et al*., 2002).

Similar finding were reported by Jacobsson *et al*., who determined the levels of IL-6 and IL-8 in cervical and amniotic fluid in relation to the presence of bacteria in membranes of women with preterm labour. They found that the levels of IL-6 in cervical fluid and IL-6 and IL-8 in amniotic fluid were higher in women with preterm labour in whom bacteria could be detected in the chorioamniotic membranes (Jacobsson *et al*., 2005).

1.3.3.4. Intra-amniotic infection

Substantial evidence exists for support that intra-amniotic infection and the immune response induced by infectious agents contribute to the initiation of pregnancy complications such as preterm labour, PPROM and premature birth (Hadar *et al*., 2006). In patients with preterm labour, approximately 10% have microbial invasion of the amniotic cavity. These patients are at increased risk of future preterm delivery, clinical chorioamnionitis, neonatal morbidity, bronchopulmonary dysplasia and cerebral palsy (Gomez *et al*., 2005). The complex mechanism leading to preterm labour caused by intra-amniotic infection needs to be elucidated. Current evidence suggests that intra-amniotic infection initiates an immune response leading to increased production of cytokines, prostaglandins and metalloproteinase's that in turn leads to cervical softening, rupture of fetal membranes, uterine contractions and preterm birth (Hermansen and Hermansen, 2006).

The most frequent organism detected in the amniotic cavity is *Ureaplasma urealyticum*. The host response towards these organisms in amniotic fluid, maternal and fetal compartments has been linked to preterm labour and PPROM. Patients with positive amniotic *Ureaplasma urealyticum* cultures have elevated levels of pro-inflammatory cytokines (TNFα, IL-1β, IL-6) in their amniotic fluid, increased plasma concentration of IL-6 in umbilical cord blood, a higher incidence of developing chorioamnionitis, increased risk of preterm delivery and adverse perinatal outcome, in contrast to patients with a sterile amniotic environment (Gomez *et al*., 2005).

1.4. Inflammation as a reason for preterm labour

1.4.1. Innate immune receptors of the genital tract

The first line of defense against microbial infection is the innate immune system, whereas the adaptive immune system develops about four-seven days later. The innate immune system provides protection against a broad diversity of pathogens, using pattern recognition receptors (PRRs) to recognise conserved microbial components known as pathogen-associated molecular patterns (PAMPs). The PRRs include, among others, members of the Toll-like receptors (TLRs)

(Albiger *et al*., 2007), which are important components of the innate immune system (Kim *et al*., 2006). The family of TLRs elicits intracellular activation of NK-κB and several others kinases, initiating the release and production of cytokine and anti-microbial peptides. These peptides are expressed in the endometrium and production is induced after intrauterine infection in preterm and term pregnancies, supporting the indirect involvement of the innate immunity (Hagberg *et al*., 2005).

To date, 10 different TLRs have been identified in humans (Ma *et al*., 2007). TLR-1, -2, -3, -5 and -6 are found in epithelia from the vagina, ecto- and endocervix, endometrium and uterine tubes. TLR-4 has been identified in the endocervix, endometrium, fallopian tubes and ectocervix (Romero *et al*., 2006a). TLR-2 mostly recognises products of Gram-positive bacteria, such as peptidoglycans and products of Mycoplasma and fungi, whereas TLR-4 distinguishes bacterial endotoxin from components of Gram-negative bacterial cell wall. The expression of TLR-2 and -4, in combination with activation of NK-κB, is significantly elevated in preterm deliveries with chorioamnionitis, demonstrating that the innate immune system manifests an inflammatory response during normal spontaneous labour (Kim *et al*., 2006). In the first trimester, apoptosis of cytotrophoblast (observed in miscarriage, IUGR and pre-eclampsia (Romero *et al*., 2006a)) is promoted by TLR-2-dependent signaling, while TLR-4 signaling stimulates production of cytokines. These observations suggest that intrauterine infection may lead to either trophoblast apoptosis or synthesis of cytokines that recruit immune cells and maintain trophoblast survival (Ma *et al*., 2007).

1.4.2. The role of pro-inflammatory cytokines

An increasing body of data implicates the role of cytokines in the mechanisms underlying preterm parturition linked to intrauterine infection. Pro-inflammatory cytokines including IL-1, TNFα, IL-6 and IL-8 in the intrauterine tissue are secreted in response to infection, and increased amounts are found in the amniotic fluid of women with infection-related preterm labour. The presence of sub-clinical intrauterine infection, implicated in a proportion of preterm birth, is associated with increased release of pro-inflammatory cytokines in the amniotic fluid leading to activation and synthesis of prostaglandins that can provoke uterine contractions (Makhseed *et al*.,

2003). The release of prostaglandins can further display uterotonic effects and increase the production of matrix metalloproteinases (MMPs), which are implicated in connective tissue degradation in the amnionchorion and cervix, related to membrane rupture and cervical ripening (Hernandez-Guerrero *et al*., 2003). IL-8 acts as a chemotactic factor attracting neutrophils to the cervix, stimulating the synthesis of proteases such as collagenase and elastase and promoting uterine changes in the cervix during labour (Gonzalez Bosquet *et al*., 2005).

The first cytokine to be associated with spontaneous preterm labour related to infection is IL-1. Evidence supporting this include: (a) human decidua produces IL-1 in response to bacterial products; (b) production of prostaglandin by the human amnion and decidua are stimulated by IL-1; (c) IL-1 in the amniotic fluid of women with preterm labour and infection has increased concentrations and bioactivity; (d) myometrial contractions can be stimulated by IL-1; and (e) administration of IL-1 to pregnant animals induces preterm labour and preterm birth, which can be blocked by administering IL-1 receptor antagonist (IL-1ra).

The roles of TNFα supporting the mechanism of preterm labour include: (a) prostaglandin production in the human amnion, decidua and myometrium are stimulated by TNFα; (b) TNFα is produced by human decidua in response to bacterial products; (c) the activity and concentrations of TNFα in the amniotic fluid are elevated in preterm labour and intra-amniotic infection; (d) during labour TNFα levels are higher in women with PPROM and intra-amniotic infection; (e) the production of MMPs is increased by TNFα; (f) in the cervix, TNFα stimulates changes similar to cervical ripening; and (g) in animal models, TNFα is associated with bacterial-induced preterm parturition (Romero *et al*., 2006a).

IL-6 is the strongest cytokine associated with preterm labour in the setting of intra-amniotic infection, produced by a selection of cells in response to bacterial infection, viruses and second messenger agonists. The production of IL-6 is increased by TNFα and IL-1β, and higher levels are found in umbilical cord blood, representing an independent risk factor for spontaneous preterm delivery and also neonatal morbidity (Jamie *et al*., 2005).

1.4.3. The role of anti-inflammatory cytokines

Anti-inflammatory cytokines such as IL-10, IL-4 and IL-1ra regulate the effect of the pro-inflammatory cytokines during pregnancy. The two most potent cytokines are IL-4 and IL-10, produced by the T_{H2} lymphocytes. These cytokines stimulate differentiation of T lymphocytes via the T_{H2} pathway, blocking production of interferon (IFN)-γ that regulates pro-inflammatory cytokine synthesis. IL-4 and IL-10 activate B-lymphocytes leading to B-cell proliferation and antibody production during pregnancy. The genes encoding IL-4 and IL-10 are both polymorphic and can lead to altered cytokine levels in different individuals. These discrepancies may alter the degree of the anti-inflammatory immune response during pregnancy and induce susceptibility to PPROM, preterm labour and subsequent preterm birth (Kalish *et al*., 2004). These cytokines reduce the production of prostaglandin by lipopolysaccharide (LPS)-stimulated human monocytes and neutrophils, thereby inhibiting the production of COX-2. Consequently, low circulating levels of IL-4 and IL-10 may initiate labour (Annells *et al*., 2004).

In response to an alteration in the vaginal flora, both, the levels of IL-1β and IL-1ra increase. IL-1ra, a competitive inhibitor of IL-1β, modulates the action of the pro-inflammatory response, destroying pathogenic microorganism while structural tissue and function are maintained (Genc *et al*., 2004a). Studies performed in animals demonstrated that IL-1β-induced preterm labour is inhibited by IL-1ra. Furthermore, *in vitro* studies established that IL-1ra inhibits the production of prostaglandin in the amnion and chorion cells stimulated by IL-1β (Murtha *et al*., 2006). Thus, an imbalance between IL-1β and IL-1ra can result in a persistent pro-inflammatory response related to adverse clinical outcomes (Genc *et al*., 2004a).

1.5. Molecular studies

1.5.1. Pro-inflammatory cytokines

1.5.1.1. Tumour necrosis factor-α

A number of DNA-based polymorphisms in the *TNF*α gene have been identified; of these the most extensively researched are the G/A promoter variant at position -308 relative to the ATG start site. The substitution of the guanine (G) for an adenine (A) nucleotide has been shown to increase the production of this cytokine, thereby increasing susceptibility and severity of septic shock. Thus individuals harbouring the *TNF*α (-308A) (*TNF*2) allele react upon stimulation by infection with an intense inflammatory response and demonstrate an increased risk of developing preterm labour and delivery (Speer *et al*., 2006).

Macones *et al*., demonstrated an association between the *TNF*2 allele and preterm birth in the presence of an environmental stimuli, BV, with an increased odds of preterm birth compared to those without the carrier status (OR 6.1, 95% CI 1.9-21.0) (Macones *et al*., 2004). Similar results were obtained by Boston *et al*., suggesting that the *TNF*2 allele (genetically determined) influences a local TNFα response to a change in the vaginal flora (Genc *et al*., 2006).

1.5.1.2. Interleukin-1β

The production of pro-inflammatory cytokines, such IL-1β, can initiate infection-related preterm labour. The infusion of IL-1β in the amniotic cavity of a non-human primate pregnancy model results in the synthesis of TNFα and prostaglandins leading to uterine contractions. The gene encoding IL-1β contains, among others, a polymorphic site at position +3953 C/G which is associated with an increase in expression of the protein *in vitro* (Genc *et al*., 2002).

In a recent study, Edwards *et al*., demonstrated an association between increased levels of IL-1β and spontaneous preterm delivery (P=0.001) and preterm delivery (P=0.017). Carriage of the maternal *IL*-1β polymorphism was not associated with preterm delivery (P=0.43) or with cervical

levels (*P*=0.35). No association was demonstrated with the fetal genotype and cervical levels in relation to preterm delivery (Edwards *et al*., 2006). Engel *et al*., demonstrated that two other polymorphisms at position -581 C/T and -1061 T/C, were associated with 1.7 (0.9-3.2) times higher risk of spontaneous preterm delivery in women with the +3953C/-581C/-1061T haplotype and individuals with the +3953C/-581T/-1061C haplotype had an 2.1 (0.9-5.2) times higher risk of spontaneous preterm delivery. In hierarchical regression, carrier status of the *IL*-1β -581C or *IL*-1β -1061T was not associated with spontaneous preterm delivery (Engel *et al*., 2005).

1.5.1.3. Interleukin-6

IL-6 is an important cytokine involved in the host response to infection, which activates the acute phase response, stimulates T lymphocytes, and induces differentiation of B-lymphocytes and production of C- reactive protein. The DNA polymorphism located in the promoter region of the *IL*-6 gene at position -174, correlates with IL-6 synthesis. The substitution of a cytosine (C) for a guanine (G) nucleotide at position -174, decreases promoter activity. Individuals with a G/G or G/C genotype produce "normal" levels, whereas the C/C genotype is associated with lower levels and furthermore, has been linked to the severity of inflammatory conditions such as juvenile chronic arthritis and end-stage renal disease (Simhan *et al*., 2003).

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Simhan and colleagues investigated the relationship between the *IL*-6 promoter variant in 156 controls and 51 cases with spontaneous preterm birth before 34 weeks gestation. In the control group the C/C genotype was present in 30 individuals (19.2%) and in two individuals (6.3%) of the preterm birth group (OR 0.17, 95% CI 0.04-0.74). In the control group of white women, the C/C genotype was present in 30 (27.2%) and only 2 (5.2%) of cases. There was no C/C genotype detected in the African American women, with the racial difference statistically significant (*P*<0.001). The -174 C/C genotype was less common in women with spontaneous preterm birth before 34 weeks (Simhan *et al*., 2003). The finding of this study contradicts with the findings of Jamie *et al*., who did not demonstrate an association with the -174 C allele and the incidence of preterm delivery at 23-32 weeks' gestation. In this study the cervical fluid levels were not elevated and the presence of the maternal -174 C allele was not associated with IL-6 cervical fluid levels or the risk of spontaneous preterm birth (Jamie *et al*., 2005).

1.5.2. Anti-inflammatory cytokines

1.5.2.1. Interleukin-1ra

The gene encoding IL-1ra displays different alleles of a fragment length polymorphism in intron two and is associated with varying levels of the cytokine (Witkin e*t al*., 2003). Individuals with the *IL*-1*RN* allele 2 (*IL*-1*RN**2) have increased levels of the cytokine (Genc *et al*., 2002). Patients with chronic pro-inflammatory disorders who habours the *IL*-1*RN**2 allele experience delayed and more severe pro-inflammatory immune responses than those who do not, therefore the increased production of IL-1β in individuals with *IL*-1*RN**2 might increase vulnerability to preterm birth (Witkin *et al*., 2003).

Murtha *et al*., examined the relationship between preterm labour before 37 weeks [or PPROM] and the polymorphism in the *IL*-1*RN* gene. In the cohort of 95 cases, 26.8% had at least one copy of allele 2, in contrast to 12.4% in the control group (P<0.0004). The results implied that one copy of the maternal *IL*-1*RN* allele might be linked to an increased risk of preterm birth (Murtha *et al*., 2006).

Genc *et al*., evaluated the association between *IL*-1*RN**2 and preterm birth in vaginal samples of 212 women (18-22 weeks). The initial results showed that *IL*-1*RN**2 was linked to an increase in vaginal pH in black (P<0.001) and white women (P=0.005). The response of IL-1β in the presence of infection was reduced (P<0.01) with a decrease rate of spontaneous preterm deliveries (6% versus 18%, P=0.02). In conclusion, carrier status of *IL*-1*RN**2 was linked with a blunted IL-1β response to irregular vaginal flora, possibly suggesting a reduce susceptibility to infection-related preterm birth (Genc *et al*., 2004a).

1.5.2.2. Interleukin-4

During normal pregnancy, the levels of IL-4 increase. The single nucleotide change representing a cytosine (*IL*-4*C) to a thymine (*IL*-4*T) substitution in the promoter region of the gene is associated with increased transcriptional activity leading to increased production of the cytokine.

The presence of an *IL*-4*T allele has been linked to increased serum immunoglobulin E (IgE) levels and increased severity of asthma. The *IL*-4*T carrier also has a higher rate and increased risk of childhood respiratory syncytial viral infections, caused by the reduction in pro-inflammatory cytokine activity.

Kalish *et al*., investigated the association between this *IL*-4 -590 polymorphism and pregnancy outcome in multifetal gestations including 73 mother-infant pairs. The frequency of the interleukin-4 T allele was higher in spontaneous preterm birth compared to term pregnancies (36.2% vs. 18.2%, *P*=0.02, OR 2.6, 95% CI, 1.1-5.9). In addition, 20.7% of individuals with spontaneous preterm birth were homozygous for the *IL*-4*T allele, compared to 2.3% of individuals with term pregnancies (*P*=0.01, OR 11.2, 95% CI, 1.2-69.5) (Kalish *et al*., 2004).

1.5.2.3. Interleukin-10

IL-10 is an important cytokine for the regulation and maintenance of pregnancy. In the term placenta, the production of IL-10 is reduced in contrast to first and second trimester tissue. The physiological initiation at labour underlies the down regulation of IL-10 (Romero *et al*., 2006a).

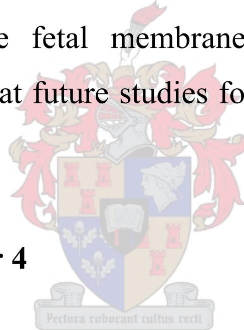
Various polymorphisms in the *IL*-10 gene have been linked to a change in transcriptional levels and the best documented of these are the promoter variants viz, -1082 G/A, -819 C/T and -592 C/A. Annells *et al*., demonstrated the presence of different genetic risk factors for preterm birth at different gestational periods. In their study, no association with the *IL*-10 G-C-C haplotype in labour before 35 weeks (*P*=0.1) could be demonstrated, while the *IL*-10 A-T-A haplotype was associated with early preterm birth before 29 weeks (*P*=0.04, OR 2.1, 95% CI, 1.0-4.1) (Annells *et al*., 2004).

1.5.3. Other regulators

1.5.3.1. Matrix metalloproteinase

The rupture of fetal membranes involves MMP-mediated interstitial collagen degradation (Crider *et al*., 2005) and has therefore been researched in PPROM. Fujimoto *et al*., investigated African American women for the promoter variant of the *MMP*-1 gene at position -1067, identified as an insertion of a G allele (2G), which is found to increase gene expression in PPROM and preterm birth (Fujimoto *et al*., 2002 and Crider *et al*., 2005). Fetal carriage of the 2G allele and PPROM showed a significant association (*P*=0.028, OR 2.29, 95% CI 1.09-4.82) and was associated with a stronger promoter activity in amniotic cells.

In pregnancies complicated by preterm labour, MMPs activate a cytokine-mediated inflammatory response, leading to rupture of the fetal membranes and cervical dilation or ripening. Consequently, it has been proposed that future studies focus on *MMP*s as candidate markers for preterm labour (Orsi *et al*., 2007).



1.5.3.2. Toll-like receptor 4

TLR4 is the main endotoxin-signal receptor, which initiates the innate immune response against Gram-negative bacteria. A DNA polymorphism in the *TLR*4 gene, Asp299Gly, has been linked to severe respiratory syncytial viral diseases in infants and increased risk of acquiring gram-negative infection in acutely-ill hospital patients.

Lorenz *et al*., reported the investigation of two *TLR*4 polymorphisms, viz, Asp299Gly and Thr399Ile: the study group consisted of 351 term infants and 440 premature infants (gestational age <35 wk; 282 singletons, 158 multiples) and 94 maternal individuals. The two polymorphisms investigated were shown to be in linkage disequilibrium. Allelic variation could be associated with increased risk of premature birth, specifically with PPROM (*P*=0.021) compared with the preterm birth group (*P*=0.045) (Lorenz *et al*., 2002).

1.5.3.3. Beta-2-adrenergic receptor

The human beta-2-adrenergic receptor (β_2AR) is a common therapeutic agent for the treatment of many disease conditions, with various coding polymorphisms that can alter receptor function. In the coding region of the gene at amino acid position 16, the arginine (Arg) residue is replaced with a glycine (Gly), with increase desensitisation *in vitro* upon stimulation. At position 27, a glutamine (Gln) residue is replaced with a glutamate (Glu), causing decreased desensitisation and *in vitro* down-regulation (Landau *et al*., 2002). These polymorphisms, documented as Arg16Gly and Gln27Glu, respectively, have been shown to be associated with increased risk of developing asthma, diabetes mellitus and hypertension (Ozkur *et al*., 2002).

In a case-control study reported in 2002, Landau *et al*., investigated the Arg16Gly and Gln27Glu genotype distribution in Hispanic women. The study consisted of 28 women who delivered spontaneously before 37 weeks of gestation and 251 control individuals with no history of preterm birth. No association was demonstrated with the Gln27Glu polymorphism and preterm labour. Only one individual in the preterm labour group was homozygous for the Arg16Arg genotype in comparison with 79 individuals in the control group (*P*=0.01, OR 0.08, 95% CI 0.01-0.58). The Arg16 allele frequency was significantly lower in the preterm labour group (29%) compared to controls (50%, *P*=0.002). This observation could possibly demonstrate a protective function in preterm delivery, due to the down-regulation of the β_2AR.

Conflicting results were reported by Ozkur *et al*, who had examined 80 cases of preterm labour and 76 control individuals. The Gln27Glu polymorphism was significantly associated with preterm labor (*P*=0.001). The allele frequency of Gly16 and Glu27 were elevated in cases vs. controls (0.54 vs. 0.48 and 0.42 vs. 0.26, respectively). The odds of developing preterm labour were 2-fold greater for the Glu 27 allele (OR 2.14, 95% CI, 1.32-3.46; *P*=0.002). The contradictory findings at the Arg16Gly locus could be due to the different populations examined in these studies (Hispanic vs. Turkish women), exclusion criteria or small sample size (preterm labour 28 vs. 80) (Ozkur *et al*., 2002).

1.5.3.4. Vascular endothelial growth factor

Vascular endothelial growth factor (VEGF) is a major angiogenic factor, regulating endothelial cell proliferation. VEGF is expressed during gestation, with strong immunoreactivity in both the amnion and chorion, and at increased levels at term spontaneous labour compared with either term or not in labour (Papazoglou *et al*., 2004). Identified polymorphisms in the *VEGF* gene include -1879 G/A, -1498 T/C, -1190 G/A, -1154 G/A, -634 G/C, -7 C/T and 936 C/T, of which the -634 C/G locus has been linked to increased levels of serum VEGF (Crider *et al*., 2005).

Papazoglou *et al*., investigated polymorphisms -634 G/C and 936 C/T in spontaneous preterm birth. The case-control study included 54 women with preterm deliveries and 79 women with two term births with no history of obstetric complications. No significant association was demonstrated with the -634 G/C polymorphism and spontaneous preterm birth. In contrast, either the homozygous C/C or heterozygous C/T 936 genotype status could be associated with spontaneous preterm birth (*P*=0.0009, OR 2.05, 95% CI 1.37-3.06) (Papazoglou *et al*., 2004).

1.6. Pregnancy-related proteins

Members of pregnancy-related proteins are produced in measurable amounts during pregnancy and can include maternal, fetal or feto-maternal proteins. The proteins differ both in function and structure but inter-relate to play an important role in the development of the fetus and placenta and / or to sustain pregnancy. To date, 56 different pregnancy-related proteins have been identified and sub-grouped into different categories according to location: fetal-, pregnancy-, soluble placental tissue- and membrane-associated placental proteins (Than *et al*., 1999).

The study described in this dissertation focused on the soluble placental protein 13 (PP13), which has been implicated as a promising serum biomarker for IUGR, pre-eclampsia and preterm delivery (Burger *et al*., 2004).

1.6.1. Placental Protein-13 (PP-13)

The gene, “lectin, galactoside-binding, soluble 13” (*LGALS*13), is located on human chromosome 19q13.1 in close vicinity to the galectin -10,-7,-4 and placental protein 13-like genes (Than *et al*., 2004a). The coding regions of *LGALS*13 are represented by four exons, with exon three encoding the critical carbohydrate recognition domain (CRD) (Visegrady *et al*., 2001).

The protein, PP13 consists of two identical 16kDa subunits held together by disulphide bonds. During pregnancy PP13 is highly expressed in the placenta and tissues such as the fetal liver and spleen (Than *et al*., 2004a).

Similar to its homologous counterpart, Charcot-Leyden crystal protein/galectin-10, PP13 exhibits weak lysophospholipase activity [confirmed by ^{13}P-NMR], which is not found in other galectins. Sugar binding assays revealed that PP13 strongly binds N-acetyl-lactosamine, mannose and N-acetyl-glucosamine residues, which are widely expressed in human placenta. Affinity chromatography, PAGE and MALDI-TOF mass spectrometry demonstrated that annexin II and β/γ actin specifically bind to PP13 in the placenta and fetal hepatic cells. Perinuclear staining of syncytiotrophoblast brush border membrane (Figure 4) established PP13’s externalization to the outer cell surface (Than *et al*., 2004a).

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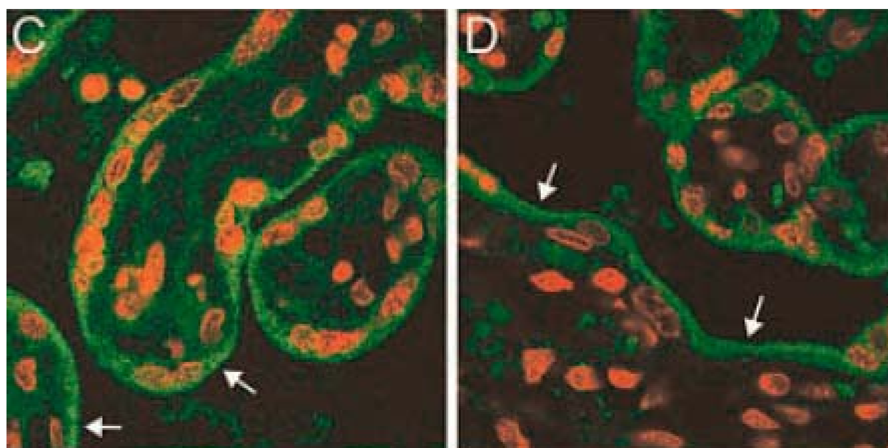


Figure 4: Intense PP13 staining (green) at the brush border membrane of the syncytiotrophoblasts (arrows) and also a discrete perinuclear labelling of the syncytiotrophoblasts with both the monoclonal (C) and polyclonal (D) antibodies to PP13 (from Than et al., 2004a).

Due to the localisation and specific complex formation, it is believed that PP13 is excreted into the maternal circulation by means of ectocytosis of microvesicles containing actin, annexin II and PP13 (Than *et al*., 2004a). Since other galectins demonstrate immunobiological functions, in the same way PP13 and its homologues may have immune functions at the feto-maternal interface (Than *et al*., 2004b).

1.6.1.1. Biological functions of PP-13

The galectins are considered important for cell adhesion functions such as cell-cell and cell-matrix interactions (Rabinovich *et al*., 2002). The binding capability of PP13 for sugar residues in the placenta and localisation to the outer surface of the invading trophoblasts at implantation, suggest that PP13 may also play a role in implantation.

The weak lysophospholipase activity accompanied by calcium mobilisation, including free fatty acid release and elevation of prostaglandins, imply a potential role in the regulation of vasoconstriction / vasodilation balance and maternal artery remodeling (Than *et al*., 2005). The strong cytolytic and membrane perturbing properties of lysophospholipids, suggest that the lysophospholipase activity of PP13 may present a protective function during implantation in pregnancy and the maintenance thereof (Than *et al*., 1999).

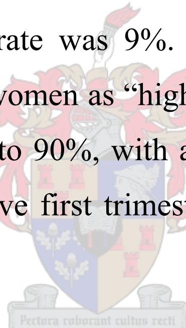
1.6.1.2. Placental vasculopathy

During normal pregnancy, the level of PP13 slowly increases. In the 1st trimester reduced levels of PP13 were observed in pregnancy subsequently complicated by IUGR and pre-eclampsia (predominantly the early form). However, as pregnancy reached the 2nd and 3rd trimesters, the levels of PP13 increase in pre-eclampsia, IUGR and preterm delivery, compared to uncomplicated pregnancies. Abnormal PP13 levels are associated with pregnancy complications such as placental insufficiency related to LBW, gestational hypertension, proteinuria, newborn mortality and morbidity. These results demonstrate the significance and potential use of PP13 as a biomarker to detect placental insufficiency. The unusual pattern of PP13 levels during complicated pregnancy may be attributed to impaired synthesis, impaired passage from the

placenta to serum, impaired primary structure or a combination of these factors (Burger *et al*., 2004).

1.6.1.3. Clinical studies

Nicolaides *et al*., conducted a case-control prospective study evaluating the *combined* use of Doppler ultrasound and serum PP13 measurement in pregnant women. The pulsatility index (PI) of blood flow in the uterine arteries and the maternal serum concentration of PP13 were measured in 10 individuals who subsequently developed PE (>34 weeks) and in 423 control individuals. In the control group, PP13 and PI were measured and expressed in multiples of gestation-specific median value (MoM). In the patient group, the median uterine artery PI was higher (1.43 MoM, $P<0.001$) and the median serum PP13 level lower (0.07 MoM, $P<0.001$). The screening false-positive rate was calculated to be 12% for PP13 and 31% for uterine artery PI; with a combination of the two methods, the rate was 9%. Screening by PP13 measurement only, identified 14% of the 10 pre-eclamptic women as "high risk" - combining this data with Doppler ultrasound, increased the detection rate to 90%, with an overall false-positive rate of 6%. This combined assessment resulted in effective first trimester screening for pre-eclampsia before 34 weeks (Nicolaides *et al*., 2006).



Similar results were reported by Spencer *et al*., who used first trimester PP13 levels in pre-eclamptic patients combined with second trimester Doppler assessment (Spencer *et al*., 2007). They performed a nested case-control study involving 73 controls and 12 pre-eclampsia cases of which 5 represented early pre-eclampsia (with their delivery induced at 35 weeks) and 7 those in which delivery was not induced. The median PP13 levels in controls and all cases were 295.9 and 212.6 pg/mL, respectively, and in early pre-eclampsia, 171.2 pg/mL [with MoM 1.00, 0.94 and 0.63 ($P<0.001$)]. The detection rate for pre-eclampsia was not improved using additional markers such as PI, pregnancy-associated plasma protein-A (PAPP-A), Activin, Inhibin or β-hCG in the 22-24 week period. The use of late second trimester PP13 measurement alone, did not improve the prediction of pre-eclampsia; neither did the use of Doppler or other biochemical markers (Spencer *et al*., 2007).

1.7. Treatment of preterm labour

The main objective in the treatment of preterm labour is to prolong pregnancy in order to improve neonatal outcome. This is usually achieved by admitting the mother to hospital, followed by antenatal corticosteroid administration to enhance lung maturation (Anotayanonth *et al*., 2004).

1.7.1. Antibiotics

During bacterial infection the biosynthesis of prostaglandins or substances such as IL-1, TNF and platelet activating factor are stimulated, which may lead to preterm delivery (Kenyon *et al*., 2003). In a Cochrane Collaborative group review by King and Flenady including 11 trials with 7428 women randomised to receiving either antibiotics or placebo, they concluded that the use of antibiotics for inhibiting preterm labour with intact membranes might have minimal effectiveness. There was no improvement in the reduction of preterm birth, delaying delivery for 48 hours (hrs) or for seven days, incidence of LBW, perinatal mortality or neonatal morbidity. No differences were observed in neonatal outcome wr.t. respiratory distress syndrome, days on a ventilator, more than 28 days of oxygen supplementation, necrotising enterocolitis or intraventricular hemorrhage. However, maternal benefits included a reduction in infection and a clinically insignificant increase in gestation (King and Flenady, 2002 and Newton, 2005).

Kenyon *et al*., in 2003, examined 22 trials involving the administration of antibiotic treatment to women who had developed PPROM before 37 weeks, to evaluate the long-term effects on maternal infectious morbidity, fetal and neonatal morbidity and mortality, and childhood development. Management with antibiotic was associated with a significant reduction in chorioamnionitis and infants born within 48hrs. A reduction in neonatal infection, use of surfactant, oxygen therapy and abnormal cerebral ultrasound scan prior to hospital discharge was observed. The routine use of antibiotics for the treatment of PPROM is supported due to the delayed delivery and reduction of neonatal morbidity but not perinatal mortality.

1.7.2. Betamimetics

Betamimetics such as ritodrine, salbutamol and terbutaline are β-adrenergic drugs acting on both β_1 and β_2 receptors. Uterine myocytes contains β_2 receptors and facilitate myometrial relaxation by stimulation of cyclic adenosine monophosphate (AMP) (Mohan and Bennett, 2006). This prevents the release of calcium from the sarcoplasmic reticulum, causing muscle relaxation (Chandraharan and Arulkumaran, 2005). Although both β_1 and β_2 receptors are localised within the myometrium of the uterus, they are also found in other human tissues. It is this lack of uterine specificity that can potentially cause undesirable effects (Mohan and Bennett, 2006 and Oei, 2006).

Maternal side effects to betamimetic treatment include tachycardia, tachypnoea, hypertension, nausea and vomiting, hyperglycemia and pulmonary oedema (Dodd *et al*., 2006). Of these, pulmonary oedema is the most serious, with an estimated incidence of 1:350 to 1:400 treated patients (Oei, 2006). Furthermore, betamimetics are able to cross the placenta and cause fetal tachycardia, hypoglycemia and hyperinsulinism after birth (Anotayanonth *et al*., 2004). A meta-analysis has shown that betamimetics are effective in delaying delivery for 24hrs, 48hrs or seven days compared with placebo but have no beneficial effect on neonatal outcome and preterm delivery rates (Anotayanonth *et al*., 2004 and Mohan and Bennett, 2006).

1.7.3. Nitric oxide donors

Nitric oxide (NO) is a potent endogenous compound that (i) causes non-striated muscles to relax and (ii) acts synergistically with progesterone to inhibit uterine contractility. Nitric oxide activates the enzyme guanylate cyclase by circulating freely into the vascular smooth muscle catalysing the conversion from guanosine triphosphate to 3'5'-guanosine monophosphate (cGMP), which leads to calcium-mediated relaxation (Leszeczynska-Gorzelak *et al*., 2001), preventing actin-myosin interaction (Chandraharan and Arulkumaran, 2005a). The therapeutic doses for nitric oxide are used to facilitate external cephalic version, difficulty with vaginal or caesarean section delivery and manual exploration of the uterus, and finally as a tocolytic agent (Helmer, 2005). The stimulation of uterine contractions at term is related to a decrease production of nitric

oxide in the uterus and placenta, initiating labour. Nitric oxide is thought to play a role in cervical ripening and the preparation of the uterus for labour, since the production of nitric oxide in the cervix increases near the end of gestation. Consequently, nitroglycerine as a donor of nitric oxide has been investigated as treatment for preterm labour and relaxation of the uterus because decreased synthesis of nitric oxide has been linked with the initiation of uterine contractions and the incidence of labour prematurely, or at term (Leszeczynska-Gorzelak *et al*., 2001).

In a Cochrane review, Duckitt and Thorton evaluated five randomised controlled trials examining the use of nitroglycerine as a donor of nitric oxide. Nitric oxide reduced the risk of delivery before 37 weeks (RR 0.69, 95% Cl 0.53-0.888) but did not delay delivery before 32-34 weeks. There was no improvement in neonatal outcome when compared with placebo, no treatment or alternative tocolysis such as ritodrine, albuterol and magnesium sulphate. Adverse side effects in the nitric oxide group were relatively reduced when compared to the other tocolysis, but significantly more women experienced headaches (RR 0.47, 95% Cl 0.37-0.61) in the nitric oxide treated group. The authors concluded that there is currently insufficient evidence supporting the use of nitric oxide as treatment for threatened preterm labour (Duckitt and Thorton, 2002). Bisits *et al*., performed a multicentered, multinational, randomised controlled trial with women who tested positive for fetal fibronectin or ruptured membranes between 24 and 35 weeks. These women were either given betamimetics (117 women) or nitroglycerine (121 women). After two hrs of treatment, 27% of women receiving betamimetics had moderate or stronger contractions compared to 53% in the nitroglycerine group. Rescue tocolysis was administered to 35% of women in the nitroglycerine group. If the requirements for betamimetic rescue were regarded as treatment failure, then nitroglycerine was not an effective tocolysis compared to betamimetics (Bisits *et al*., 2004). The infant follow-up study found no difference in psychometric performance of infants at 18 months of age enrolled in either the nitroglycerine (81 infants) and betamimetic (75 infants) arms of the study. Infant assessment was based on the Griffiths Mental development scale (Gill *et al*., 2006).

In a recent randomised double-blind placebo controlled trial, Smith *et al*., found that the use of transdermal nitroglycerine patches reduced birth prior to 28 weeks (RR 0.50, 95% CI 0.23-1.09). They suggest that the improvement in neonatal outcome was not due to a difference in delivery

within 48 hrs or the administration of corticosteroids but rather due to the maintenance of pregnancy and the potential non-tocolytic effect of nitroglycerine, having a direct effect on uterine blood flow of the placenta (Smith *et al*., 2007).

1.7.4. Prostaglandin synthetase inhibitors

Prostaglandin production occurs in the myometrium and other gestational tissues and is implicated in cervical ripening, membrane rupture and uterine contractility (Groom *et al*., 2005). Prostaglandins are formed from free arachidonic acid (AA) catalyzed by cyclo-oxygenase (COX) enzyme. This conversion, mediated by COX enzymes, is the committing step of prostaglandin synthesis (Mohan and Bennett, 2006).

The first most widely used non-steroidal anti-inflammatory drug (NSAID) was indomethacin, a reversible COX inhibitor, influencing both COX-1 and COX-2 (Mohan and Bennett, 2006). However, the use of indomethacin as a tocolytic drug for the prevention of preterm labour included fetal side effects such as oligohydramnios, renal failure, and premature closure of the ductus arteriosus associated with pulmonary hypertension, patent ductus arteriosus, necrotising enterocolitis and intraventricular haemorrhage (King *et al*., 2005). Although indomethacin delays pregnancy for more than seven days, the use of this drug in the clinical setting is limited, due to harmful effects on the fetus (Mohan and Bennett, 2006).

During the onset of labour, at both term and preterm, the expression of COX-2 increases while COX-1 remains constant. COX-2 inhibitors lead to a decrease production of prostaglandin E_2 in human fetal membranes but not COX-1. This demonstrates the importance of COX-2 controlling the production of prostaglandin and consequently labour. This regulation step led to the development of COX-2 inhibitors in the prevention of preterm labour (Groom *et al*., 2005). Although it was thought that COX-2 inhibitors would display fewer side effects, the use of this drug has been found to be associated with fetal ductal closure and reduced renal blood flow (Caritis, 2005). Following a randomised, double-blind, placebo-controlled trial, Groom *et al*., concluded that the use of rofecoxib, a COX-2 inhibitor, was not effective in preterm labour management and was associated with a greater risk of delivery before 37 weeks. The agent also

demonstrated considerable but reversible effects on fetal renal function and ductus arteriosus. On these grounds, the author does not support the use of this drug in the management of preterm labour (Groom *et al*., 2005).

1.7.5. Calcium channel blockers

Calcium antagonists such as nifedipine and nicardipine were developed to treat coronary heart disease, chronic stable angina pectoris and hypertension. These compounds are identified structurally as dihydropyridines acting on the L-type channels, mostly found in cardiac and smooth muscle. Binding to these types of channels slows down the inward action potential reducing the intracellular amount of calcium leading to decreased myocardial contractions. The physiological changes include the lowering of blood pressure, increased heart rate and contractility as well as increased cardiac output mainly due to vasodilation. Adverse side effects include excessive vasodilation causing dizziness, hypotension, headache, flushing and oedema (Oei, 2006).

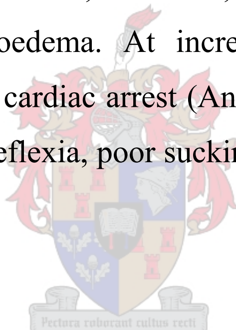
Nifedipine is the most common calcium antagonist used as tocolysis for preterm labour. When administered, nifedipine blocks the influx of extracellular calcium into myometrial cells (Mohan and Bennett, 2006) by binding to the inside of the myometrial L-type voltage-dependent calcium channels, keeping them closed and inhibiting uterine contractility (Oei, 2006). In human trials there is no significant evidence to support (i) any adverse effect on the fetus and (ii) that antenatal nifedipine exposure may offer some protection against neonatal morbidity and mortality. The amounts of nifedipine deposited in breast milk are less than 5% of the therapeutic dose. Administration of calcium antagonists does not have any teratogenic effect on the fetus (Mohan and Bennett, 2006). Maternal side effects reflect the vasodilation caused by nifedipine and mostly include headache and facial flushes, disappearing within 24 hrs. Additional side effects include hypotension, reflex tachycardia, dizziness, nausea and increased levels of liver transaminases (van Geijn *et al*., 2005).

In a Cochrane review evaluating the use of calcium channel blockers as tocolysis for preterm labour, it was demonstrated that nifedipine was more effective than betamimetics in delaying

delivery and reducing the risk of neonatal morbidities. The incidence of respiratory distress syndrome, intraventricular haemorrhage, necrotising enterocolitis and neonatal jaundice were also reduced in the nifedipine group compared to betamimetic group. Unfortunately, in this review no comparison was made between nifedipine and placebo or no treatment groups (King *et al*., 2003).

1.7.6. Magnesium sulphate

Magnesium sulphate acts as a calcium antagonist inhibiting the movement of calcium ions through the voltage-gated calcium channels. The release of calcium ions from the sarcoplasmic reticulum is prevented and leads to decreased activity of the calmodulin-dependent myosin light-chain kinase. The interaction between actin-myosin is inhibited; hence muscle relaxation occurs (Chandraharan and Arulkumaran, 2005a). Side effects of magnesium sulphate administrations may include flushing, a sensation of warmth, headache, blurring of vision, drowsiness, nausea, palpitations and rarely, pulmonary oedema. At increased therapeutic levels it can cause respiratory depression, respiratory and cardiac arrest (Anwar *et al*., 2006). Neonatal effects such as hypermagnesaemia can cause hyporeflexia, poor sucking and respiratory depression (Crowther *et* al., 2002).



1.7.7. Oxytocin antagonist

Oxytocin production occurs in the hypothalamus and in other peripheral tissues including choriodecidua (Gullam *et al*., 2005). The receptor binding sites for oxytocin are localised mainly in the uterine smooth muscle and the myoepithelial cells of the mammary gland (Bernal and TambyRaja, 2000). The effects of oxytocin are regulated through a transmembrane receptor coupled to G-proteins that activate phospholipase C, resulting in an increase of intracellular calcium and increased activity of protein kinase C. These regulation patterns lead to myometrial smooth muscle contraction and furthermore, oxytocin stimulates the release of prostaglandins that are responsible for cervical ripening and enhancing smooth muscle contractions (Mohan and Benett, 2006). During both term and preterm labour, the expression of oxytocin receptors increases and for this reason oxytocin antagonists were studied and developed as a tocolytic agent in the prevention of preterm labour. Of all the oxytocin antagonists under study, atosiban has

been the most extensively studied in animal, preclinical and clinical trials as tocolysis for preterm labour (King, 2004). Atosiban binds to the oxytocin receptor and interrupts the action of this receptor on G-proteins, which delays the activity of the phospholipase C enzyme, inhibiting the release of calcium from the sarcoplasmic reticulum. The end result is down-regulation of intracellular calcium leading to muscle relaxation (Chandraharan and Arulkumaran, 2005a).

In pregnant women the amount of bound plasma protein is 46-48%, and has limited crossover into fetal circulation with no direct effects on the fetus (Mohan and Benett, 2006). On the basis of adequate evidence, it was recommended that atosiban is just as effective as betamimetics in delaying preterm labour with minimal side effects including chest pain, palpitations, tachycardia, hypotension, nausea, vomiting and headache (Chandraharan and Arulkumaran, 2005b). A Korean multicentred, parallel group, randomised, single blind study of the safety and efficacy of atosiban versus ritodrine found that the efficacy after seven days was significantly better in the atosiban group than in the ritodrine group (60.3 versus 34.9%), but not at 48 hrs (68.3 versus 58.7%). Maternal side effects were less common in the atosiban group (7.9 versus 70.8%; *P*=0.0001), with fewer earlier drug terminations due to adverse effects (0 versus 20.0%; *P*=0.0001). There was no difference in the treatments w.r.t. neonatal morbidities, despite the fact that infection, intraventricular haemorrhage, respiratory distress syndrome and patent ductus arteriosus were reported more frequently in the atosiban group. The authors concluded that the efficacy and safety of atosiban as a tocolytic treatment for preterm labour were superior to those of ritodrine (Shim *et al*., 2006).

A randomised controlled trial performed in 2005 by Kashanian *et al*., compared the use of atosiban and nifedipine for treatment of preterm labour and found no significant difference between the two groups. Effectiveness in delaying delivery for 48 hrs was 82.5% in the atosiban and 75% in the nifedipine groups (*P*=1.000), and delaying for more than seven days was 75% in the atosiban and 65% in the nifedipine group. Maternal side effects were 17.5% in the atosiban and 40% in the nifedipine group. Atosiban was found to be more effective and safe for the treatment of preterm labour, especially in patients with heart disease and multi-fetal pregnancies.

1.8. Preterm labour: Where we stand now?

Despite genetic research in perinatal medicine, current investigations are analytically limited in regards to performance and output compared to the field of autoimmunity, oncology and cardiovascular disorders. These boundaries are brought about due to the moderately small size of our research field, influencing funding, expertise and analytical capacity (Orsi *et al*., 2007).

Although neonatal and maternal care has improved, the rate of preterm birth has increased slowly (Pennell *et al*., 2007). Worldwide, infection is the single most frequent cause of spontaneous preterm labour, with HIV globally becoming an increasing problem, particularly in Africa (Steer, 2005).

Recent advances in the field of clinical genetics in the hope to identify maternal risk, predictive and management strategies have been limited by heterogeneity, as well as the complex nature of genetic and environmental predispositions (Lyndon *et al*., 2006).

1.9. Aim and Objectives

The present study was designed to incorporate the clinical setting and investigate potential genetic associations with preterm labour. The study cohort comprised consecutive first-time booking low-risk pregnant women from a restricted geographical region.

This data set will be scrutinised by clinicians and policy-makers at Paarl Hospital who will be able to compare these data with their previous hospital records, to enable them to identify current trends, strengths and weaknesses in their services, facilities and resources. Ultimately, this will impact on their planning for the future.

The aim of this project was to establish a predictive marker profile for pregnancy outcome.

This will be achieved by:

1. Evaluating whether selected polymorphisms in pro-inflammatory and anti-inflammatory genes predispose to poor pregnancy outcome.
2. Investigating whether sequence variants in the PP13 encoding gene impact on pregnancy outcome.
3. Use appropriate statistical tools to evaluate 1 and 2.

2. Material and Methods

2.1. Materials

2.1.1. Patients

The project was approved by the Institutional Review and Ethics board (N04/09/147) (Appendix 1) and study participants provided written consent (Appendix 2 and 3). The study cohort comprised 421 pregnant females presenting at the Paarl Hospital Obstetric clinic (Western Cape region of South Africa) during the period 2005 - 2007. To reduce genetic heterogeneity, patient recruitment focussed on the South African Coloured (San, Khoi, Madagascan, Javanese and European ancestry: Loubser *et al.*, 1999) [N=306] and Black (Xhosa speaking) [N=115] population groups. These two population groups are served within the Paarl and Tygerberg Hospital catchment areas.

Gestational assessment was made using current clinical protocols such as menstrual dates, clinical examination and early ultrasound. Exclusion criteria included women with multiple gravidity, known uterine or cervical abnormalities and HIV positive status.

To simplify the genotyping analysis, the following complication subgroups were combined: (i) preterm labour with PPROM, and (ii) pre-eclampsia with eclampsia and PIH (representing hypertension).

2.1.2. Controls

The control cohort comprised individuals within the study cohort who delivered at term, with a healthy pregnancy outcome.

2.2. Methods

2.2.1. DNA Extraction

Maternal whole blood was drawn from peripheral veins and stored in BD vacutainer K2E tubes with EDTA (Belliver Industrial State, Plymouth, UK). Blood samples were kept at -20°C until required. Genomic DNA was extracted from 5mL of whole blood using a salting-out extraction method described by Miller *et al*., 1988 (Appendix 4) and dilutions prepared in sterile distilled water at a final concentration of 20ng/µL. The DNA concentration had been established using a NanoDrop® Spectrophotometer (NanoDrop Technologies, USA).

2.2.2. Polymerase Chain reaction

2.2.2.1. Oligonucleotide Primers

Reference sequences for *LGALS*13 (NM_013268.2) were obtained from the NCBI website (http://www.ncbi.nlm.nih.gov/) and primer pairs for PCR amplification (designed to encompass the promoter and intronic sequences flanking the encoding regions under investigation (Figure 5, Table 2 and Appendix 5) were designed using Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) software. The primers (synthesised by the Department of Molecular and Cell Biology, University of Cape Town) were analysed for hairpin, homo- and hetero-dimer formation using IDT® (Integrated DNA Technologies, Inc, Coralville, IO, USA) (http://www.idtdna.com/) OligoAnalyzer and sequence specificity using NCBI Basic Local Aligment Search Tool (BLAST) (http://www.ncbi.nlm.nih.gov/BLAST/).

The immunological gene variants (*IL*-4 -590, *IL*-1β +3953, *IL*-1*RN*, *IL*-10 -1082; -819; -592 and *TNF*α -308; -238; +488 were amplified using primers sets described by Kalish *et al*., 2004, Genc *et al*., 2002, Hellmig *et al*., 2005, Koch *et al*., 2001, Yamanoto-Furusho *et al*., 2004 and Manchanda *et al*., 2006, respectively (Tables 3A&B).

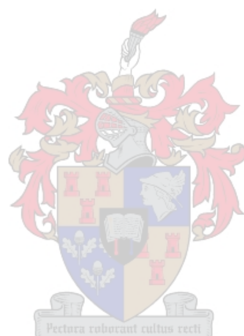


Table 2: *LGALS*13 designed primers, annealing temperatures and amplicon sizes.

Coding region	Primer	Primer Sequence [5'-3']	Tm (°C)	Ta (°C)	Fragment (bp)	Reference
LGALS 13 gene						
Exon 1	E1F	GCTGCAAGGAAATCTTGCTG	52			G Rebello, Personal Communication
	E1R	CCACACCTCAATAGCTCTAAG	52	52	292	
Exon 2	E2F	CAACCTCCTGCACCATGAG	53			
	E2R	CATCACCCACATGTAAGGTC	52	52	245	
Exon 3	E3.1F	GGCCATCAGTATTATCTGGGAG	55			
	E3.1R	GATTGCCAAAGTGCACTCGG	54	54	213	
	E3.2F	GGATGAGGATTCAGATATTGCC	53			
	E3.2R	CCCTGACGGACTACTGAG	53	53	265	
Exon 4	E4.1F	TGTACCAGGACAGAGTGGAG	54			
	E4.1R	CTATTGCAGACACACACTGA	50	52	196	
	E4.2F	GAGAGATATCTCCCTGACCTC	54			
	E4.2R	CCTGATGCCTCCCATAGAATG	54	54	258	

E (exon); F (forward); R (reverse); Tm (melting temperature); Ta (annealing temperature)

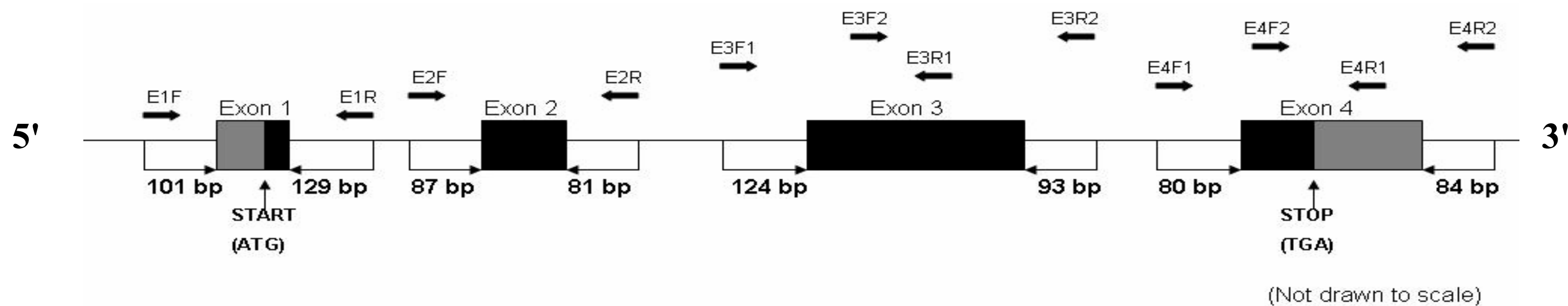


Figure 5: Graphical representation of the *LGALS*13 gene, indicating the primer sets and their relative positions used in this study. Start (ATG) and stop (TGA) codons are also indicated. Black indicates coding and the grey boxes non-coding regions.

Table 3: (A) Documented primer sequences, annealing temperatures and amplicon sizes (RFLP analysis). **(B)** Documented allele specific primer sequence, annealing temperatures and amplicon sizes (ARMS assay).

A

Polymorphism	Primer Sequence [5'-3']	Tm (°C)	Ta (°C)	Amplicon (bp)	Reference
IL-1*RN* (86 bp repeat)	F: CTCAGCAACACTCCTAT	45			
Alleles 1-5 *	R: TCCTGGTCTGCAGGTAA	47	54	(a)	Hellmig *et al*., 2005
IL-4 -590 (C/T)	F: ACTAGGCCTCACCTGATACG	54			
	R: GTTGTAATGCAGTCCTCCTG	52	57	252	Kalish *et al*., 2004
IL-1β +3954 (C/T)	F: GTTGTCATCAGACTTTGACC	50			
	R: TTCAGTTCATATGGACCAGA	48	54	250	Genc *et al*., 2002
IL-10 -1082 (A/G)	F: CCAAGACAACACTACTAAGGCTCCTTT	58			
	R: GTGGGCTAAATATCCTCAAAGTT	52	55	377	Koch *et al.,* 2001
IL-10 -819 (C/T)	F: CAACTTCTTCCACCCATCTTT	50			
-592 (C/A)	R: GTGGGCTAAATATCCTCAAAGTT	52	52	477	Koch *et al.,* 2001
TNFα +488 G/A)	F: TGCACTTTGGAGTGATCGGC	54			
	R: AACAGCTGGCTGCCTGTCT	53	55	588	Manchanda *et al.,* 2006

* 410bp (allele 1), 240bp (allele 2), 500bp (allele 3), 325bp (allele 4) or 595bp (allele 5)

B

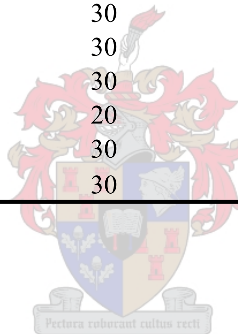
Polymorphism	Allele specific primers [5'-3']	Tm (°C)	Ta (°C)	Amplicon (bp)	Reference
TNFα -308 (G/A)	F: GGCAATAGGTTTTGAGGGGCGTGG (G)	63		216	
	R: ACCCTGGAGGCTGAACCCCGGCCT (A)	66		139	
	F: GCCCCTCCCAGTTCTAGTTCTAT ©	57		308	
	R: AAGCGGTAGTGGGCCCTGCACCTT ©	61	62		Yamamoto-Furusho *et al.,* 2004
TNFα -238 (G/A)	F: AGACCCCCCTCGGAATCG (G)	67		449	
	R: CACACTCCCATCCTCCCTGGTCT (A)	61		209	
	F: GCCCCTCCAGTTCTAGTTCTATC ©	57		608	
	R: CCGGATCATGCTTTCAGTGC ©	54	61		Yamamoto-Furusho *et al.,* 2004

Pectora roborant cultus recti

Table 4: PCR cycling conditions used to amplify each polymorphic locus.

Gene	primer (pmol)	Ta (°C)	No. cycles	Time at temperatures				
				94°C (min)	94°C (sec)	Ta°C (sec)	72°C (sec)	72°C (min)
IL-1*RN*	10	54	35X	3	20	30	30	5
IL-4 -590	10	57	32X	3	30	30	30	5
IL-1β +3953	20	54	35X	3	20	30	30	5
IL-10 -1082	10	55	35X	5	30	30	30	3
IL-10 -819/-592	10	52	35X	5	30	45	45	6
*TNF*α -308	20	62	35X	1	30	30	30	2
*TNF*α -238	20	61	35X	3	30	30	30	5
*TNF*α +488	10	55	40X	5	30	45	45	8
PP13 E1	10	52	35X	3	30	30	30	5
PP13 E2	10	52	35X	3	30	45	30	5
PP13 E3.1	10	54	35X	5	30	30	30	7
PP13 E3.2	10	53	30X	3	20	30	30	5
PP13 E4.1	10	52	35X	3	30	30	40	5
PP13 E4.2	10	54	35X	3	30	30	30	5

sec (seconds); min (minutes); X (times i.e. number of cycles)



2.2.2.2. DNA Amplification

DNA amplification was performed using the GeneAmp® PCR System 2700 (Applied Biosystems, California, USA) in a total volume of 50µL, which contained 0.5U GoTaq® DNA Polymerase (Promega, Madison, WI, USA), 1X commercial buffer and 0.25mM of each dNTP (Promega, WI, USA); or 0.5U GoTaq® Flexi DNA Polymerase (Promega, Madison, WI, USA), 1X commercial buffer, 0.5, 1 or 2mM $MgCl_2$, 0.25mM of each dNTP (Promega, WI, USA) and ~20ng of genomic DNA. The cycling conditions and primer concentration are indicated in Table 4. The polymorphisms listed in Table 2b were evaluated by the amplification refractory system polymerase chain reaction (ARMS-PCR).

2.2.3. Agarose Gel Electrophoresis

For confirmation of successful amplification, five microliters of each PCR product was mixed with 5µL of loading dye (95% Formamide, 20mM EDTA, 0.05% Xylene Cyanol, 0.05% Bromophenol Blue) and resolved by electrophoresis on 1% agarose gels in 1X TBE buffer (90mM Tris-HCl, 90mM Boric acid and 1mM EDTA, pH 8.0) for 20 minutes at 90V and stained with 0.5 µg/mL Ethidium bromide (Sigma, Missouri, USA). The gels were photographed under ultraviolet light (260nm) using a Multigenius Bio Imaging System (Syngene, Cambridge, UK).

2.2.4. Mutation Screening

2.2.4.1. Single Stranded conformational polymorphism (SSCP) and heteroduplex analysis

The amplification products selected for mutation analysis were added to SSCP loading buffer (95% formamide, 100mM NaOH, 0.25% bromphenol blue and 0.25% xylencyanol) in a 1:1 ratio and denatured at 95°C for 5 min followed by rapid quenching on ice for 3 min. A 12% non-denaturing polyacrylamide gel (99% acrylamide, 1% piperazine diacrylamide [PDA], TEMED and 10% ammonium persulfate was used to resolve bands. Electrophoresis was performed on a Pharmacia LKP 2117 Multiphor II Electrophoresis Unit at 355 V for ~120 min (4-11°C). The

single stranded conformational polymorphisms (SSCP) and heteroduplex (HD) conformation patterns were obtained by 0.1 % silver staining (Liechti-Gallati *et al*. 1999) (Appendix 6).

2.2.4.2. Sequencing

PCR amplicons displaying aberrant banding patterns following SSCP and HD analysis were subjected to semi-automated sequencing and restriction enzyme digestion, where applicable (Table 6). Prior to sequencing, the products were purified using Bioline Quick Clean (Bioline, UK) (Appendix 7) and recovery was confirmed on a 1% agarose gel. Sequencing reactions were carried out using the BigDye® Terminator V3.1 Cycle Sequencing Kit (Applied Biosystems, California, USA) and the products were analysed using an ABI 3130xl Genetic Analyzer (Applied Biosystems, California, USA). Sequencing was bi-directional, when required. Sequencing reactions were performed at the University of Stellenbosch, DNA Sequencing Facility.

2.2.4.3. Restriction Enzyme Analysis (REA)

Table 5 is a summary of the restriction enzymes and conditions used to screen each documented polymorphisms and Table 6 shows the conditions required for REA to confirm variations detected by Multiphor analysis.

Restriction enzyme sites for conformational variants of the *LGALS*13 gene were characterised using the BioEdit Sequence Alignment Editor (Isis Pharmaceutical, Carlsbad, California, USA). For each restriction, 10-15 µL of PCR product was digested overnight with 1 or 2 units of restriction enzyme in a total reaction of 20µL. Restricted products (and appropriate size ladders) were subsequently resolved on a 2 or 3.5% agarose gel (dependent on size), in 1X TBE, at 70V for 90 minutes.

Table 5: Restriction enzymes (RE) and allele description used to genotype each polymorphism.

Genes	Position and nature	RE and condition	dbSNP/Reference	Recognition site (5'-3')
IL-1β	+3954 (C/T)	*Taqα*I (37°C) [a]	rs: 1143634	T↓CGA
IL-4	-590 (C/T)	*BsmF*I (65°C) [a]	rs: 2243259	GGGAC(N)$_{10}$↓
IL-10	-1082 (A/G)	*EcoN*I (37°C) [a]	rs: 1800896	CCTNN↓NNNAGG
	-592 (C/A)	*Rsa*I (37°C) [a]	rs: 1800871	GT↓AC
*TNF*α	+488 (G/A)	*Nla*III (37°C) [a]	Mullighan *et al.* 1997	CATG↓

New England Biolabs [a]

Table 6: Restriction enzymes (RE) and allele description used to confirm variations identified in this study.

Genes	Region	Position and nature	RE and condition	dbSNP	Recognition site (5'-3')
*LGALS*13	E1	-98 (A/C), 5'UTR	*Ava*I (37°C) [a]	rs: 3764843	C↓YCGRG
	E3.1	IVS2-36 (G/A)	*Nla*IV (37°C) [a]	**novel**	GGN↓NCC
		IVS2-22 (A/G)	*Aci*I (37°C) [a]	rs: 2233706	AA↓CGTT
		IVS2-15 (G/A)	*Nla*III (37°C) [a]	**novel**	CATG↓
	E3.2	Hotspot	*Apo*I (55°C) [a]	**novel**	R↓AATTY
		IVS3+72 (T/A)	*Stu*I (37°C) [a]	rs: 2233708	AGG↓CCT

New England Biolabs [a]
E = exon

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2.2.5. Statistical Analysis

Statistical analysis was performed using the Hardy-Weinberg Equilibrium Chi squared test to compare the genotype and allelic frequency distribution in the study groups. Genotypes and alleles were considered to be in Hardy-Weinberg equilibrium if the observed frequencies did not differ significantly from the expected (P>0.05). The study cohort was divided into groups with (1) good pregnancy outcome (controls) and (2) poor pregnancy outcome (patients) for each population group (Coloured and Black [Xhosa speaking]). Subgroup analysis was subsequently performed, grouping related pregnancy complications, using 2 × 2 contingency table analyses and the Chi square test for independence using EpiInfo 6 statistical software (http://www.cdc.gov/epiinfo/Epi6/ei6.htm). Odds ratio (OR) and relative risk (RR) at 95% confidence intervals (CI) were also determined to describe the strength of association. Any

association demonstrated was confirmed by Fisher's exact test. Descriptive analyses were performed using Student's independent *t* test for continuous variables using Statistica 8 software (http://www.statsoft.co.uk/). A P value of < 0.05 was considered statistically significant. Haploview 3.32 (http://broad.mit.edu/mpg/haploview) was used to calculate haplotype associations and the measures of linkage disequilibrium (D' and r^2) between SNPs, a LOD score > 3 were considered as evidence of linkage.

2.2.6. Genotype: phenotype association

Association of genotypic and clinical information were assessed to determine whether certain loci were linked to clinical parameters which included gestational age at complication and birth weight of infant, among others.

3. Results and Discussion

3.1. Results

In the analysis of the raw data, four significant findings were obtained:

1. Clinical profiles in two distinct South African population groups serviced by the Paarl Hospital region could be established and will be used for monitoring "trends".
2. A novel *IL*-1*RN* allele was identified.
3. The following significant associations were demonstrated:
    i. Preterm labour and the *IL*-1*RN**2
    ii. Pre-eclampsia the *IL*-1*RN**2
    iii. Preterm labour and the *TNF*α-308 allele 2
    iv. Preterm labour and the *LGALS*13 221delT
4. Linkage disequilibrium was identified between *LGALS*13 intronic variants IVS2-22 and IVS3+72.

3.1.1. Participant's demographic and clinical characteristics

3.1.1.1. The Paarl Hospital patient PROFILE

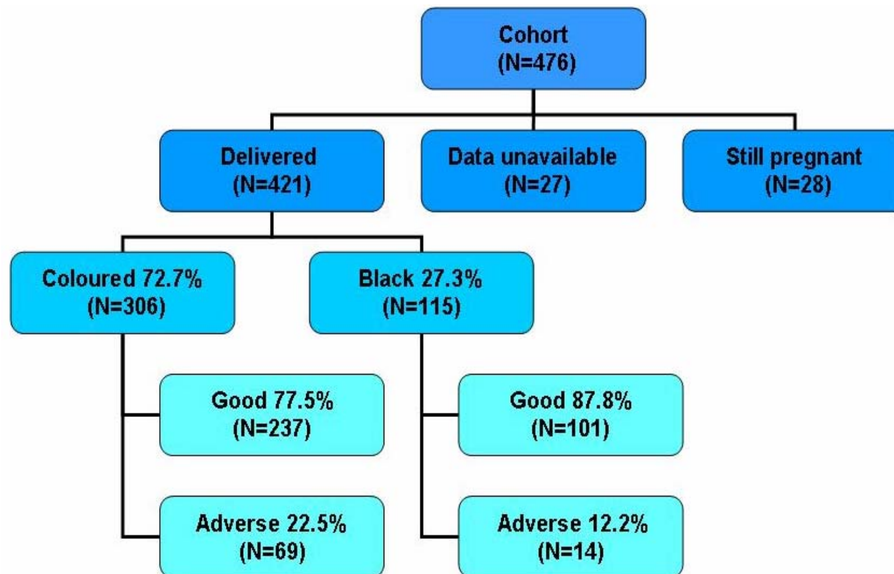
Good perinatal intensive care requires the close collaboration of obstetricians, midwives, anaesthetists, neonatologists and the availability of appropriate equipment. The frequency of pregnancy complications at special referral centres such as Tygerberg hospital mostly represents a "cluster" of cases that could not be dealt with at primary or secondary level care. Hence the incidence of complications documented at Tygerberg hospital do not accurately reflect that of the general population.

Establishing a specific profile of the spectrum of complications encountered in the primary and secondary health care setting will assist with improvement of hospital infrastructure, regarding staffing and facilities and is more "aligned" with potential patients' needs. These improved changes will inevitably have an impact on clinical practice and policy development, offering effective treatment and management programs, thereby lowering the incidence of maternal/fetal morbidity and mortality.

The "Westernisation" of lifestyle has brought about an increase in cardiovascular disease (CVD), diabetes and obesity (Mutch *et al*., 2005), possibly worsening the health status of individuals in South Africa. The current rates and forms of pregnancy complications in Coloured and Black women in the Paarl region may not be the same now as in the past. This study was design to (i) assess which complications prevail now and (ii) determine whether there is a genetic component contributing to their development.

The study cohort is shown diagrammatically in Figure 6 and reflects the population delivering at Paarl Hospital.

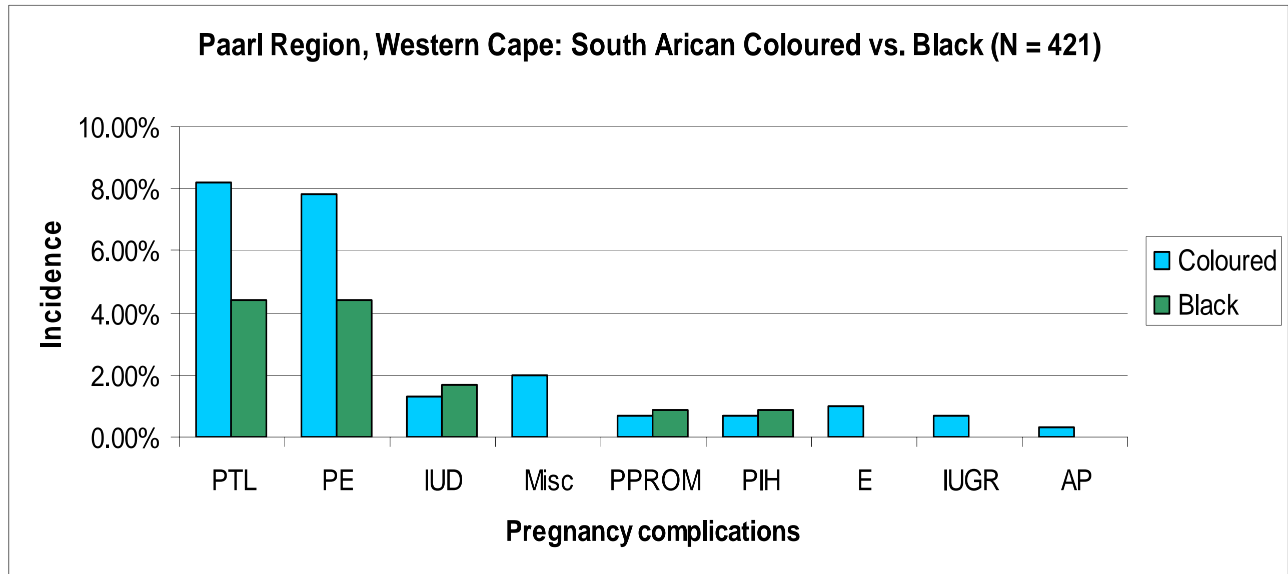
Figure 6: The initial size of the study cohort was 476 (data collected at first booking). Of these, 27 transferred to another clinic and / or were lost to follow-up. At the time of data analysis, 421 patients had delivery outcome, with 28 still pregnant. The cohort (N=421) was sub-divided according to ethnicity and subsequently, outcome of pregnancy (good or adverse).



Complications arose in 19.4% of all the participants. The adverse outcomes represented preterm labour (N=30, 7.1%), pre-eclampsia (PE) (N=29, 6.8%), intrauterine death (IUD) (N=6, 1.4%), miscarriage (N=6, 1.4%), preterm premature rupture of membranes (PPROM) (N=3, 0.7%), pregnancy-induced hypertension (PIH) (N=3, 0.7%), eclampsia (N=3, 0.7%), intrauterine growth restriction (IUGR) (N=2, 0.5%) and abruption placentae (N=1, 0.2%).

The incidence of complications in this study was higher in Coloured (22.5%) than in Black women (12.2%). In our cohort, Coloured ethnicity with its related culture appears to increase an individual's risk of developing various pregnancy pathologies. A summary of the maternal pregnancy complications is presented in Figure 7. In the Coloured group, 8.2% developed preterm labour; the presence of hypertension disorders was 7.8%, 1% and 0.7% representing pre-eclampsia, eclampsia and PIH, respectively. The prevalence of IUGR in this group was 0.7%, with the incidence of PPROM and AP, 0.7% and 0.3%, respectively. Miscarriage and intrauterine death accounted for 2.0% and 1.3% of cases, respectively. The incidence of complications in the Black group was 4.4% each for preterm labour and pre-eclampsia, 1.7% for IUD and 0.9% for PPROM and PIH, respectively.

Figure 7: Graphical presentation of the prevalence of various pregnancy complications observed at Paarl Hospital within the period 2005-2007.



PTL (preterm labour), PE (pre-eclampsia), IUD (intrauterine death), Misc (miscarriage), PPROM (preterm premature rupture of membranes), PIH (pregnancy-induced hypertension), E (eclampsia), IUGR (intrauterine growth restriction) and AP (abruption placentae).

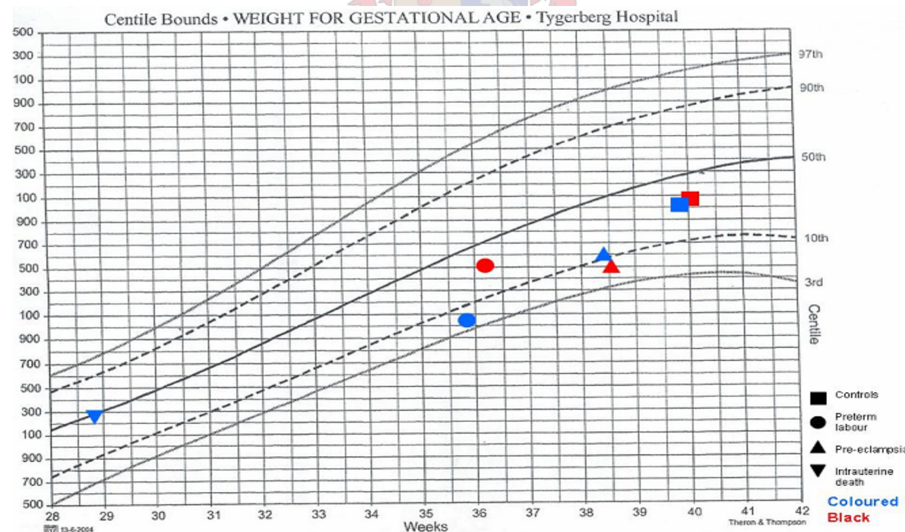
Demographic and clinical data on the cohort are summarised in Table 7. As expected, the mean gestational age and birth weight of complicated pregnancies were significantly lower than deliveries at term. There were too few Black patients in the "complications group" to perform subgroup analysis. However, there was a significant difference in the mean gestational age (P=0.022) and birth weight (P=0.003) for healthy Black vs. healthy Coloured individuals. There was no significant difference in maternal age at delivery in either healthy or complicated outcomes. In contrast, there was a significant difference in maternal age in the Coloured preterm labour group (P=0.023), representing a trend towards lower maternal age.

Table 7: Demographic and clinical characteristics of study cohort.

Characteristic	Controls		Preterm Labour		Pre-eclampsia		Intrauterine death	
	Coloured	Black	Coloured	Black	Coloured	Black	Coloured	Black
Maternal age (mean yrs [range])	N=238 21.9 [16-39]	N=101 22.7 [16-36]	N=27 20.3 [17-26]	N=6 22.0 [19-25]	N=29 22.9 [16-42]	N=6 21.0 [16-24]	N=4 22.8 [21-26]	N=2 28.5 [24-33]
Gestational age (mean wks [range])	N=226 39.7 [38-42]	N=101 39.9 [38-42]	N=27 35.7 [28-40[A]]	N=6 36.2 [28-40[A]]	N=25 38.4 [30-40]	N=6 38.5 [36-40]	N=3 28.7 [24-33]	N=2 22.0 [22]
Birth weight (mean g [range])	N=224 2959.0 [1820-4050]	N=101 3113.5 [2100-4400]	N=27 2108.1 [980-3640]	N=5 2502.0 [1780-3580]	N=24 2636.9 [1440-3700]	N=6 2543.3 [2300-2820]	N=4 1294.0 [426-3010]	N=2 503.0 [446-560]
Caesarean delivery	58/478 (12.1%)							

[A] Labour suppressed

Figure 8: Based on the results of Table 7, the mean gestational age and birth weight for each group (controls, preterm labour, pre-eclampsia and intrauterine death) were plotted on a standard growth chart. This chart had been specifically designed for the Tygerberg patient (G Theron, personal communication).



Control and preterm labour Black infants weighed more than the Coloured infants, while the trend was reversed in cases of pre-eclampsia, where the Coloured infants weighed more.

3.1.1.2. Discussion

This study has shown a particular *order* in the presentation of pregnancy-related complications in this cohort, viz, IUD (week 22-28), preterm labour (up to 36 weeks) and pre-eclampsia (up to term). Pre-eclampsia (early and late onset forms) often further complicated by AP or IUGR and the more sudden eclampsia, followed closely in frequency by preterm labour, represent the major complications at Paarl hospital.

Within this geographical region, Black women generally had healthier pregnancy outcomes compared to Coloured women. The poor pregnancy outcome in the Coloured group could be caused by a combination of genetic and environmental factors. In our study, maternal age of Coloured women was identified as a risk factor for early labour, with younger women more prone to early delivery. In a survey, smoking during pregnancy was reported by 47% of Coloured women attending public service clinics. Within this population, smoking during pregnancy was shown to significantly increase the risk of developing AP and preterm labour, with a mean birth weight reduction of 256 g (Evertt *et al*., 2005).

Based on these data, smaller public obstetric clinics serving Coloured women should be specifically equipped to (i) identify and (ii) manage and / or treat cases of preterm labour and the various forms of hypertension. Concurrently, there should be a drive to educate women (specifically Coloured women) about pregnancy complications and the link with environmental factors such as smoking and alcohol abuse. Collectively, these initiatives could minimise maternal and fetal mortality and morbidity.

The general observation of healthier Black pregnant women within the Paarl region, should be investigated to establish whether there is an underlying protective effect: either at a genetic and / or environmental level.

3.1.2. Inflammatory cytokines

Significant associations were demonstrated between pregnancy complications and selected polymorphisms within genes encoding two cytokines. These data will be presented and discussed together. Information on the remaining loci screened in this study cohort is in Appendix 8.

3.1.2.1.1. *IL*-1*RN* locus

Various *IL*-1*RN* genotypes were identified in this study (Figure 9).

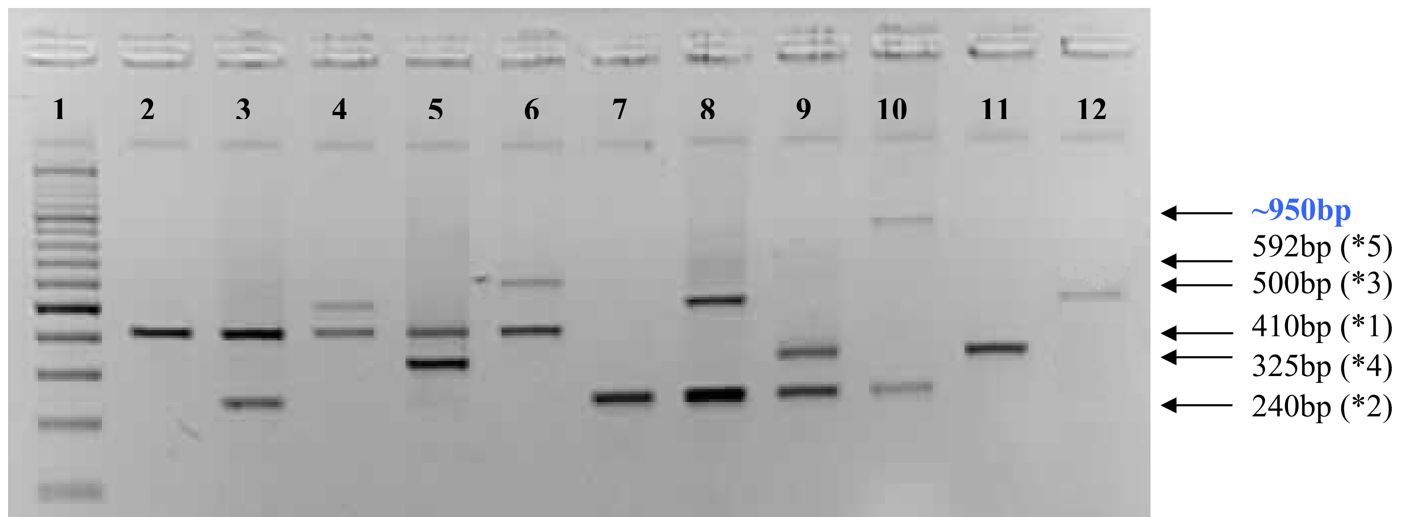


Figure 9: 2% Agarose gel reflecting the different *IL*-1*RN* genotypes as assigned by Hellmig *et al*., 2005. Lane 1: 100bp ladder (Fermentas), Lane 2: 1/1 (410bp), Lane 3: 1/2 (410bp and 240bp), Lane 4: 1/3 (410bp and 500bp), Lane 5: 1/4 (410bp and 325bp), Lane 6: 1/5 (410bp and 592bp), Lane 7: 2/2 (240bp), Lane 8: 2/3 (240bp and 500bp), Lane 9: 2/4 (240bp and 352bp), Lane 10: 2/**novel** (240bp and **~950bp**), Lane 11: 4/4 (325bp) and Lane 12: 3/3 (500bp).

The distribution of the *IL*-1*RN* genotypes in the combined study population (Coloured and Black) as well as the adverse pregnancy outcome [N=83] and subgroups (preterm labour [N=33] and hypertension [N=35]) are shown in Table 8. The remaining 15 cases represented a diverse "other complication" group of cases, such as IUGR and AP. Genotypes containing the *IL*-1*RN**3, *IL*-1*RN**4 and *IL*-1*RN**5 alleles were rare and therefore excluded from the analysis (in

keeping with international trends). Analysis was performed as previously documented (Witkin *et al*., 2003).

Table 8: Genotype frequencies determined at the *IL*-1*RN* locus. The 3 individual rows (in blue) were extracted from the dataset and further analysed, in keeping with international trends.

Genotype	Total (N=421)	Controls (N=338)	Adverse outcome (N=83)	Preterm labour (N=33)	Hypertension (N=35)
1/1	254 (60.3%)	212 (62.7%)	42 (50.6%)	15 (45.5%)	15 (42.9%)
1/2	109 (26.0%)	77 (22.8%)	32 (38.6%)	15 (45.5%)	15 (42.9%)
1/3	13 (3.1%)	12 (3.6%)	1 (1.2%)	1 (3.0%)	0
1/4	17 (4.0%)	15 (4.4%)	2 (2.4%)	1 (3.0%)	1 (2.9%)
1/5	3 (0.7%)	2 (0.6%)	1 (1.2%)	0	0
2/2	18 (4.3%)	13 (3.8%)	5 (6.0%)	1 (3.0%)	4 (11.4%)
2/3	1 (0.2%)	1 (0.3%)	0	0	0
2/4	2 (0.5%)	2 (0.6%)	0	0	0
2/novel	1 (0.2%)	1 (0.3%)	0	0	0
3/3	2 (0.5%)	2 (0.6%)	0	0	0
4/4	1 (0.2%)	1 (0.3%)	0	0	0

The novel allele in one control individual (shown in blue) is currently being characterized by automated DNA sequencing. The estimated amplicon size is ~950bp, which is equal to approximately ten 86-bp tandem repeats.

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Table 9: Genotype and allele frequencies determined for the ***IL*-1*RN*** polymorphism for the total **cohort**. Hardy Weinberg Equilibrium was determined for each individual group and is shown in the last row of the table. Statistical analysis was performed using Fisher's exact test analog to determine associations at both genotype and allele level. The corresponding *P* values are shown at the bottom of the table. Statistically significant *P* values are shown in bold.

			Total Cohort (N=381)		
Genotype	**Total (N=381)**	**Controls (N=302)**	**Adverse (N=79)**	**PTL (N=31)**	**Hypertension (N=34)**
1/1	254 (66.7%)	212 (70.2%)	42 (53.2%)	15 (48.4%)	15 (44.1%)
1/2	109 (26.6%)	77 (25.5%)	32 (40.5%)	15 (48.4%)	15 (44.1%
2/2	18 (4.7%)	13 (4.3%)	5 (6.3%)	1 (3.2%)	4 (11.8%)
1-allele	0.809	0.829	0.734	0.726	0.662
2-allele	0.191	0.171	0.266	0.274	0.338
HWE	0.376	0.229	0.945	0.486	0.996

Controls vs. Adverse: ***P*=0.017** (genotype) and ***P*=0.007** (allele); Controls vs. Preterm labour: ***P*=0.025** (genotype) and ***P*=0.043** (allele); Controls vs. Hypertension: ***P*=0.006** (genotype) and ***P*=0.0008** (allele).

Distribution of the *IL*-1*RN* genotypes for the total group is shown in Table 9 and was shown to be in Hardy-Weinberg Equilibrium (*P*>0.05). The genotype and allele frequencies in the controls compared to the adverse pregnancy outcome differed significantly (*P*=0.017 and *P*=0.0066, respectively). Carriage of the *IL*-1*RN**2 (1/2 and 2/2) were associated with an increased odds (OR 2.08, 95% CI 1.20-3.55) and relative risk (RR 1.76, 95% CI 1.20-2.60) for developing a pregnancy complication (*P*=0.004). The enrichment of the *IL*-1*RN* *2 were more prevalent in the adverse outcome, preterm labour and hypertension versus the controls groups, 26.6%, 27.4%, 33.8% and 17.1%, respectively.

Further subgroup analysis revealed the association was contained within both the preterm labour (*P*<0.05) and strengthened in the hypertension group (*P*<0.01). The presence of the *IL*-1*RN* *2 had an increase odds (OR 2.51, 95% CI 1.12-5.64) and relative risk (RR 2.28, 95% CI 1.17-4.44, *P*=0.013) for developing preterm labour. In the hypertension group, *P*=0.002, the OR was 2.98 (95% CI 1.37-6.51) and the RR was 2.64 (95% CI 1.39-4.99).

Table 10: Genotype and allele frequencies of the ***IL*-1*RN*** polymorphism in the **Coloured** population. Hardy Weinberg Equilibrium (HWE) was determined for each individual group. Statistical analysis was performed using Fisher's exact test analog to determine associations at both genotype and allele level. The corresponding *P* values are shown at the bottom of the table. Statistically significant *P* values are shown in bold.

Coloured Females (N=279)					
Genotype	**Total (N=279)**	**Controls (N=212)**	**Adverse (N=67)**	**PTL (N=26)**	**Hypertension (N=28)**
1/1	172 (61.6%)	138 (65.1%)	34 (50.7%)	12 (46.2%)	11 (39.3%)
1/2	90 (32.3%)	62 (29.2%)	28 (41.8%)	13 (50%)	13 (46.4%)
2/2	17 (6.1%)	12 (5.7%)	5 (7.5%)	1 (3.8%)	4 (14.3%)
1-allele	0.778	0.797	0.716	0.712	0.625
2-allele	0.222	0.203	0.284	0.288	0.375
HWE	0.536	0.379	0.973	0.539	0.999

Controls vs. Adverse: *P*=0.108 (genotype) and ***P*=0.050** (allele); Controls vs. Preterm labour: *P*=0.099 (genotype) and *P*=0.154 (allele); Controls vs. Hypertension: ***P*=0.021** (genotype) and ***P*=0.0036** (allele).

Table 10 is a summary of genotype and allele frequencies within the Coloured population. All the groups were found to be in Hardy Weinberg Equilibrium. An association was observed with the allele frequency in the control versus the adverse pregnancy outcome group (*P*=0.050). The carrier status frequency between controls and adverse outcome group differed significant

(*P*=0.028) with an OR of 1.86 (95% CI 1.03-3.39) and RR of 1.60 (95% CI 1.05-2.43). Subgroup analysis demonstrated no association with the preterm labour group. Carrier status of the minor allele revealed no association but an increased odds ratio of 2.18 (OR 95% CI 0.89-5.33) and relative risk of 1.99 (RR 95% CI 0.96-4.10) of developing preterm labour. A significant association was demonstrated in the hypertension group with both genotype and allele frequencies (*P*=0.021 and *P*=0.0036). Maternal carriage of the *IL*-1*RN**2 was associated (*P*=0.0083) with an increased odds (2.88, 95% CI 1.20-6.99) and relative risk (2.53, 95% CI 1.24-5.16) of developing pregnancy related hypertension.

Table 11: Genotype and allele frequencies of the ***IL*-1*RN*** polymorphism in the **Black** population. Hardy Weinberg Equilibrium (HWE) was determined for each individual group.

Black Females (N=102)					
Genotype	**Total (N=102)**	**Controls (N=90)**	**Adverse (N=12)**	**PTL (N=6)**	**Hypertension (N=6)**
1/1	82 (80.4%)	74 (82.2%)	8 (66.7%)	3 (60%)	4 (66.7%)
1/2	19 (18.6%)	15 (16.7%)	4 (33.3%)	2 (40%)	2 (33.3%)
2/2	1 (1%)	1 (1.1%)	0	0	0
1-allele	0.897	0.906	0.833	0.800	0.833
2-allele	0.103	0.094	0.167	0.200	0.167
HWE	0.996	0.971	0.787	0.855	0.887

Sample size insufficient to perform association analysis (*P*<0.05).

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Genotype frequencies of black individuals (Table 11) at the *IL*-1*RN* locus were in Hardy Weinberg Equilibrium and no significant association could be demonstrated. Subgroup analysis was not performed due to the limited sample size. In the total group, ethnic disparity was observed in the distribution of alleles. The frequency of alleles in Black individuals was 89.7% for *IL*-1*RN**1 and 10.3% for *IL*-1*RN**2 versus 77.7% and 22.3% in Coloured individuals, with a significant difference of *P*<0.001 (Black vs. Coloured).

3.1.2.1.2. *TNFα*-308 G/A variant

The *TNF*α-308 genotypes identified are illustrated in Figure 10 and the distribution of the genotype and allele frequencies for the total cohort is shown in Table 12. The control group genotypes deviated from Hardy Weinberg equilibrium (and this was also reflected in the "total

cohort" genotypes), which restricted further analysis. Within the total cohort, the A/A genotype was very rare, although heterozygosity was fairly common in each group.

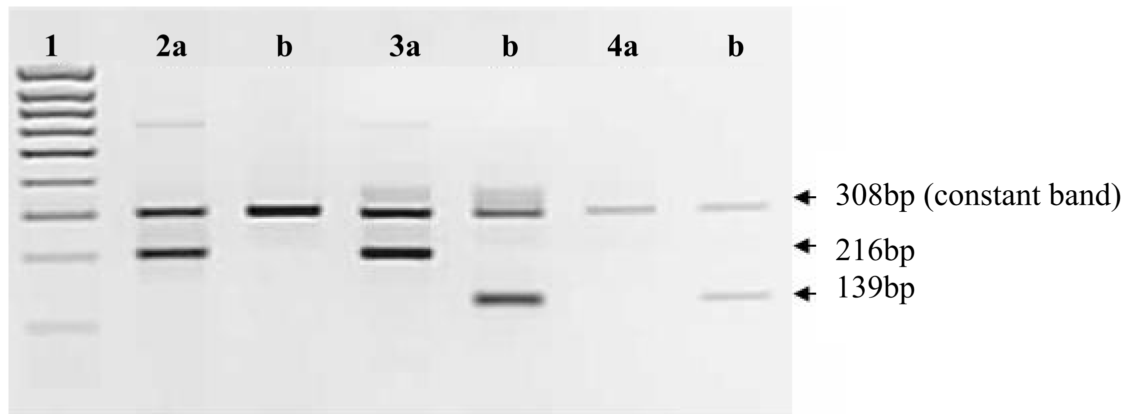


Figure 10: Agarose gel (2%) demonstrating the three genotypes of *TNF*α-308 locus detected by ARMS PCR (Yamamoto-Furusho *et al*., 2004). Lane 1 represents a 100 bp ladder (Fermentas). Lanes 2a and b represents homozygosity for the *TNF*α-308 G-allele (216 bp), Lanes 3a and b represent a heterozygous genotype (216 and 139 bp) and Lanes 4a and b represents homozygosity for the *TNF*α-308 A-allele (A at 139 bp).

Table 12: Genotype and allele frequencies of the ***TNFα*-308 G/A** polymorphism for the total **cohort**. Hardy Weinberg Equilibrium (HWE) was determined for each individual group.

Total Cohort (N=421)					
Genotype	**Total (N=421)**	**Controls (N=338)**	**Adverse (N=83)**	**PTL (N=33)**	**Hypertension (N=35)**
G/G	307 (72.9%)	253 (74.9%)	54 (65.1%)	20 (60.6%)	24 (68.6%)
G/A	113 (26.9%)	85 (25.1%)	28 (33.7%)	12 (36.4%)	11 (31.4%)
A/A	1 (0.20%)	0	1 (1.2%)	1 (3%)	0
G-allele	0.863	0.874	0.819	0.788	0.843
A-allele	0.137	0.124	0.183	0.212	0.157
HWE	**0.018**	**0.030**	0.436	0.880	0.544

Total cohort and controls did not reach HWE significance (*P*<0.05).

Table 13 shows the frequencies of the genotypes and alleles in the Coloured population. All genotype distributions were in Hardy Weinberg Equilibrium. The segregation of the *TNF*α-308 A-allele (*TNF*2) in the adverse outcome (16.7%), preterm labour (20.4%) and hypertension (15.5%) groups were higher than controls (10.1%). A significant difference was observed in the comparison of control versus adverse pregnancy outcome, in terms of the genotype and allele frequencies (*P*=0.033 and *P*=0.035, respectively). The occurrence of both G/A and A/A genotypes showed an association with adverse pregnancy outcome (OR 1.84, 95% CI 0.96-3.47

and RR 1.58, 95% CI 1.03-2.43, *P*=0.043). Statistical analysis revealed a significant association between the preterm labour and *TNF*2 at both genotype (*P*=0.003) and allele (*P*=0.024) level. The presence of *TNF*2 was associated (*P*=0.047) with an increased odds (2.32, 95% CI 0.92-5.78) and relative risk (2.09, 95% CI 1.01-4.31) for preterm labour. The association did not extend to the hypertension group (*P*=0.210).

Table 13: Genotype and allele frequencies of the ***TNFα*-308 G/A** polymorphism in the **Coloured** population. Hardy Weinberg Equilibrium (HWE) was determined for each individual group. Statistical analysis was performed using Fisher's exact test analog to determine associations at both genotype and allele level. The corresponding *P* values are shown at the bottom of the table. Statistically significant *P* values are shown in bold.

Coloured Females (N=306)					
Genotype	**Total (N=306)**	**Controls (N=237)**	**Adverse (N=69)**	**PTL (N=27)**	**Hypertension (N=29)**
G/G	236 (77.1%)	189 (79.7%)	47 (68.1%)	17 (63%)	20 (69%)
G/A	69 (22.6%)	48 (20.3%)	21 (30.4%)	9 (33.3%)	9 (31%)
A/A	1 (0.30%)	0	1 (1.5%)	1 (3.7%)	0
G-allele	0.884	0.899	0.833	0.796	0.845
A-allele	0.116	0.101	0.167	0.204	0.155
HWE	0.221	0.222	0.729	0.990	0.613

Controls vs. Adverse: ***P*=0.033** (genotype) and ***P*=0.035** (allele); Controls vs. Preterm labour: ***P*=0.003** (genotype) and ***P*=0.024** (allele); Controls vs. Hypertension: *P*=0.210 (allele).

Genotype and allele frequencies of the Black individuals are shown in Table 14 and no significant association could be demonstrated in the adverse pregnancy group. The distribution of GG and GA genotypes differed significantly in Black versus Coloured individuals, 61.7% and 38.3% versus 77.1% and 22.6%, respectively (*P*<0.01).

Table 14: Genotype and allele frequencies of the ***TNFα*-308 G/A** polymorphism in the **Black** population. Hardy Weinberg Equilibrium (HWE) was determined for each individual group.

Black Females (N=115)					
Genotype	**Total (N=115)**	**Controls (N=101)**	**Adverse (N=14)**	**PTL (N=6)**	**Hypertension (N=6)**
GG	71 (61.7%)	64 (63.4%)	7 (50%)	3 (50%)	4 (66.7%)
GA	44 (38.3%)	37 (36.6%)	7 (50%)	3 (50%)	2 (33.3%)
AA	0	0	0	0	0
G-allele	0.809	0.817	0.750	0.750	0.833
A-allele	0.191	0.183	0.250	0.250	0.167
HWE	**0.004**	0.079	0.459	0.717	0.887

Sample size insufficient to perform association analysis (*P*<0.05).

3.1.2.2. Discussion

In this cohort, maternal carriage of the *IL*-1*RN**2-allele could be associated with adverse pregnancy outcome. Upon closer inspection of the genotype distribution in the adverse outcome group, the association was restricted to both the preterm labour and particularly in the hypertension group. After adjusting for ethnicity, the association was only demonstrated in the hypertension group of Coloured individuals. The presence *TNF*α-308 A-allele was associated with overall adverse pregnancy outcome and preterm labour in the Coloured group.

Strengths of the study included the analysis of 421 individuals in two distinct population groups for the comparison of allele and genotype distribution at different loci. All the immunological variants were detected either by allele-specific or restriction enzyme analysis, with reproducible results. Consequently, these results can be applied as a general population reference dataset.

However, as expected in studies like these, the sample size of the different complication subgroups becomes restricted; in addition to the inclusion criteria limited to low risk primigravid pregnant women. This study also lacks measured TNFα and IL-1 levels to correlate with the genotypes. In relation to patient information, data on "infection" were not available. Another limitation was the exclusion of corresponding fetal samples to establish fetal genotype status.

Homozygosity for *IL*-1*RN**2 has been associated with various inflammatory conditions including inflammatory bowl disease, alopecia areata, psoriasis, lichen sclerosus, lupus erythematosus, vulvar vestibulitis and multiple sclerosis (Witkin *et al*., 2002). The regulation of both IL-1β and IL-1ra production has been associated with *IL*-1*RN* genotype, with conflicting results. Mononuclear cells positive for the *IL*-1*RN**2 produced higher levels of IL-1β after stimulation *in vitro*. In second trimester amniotic fluid, the amounts of IL-1β and IL-1ra are increased leading to an increased IL-1β and IL-1ra ratio in fetal samples containing the *IL*-1*RN**2. In relation to maternal carrier status, the vaginal concentration of IL-1ra is elevated with an increase isolation rate of *Ureaplasma urealyticum*. These interpretations are consistent with the results of *IL*-1*RN**2 and the variety of inflammatory conditions (Witkin *et al*., 2002 and Nguyen *et al*., 2004). In contrast to these results, two studies had reported that *IL*-1*RN**2 leads to a decrease in

production of circulating levels (Rider *et al*., 2000 and Vamvakopoulos *et al*., 2002). The functional effect of the *IL*-1*RN* polymorphism on IL-1β, which is not fully understood, could be explained by linkage disequilibrium with another locus in the *IL*-1 gene complex regulating the production of IL-1β (Nguyen *et al*., 2004).

Similar results were found by Genc *et al*., who demonstrated a blunted IL-1β response in maternal carriage of the *IL*-1*RN**2, with a reduced probability of spontaneous preterm delivery. In this study, Genc *et al*., demonstrated association between preterm labour and *IL*-1*RN**1 (Genc *et al*., 2004a), compared to our current study demonstrating an association with the *IL*-1*RN**2 and preterm labour. In the Genc study, the association established in the total group could be explained by the difference in the carrier and non-carrier frequency in the different ethnicities; White (43.7% and 56.3%), Hispanic (44.3% and 55.7%) and Black (10.9% and 89.1%) individuals (Genc *et al*., 2004a). The association with *IL*-1*RN**1 in the total group most likely rests on the frequencies in the Black individuals. The common *IL*-1*RN* allele appears to be more frequent in the Black population, as seen in our study and others (Genc *et al*., 2002 and Nguyen *et al*., 2004).

The results of our study concurs with the evaluation of the maternal genotype by Murtha *et al*., (cases, N=95) who described an association between preterm birth and the *IL*-1*RN**2 in all ethnicities (*P*=0.0004) and white (*P*=0.001) individuals (Murtha *et al*., 2006). Although the association in our study was based on a much smaller sample size, in the case of the Coloured cohort, a non-homogenous population, the level of significance supports the observation found by Murtha and colleagues. Similar *IL*-1*RN**2 frequencies in the controls vs. preterm labour of the black individuals was observed in our study compared to Murtha *et al*., (0.094 vs. 0.200 versus 0.097 vs. 0.174). However, the sample size of our Black preterm labour group was too small to be analyzed for ethnic-specific differences.

The production of cytokines by the fetal component of the placenta can lead to the initiation of myometrial contractions. For that reason, fetal contribution can initiate preterm labour (Witkin *et al*., 2003). Witkin *et al*., evaluated the concentrations of IL-1β and IL-1ra in amniotic fluid in conjunction with *IL*-1*RN* fetal genotypes in white Europeans. A significant association was

demonstrated in birth before 37 weeks (*P*<0.0001) and fetuses carrying the *IL*-1*RN**2, illustrating the importance of corresponding fetal samples. Possession of the *IL*-1*RN**1 in homozygous state was linked to low levels of each, IL-1β (*P*=0.0006) and IL-1ra (*P*=0.01) in relation to one or two copies of *IL*-1*RN**2. The increase in the pro-inflammatory response in the presence of the *IL*-1*RN* fetal genotype demonstrates the relationship of IL-1β mediated preterm labour and augments the importance of fetal samples and measured cytokines (Witkin *et al*., 2003). However, this association was based on a much smaller patient size (N=7) than our study and the collection of fetal DNA was done by amniocentesis, which increases the risk of preterm delivery (Medda *et al*., 2003). As in many other studies, our investigation mainly focused on the maternal genotype.

In vitro studies have demonstrated that IL-1ra production during the pre-implantation stage of human embryos inhibits implantation into the uterine decidua. Individuals in possession of the *IL*-1*RN**2 genotype or with elevated production, may be at a selective disadvantage at the start of pregnancy and at increase risk for early-stage pregnancy loss (Witkin *et al*., 2002). In pre-eclamptic patients, levels of circulating IL-1ra are noticeably increased, but with no correlation to disease severity (Faisel *et al*., 2003). Pregnancy-related conditions such as pre-eclampsia and preterm labour, are thought to mainly originate from abnormalities in (a) implantation, which is immunologically regulated (Roberts and Cooper, 2001), and (b) development of the placenta in early pregnancy (Norwitz, 2006). The results from our study concur with Faisel *et al*., who demonstrated an association with the *IL*-1*RN**2 and pre-eclampsia in the Finnish population. In the setting of their case-control study, comparative analysis did not reach statistical significance. Significance was only demonstrated when a control group, representing an existing reference database of the general population, was used in the analysis (Faisel *et al*., 2003).

Another *IL*-1*RN**2 case control association study was reported by Hefler *et al*., (2001). Their study population was of Hispanic decent (cases, N=59) and the frequency of the IL *IL*-1*RN**2 was much lower compared to those determined by Nguyen *et al*., (Hefler et al., 2001 and Nguyen et al., 2004). However, *IL*-1*RN**2 was associated with a higher systolic blood pressure (BP) in early onset pre-eclampsia. A general problem in association studies is the choice and

stratification of controls, which could inevitably create or eradicate an association (Faisel *et al*., 2003).

The process leading to preterm labour is brought forward by a combination of different pathophysiologic actions, such as intrauterine infection. Preterm labour is often preceded by pro-inflammatory cytokines secreted by both the fetal and maternal counterparts, in response to infection (Makhseed *et al*., 2003). Previous research focused generally on single nucleotide polymorphisms (SNPs) within pro-inflammatory cytokine genes (Annells *et al*., 2005). The level of heterogeneity within genes in the pathway of infectious and inflammatory response complicates the search for genetic variants leading to inflammation-related preterm birth (Amory *et al*., 2004). In our study we demonstrated an association with the *TNF*α -308 G/A polymorphism, which concurs with the findings by Moore and colleagues. The investigation by Moore *et al*., was performed in a mixed ethnic group, and subgroup analysis was not performed in the different ethnic groups due to inadequate cohort size (Moore *et al*., 2004). Macones *et al*., demonstrated a similar trend between, *TNF*2 allele and spontaneous preterm birth. The significance by Macones *et al*., was based on a larger sample size (N=125), compared to Moore *et al*., and our cohort. The Macones study included clinical diagnosis of bacterial vaginosis (BV), lacking in our study. The presence of symptomatic BV and *TNF*2 allele increased the odds of developing preterm birth by 6-fold, illustrating the importance of interaction between genetic and environmental factors and the etiology of spontaneous preterm birth (Macones *et al*., 2004). The findings of our study contrast with three other studies, which indicated no association with the *TNF*2 allele (Amory *et al*., 2004, Annells *et al*., 2004 and Valdez *et al*., 2004). The statistical power of association in our study, compared to investigations by other two larger studies showing no association (Menon *et al*., 2006) should be addressed by further sampling of patients. The lack of association with the *TNF*2 allele in previous studies could be related to the possibility that *TNF*α -308 variant is not functionally significant, or is in linkage disequilibrium with other polymorphisms (e.g. lymphotoxin alpha and HLA genes) (Annells *et al*., 2005) or part of an extended haplotype (Amory *et al*., 2004). A meta-analysis by Menon *et al*., suggest that maternal possession of the minor allele is not a strong risk factor for preterm birth, and if this SNP contributes, it is probably due to other interacting factors including fetal involvement (Menon *et al*., 2006), which was not assessed in our study.

In conclusion, the present findings possibly suggest that *TNF*α -308 and *IL-1RN* gene variants in our population, may confer predisposition to complications such as preterm labour and preeclampsia. These results should be treated with caution, as they need validation. The maintenance of normal pregnancy is characterised by low levels of TNFα. This cytokine is involved in remodeling of the cervix and fetal membranes by stimulating the production of MMP-1 and -9. During normal functioning, TNFα stimulates trophoblast differentiation, invasion and adhesion, implantation, placental development as well as growth and remodeling of fetal membranes. However, a change in circulating levels may activate endocrine function inhibition, protease activation and degrading of extracellular matrix. Dysfunctional regulation caused by polymorphisms in this gene, may possibly result in pregnancy complications such as preterm labour (Crider *et al*., 2005). IL-1ra functions as a competitive inhibitor of IL-1β, a key cytokine modulating the pro-inflammatory cascade (Genc *et al*., 2004a). The imbalance between both IL-1β and IL-1ra has been implicated in the development of infection-related preterm labour (Genc *et al*., 2004b). Conflicting results regarding functional effect, describe previously, need to be resolved. Thus, if IL-1RN*2 is associated with decrease circulating levels, then more IL-1β will be available, increasing prostaglandin production and subsequently leading to myometrial contractions (Genc *et al*., 2004b).

The association demonstrated in our study, was based on an "admixed population", which may introduce further complexity. Clinicians have to rely on patients self-reporting their ethnicity on documentation. The combined effects of these factors increase heterogeneity and complexity within the study and probably lessen any association established.

Confirmation of these associations may identify those individuals at risk of specific pregnancy complications and help clarify the possible mechanisms of genetic and biochemical pathways which lead to these adverse pregnancy outcomes in the South African Coloured population. Additionally, establishing a marker profile for women at risk will further assist clinicians to asses the problem before any clinical symptoms arise and thereby initiate timeous treatment and management to minimise adverse pregnancy and neonatal outcome.

3.1.3. *LGALS*13 variants

A total of ten DNA sequence variants were identified in the screening of the *LGALS*13 gene, of which seven are novel and three are previously-documented polymorphisms (Table 15). A diagram of the *LGALS*13 gene, with the sequence variants identified, is shown in Appendix 9.

Table 15: List of novel and previously documented (dbSNP database) sequence variants identified in our study.

*LGALS*13 gene variants	Region	Amino acid change	Number[A] of individuals (N=421)
-98 A/C (rs: 3764843)	Promoter	-	201
IVS2-36 G/A (novel)	Intron 2	-	31
IVS2-22 A/G (rs:2233706)	Intron 2	-	121
IVS2-15 G/A (novel)	Intron 2	-	8
130 A→G (novel)	Exon 3	M44V	2
221delT (novel)	Exon 3	L74W	14
Hotspot (novel)	Exon 3	T77N, T78L, D79H	30
260 A→G (novel)	Exon 3	K87R	1
IVS3+72 T/A (rs:2233708)	Intron 3	-	120
351 G→A (novel)	Exon 4	P117P	1

[A] Number of individuals with heterozygous and / or homozygous variant genotypes.

In this thesis detailed information will be reported on the following three of the identified *LGALS*13 sequence variants:

- IVS2-22 A/G
- IVS3+72 T/A
- 221delT/L74W (novel)

Information on the remaining loci screened in this study cohort is in Appendix 10.

3.1.3.1. Linkage disequilibrium of the IVS2-22 and IVS3+72 variants

Conformational variants were detected by Multiphor SSCP/HD analysis (Figure 12a and 14a). These variants were further characterised by automated sequencing (Figure 12b and 14b), followed by restriction enzyme analysis for confirmation (Figure 13 and 15).

The IVS2-22 locus falls within a cluster of sequence variants identified within close proximity to the exon 5' 3' boundary (Figure 11).

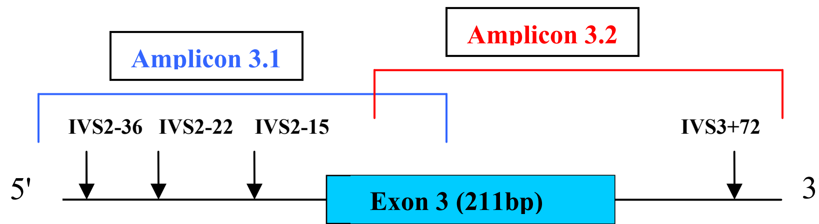
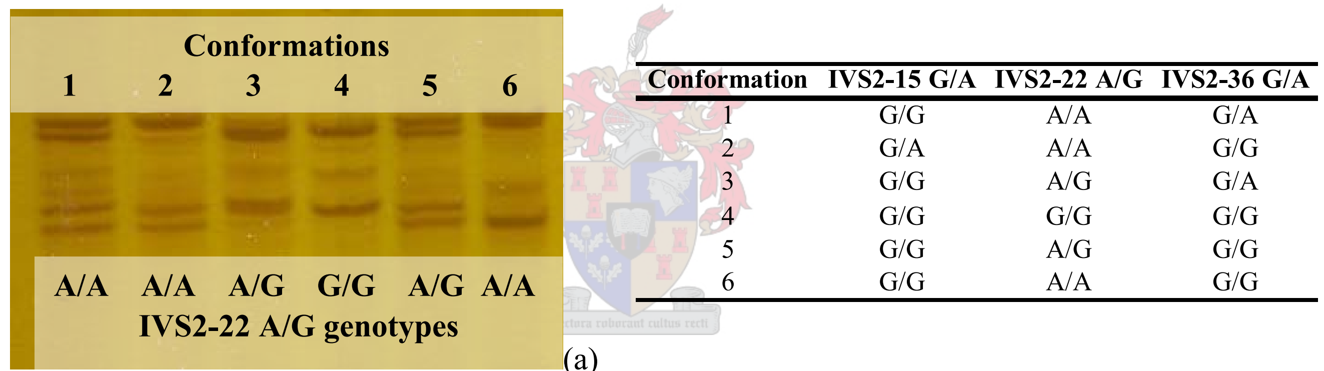


Figure 11: Illustration of the cluster of variants identified in the 5' flanking region of exon three. Amplification of exon three was performed separately (two amplicons) to keep fragment size under 300bp for effective SSCP/HD analysis.

The different multiphor conformations shown in Figure 12a reflect the status of the three polymorphic loci upstream of exon three.



Conformation	IVS2-15 G/A	IVS2-22 A/G	IVS2-36 G/A
1	G/G	A/A	G/A
2	G/A	A/A	G/G
3	G/G	A/G	G/A
4	G/G	G/G	G/G
5	G/G	A/G	G/G
6	G/G	A/A	G/G

(a)

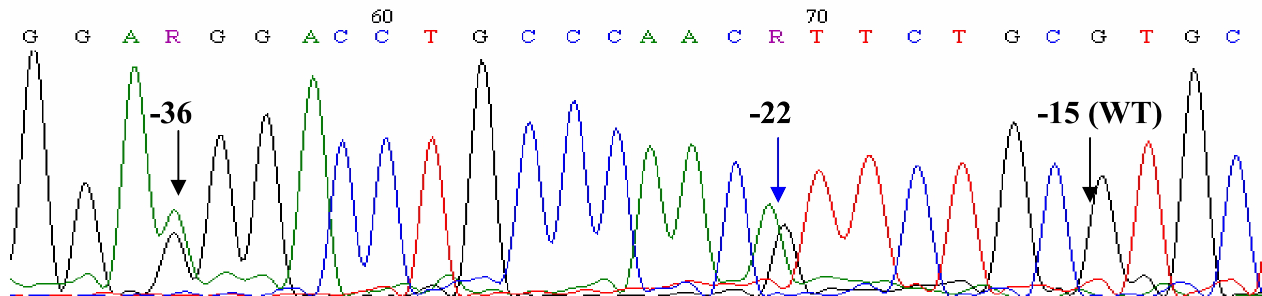


Figure 12: (a) Multiphor SSCP/HD gel illustrating the different conformations of the IVS2-22A/G variant detected in only the SSCP component of the gel. The different conformations observed for the IVS2-22 locus are due to the additional variants identified within the same genomic amplicon (b) Electropherogram depicting the individual genotypes within the exon 3.1 genomic amplicon. Heterozygous status is shown at loci IVS2-36 and IVS2-22, and homozygous status for variant IVS2-15. Sequence is shown in the forward (5'→3') orientation.

(b)

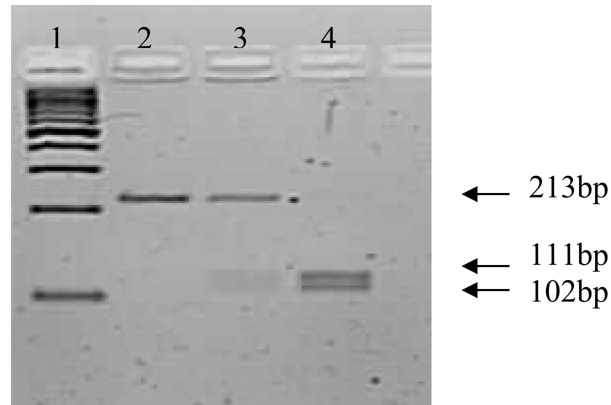


Figure 13: Agarose gel (3.5%) demonstrating the three genotypes of the IVS2-22 A/G variant, detected by restriction enzyme analysis with *Aci*I (37˚C). Lane 1 represents a 100 bp ladder (Fermentas). Lane 2 represents the A/A genotype at 213bp, Lane 3 the heterozygous genotype (A/G) and Lane 4 corresponding to the homozygous G/G genotype at 102 and 111bp.

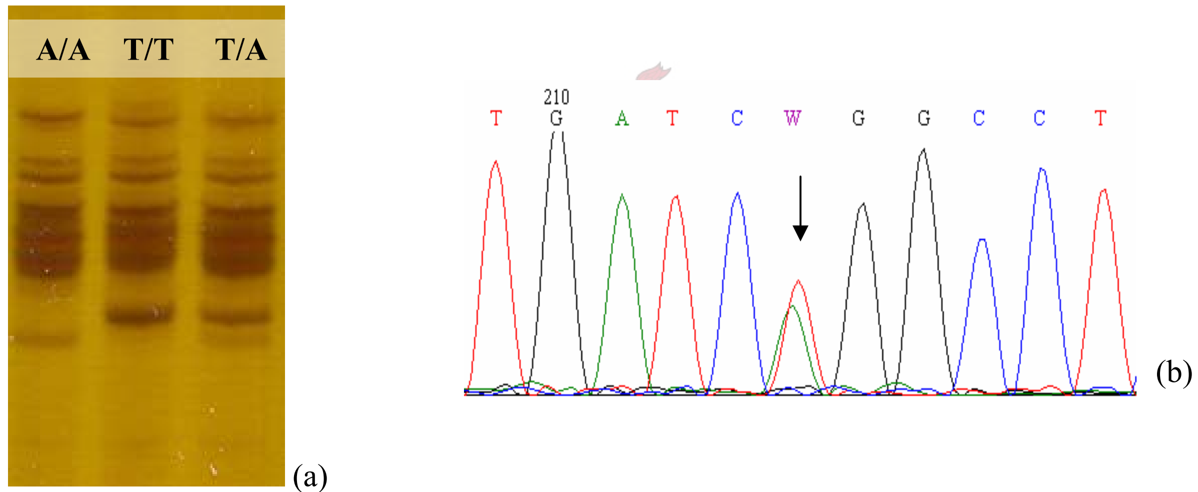


Figure 14: (a) Three conformational banding patterns for the IVS3+72T/A variant detected by Multiphor SSCP/HD analysis and (b) the corresponding electropherogram demonstrating the heterozygous form of the variant (arrowed) sequenced in the forward (5'→3') orientation.

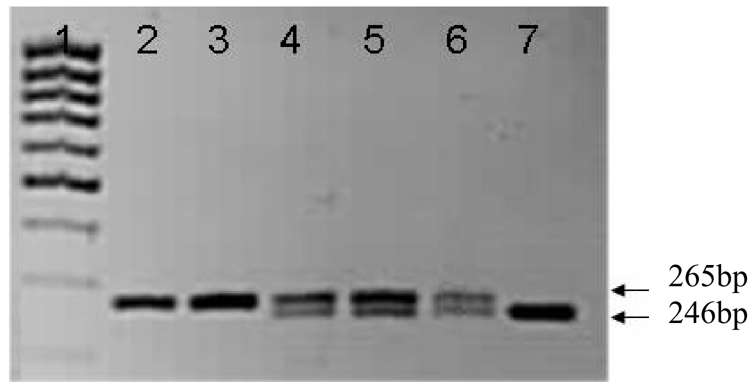


Figure 15: Agarose gel (2%) demonstrating the different genotypes of the IVS2+72 T/A variant, detected by restriction enzyme analysis with *Stu*I (37˚C). Lane 1 is a 100 bp ladder (Fermentas). Lanes 2 and 3 corresponding to the T/T genotype at 265bp, Lanes 4-6 the heterozygous genotype (T/A) and Lane 7 representing the homozygous A/A genotype at 246bp.

Tables 16 and 17 summarise the genotype and allele frequencies of two previously documented intronic IVS2-22A/G (dbSNP: rs 2233706) and IVS3+72T/A (dbSNP: rs 2233708) variants identified in the total cohort. No significant difference was demonstrated in the different subgroups compared to controls.

Pectora roborant cultus recti

Table 16: Observed genotype and allele frequencies at the **IVS2-22 A/G** locus in total **cohort**. Hardy Weinberg Equilibrium (HWE) was determined for each individual group. Statistical analysis was performed using Fisher's exact test analog to determine associations at both genotype and allele level. The corresponding *P* values are shown at the bottom of the table.

Total Cohort (N=421)					
Genotype	**Total (N=421)**	**Controls (N=338)**	**Adverse (N=83)**	**PTL (N=33)**	**Hypertension (N=35)**
A/A	300 (71.4%)	233 (68.9%)	67 (80.7%)	28 (84.8%)	26 (74.3%)
A/G	111 (26.4%)	97 (28.7%)	14 (16.9%)	4 (12.2%)	8 (22.9%)
G/G	10 (2.4%)	8 (2.4%)	2 (2.4%)	1 (3%)	1 (2.8%)
A-allele	0.844	0.833	0.892	0.909	0.857
G-allele	0.156	0.167	0.108	0.091	0.143
HWE	0.997	0.853	0.509	0.309	0.925

Controls vs. Adverse: *P*=0.089 (genotype) and *P*=0.061 (allele); Controls vs. Preterm labour: *P*=0.124 (genotype) and *P*=0.107 (allele); Controls vs. Hypertension: *P*=0.761 (genotype) and *P*=0.602 (allele).

Table 17: Observed genotype and allele frequencies at the **IVS3+72 T/A** locus in total **cohort**. Hardy Weinberg Equilibrium (HWE) was determined for each individual group. Statistical analysis was performed using Fisher's exact test analog to determine associations at both genotype and allele level. The corresponding *P* values are shown at the bottom of the table.

Total Cohort (N=421)					
Genotype	**Total (N=421)**	**Controls (N=338)**	**Adverse (N=83)**	**PTL (N=33)**	**Hypertension (N=35)**
TT	300 (71.3%)	234 (69.2%)	66 (79.5%)	26 (78.8%)	26 (74.3%)
TA	113 (26.8%)	98 (29%)	15 (18.7%)	6 (18.2%)	8 (22.8%)
AA	8 (1.9%)	6 (1.8%)	2 (2.4%)	1 (3%)	1 (2.9%)
T-allele	0.848	0.837	0.886	0.879	0.857
A-allele	0.152	0.163	0.114	0.121	0.143
HWE	0.827	0.499	0.614	0.702	0.925

Controls vs. Adverse: *P*=0.129 (genotype) and *P*=0.122 (allele); Controls vs. Preterm labour: *P*=0.387 (genotype) and *P*=0.379 (allele); Controls vs. Hypertension: *P*=0.693 (genotype) and *P*=0.667 (allele).

A total of 478 typings were performed. Almost identical typing was scored in most cases, suggesting the possibility of linkage disequilibrium. The IVS2-22A/G and IVS3+72T/A are located 305bp apart, on either side of exon three. In 97.1% of typings, IVS2-22 A/A, A/G and G/G genotypes corresponded to IVS3+72T/A genotypes T/T, T/A and A/A, respectively.

The frequencies of observed A-T, A-A, G-A and G-T haplotypes were 84.4%, 0.5%, 14.1% and 0.9%, respectively. The measure of linkage disequilibrium between the two observed loci was 0.96, measured as D', with r^2>0.8 and a maximum LOD score >3 (Table 18). These findings provide genetic (and possibly evolutionary) evidence for the significance of the region encompassed by these two loci.

Table 18: Linkage disequilibrium parameters determined at the IVS2-22A/G and IVS3+72T/A loci.

Locus 1	Locus 2	D'	LOD	r^2	*P*
IVS-22A/G	IVS+72T/A	0.958	109.88	0.887	0.000

3.1.3.2. 221delT

A novel sequence variant was identified in the coding region of *LGALS*13 exon three, apparent in both the SSCP and HD component of the gel and characterised by subsequent automated sequencing (Figure 16a&b).

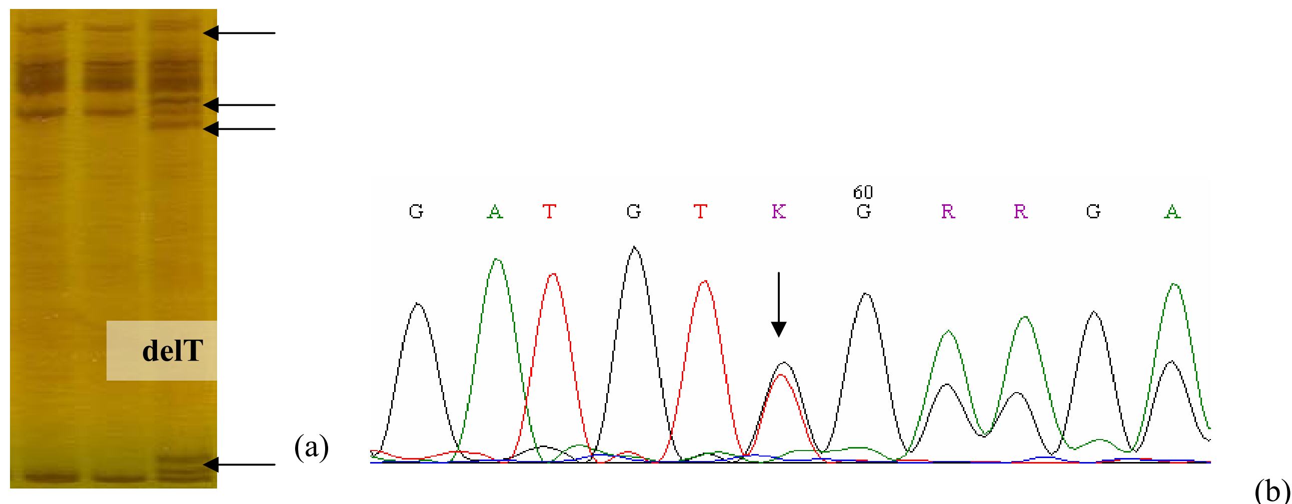


Figure 16: (a) Multiphor SSCP/HD conformation demonstrating the heterozygous form of the 221delT, detected in both the SSCP and HD component of the gel. (b) The corresponding electropherogram demonstrating the heterozygous form (arrowed) of the single base deletion sequenced in the forward (5'→3') orientation. Note the doubling in sequence after the deletion site.

The polymorphism causes a deletion of a single thymine base at nucleotide position 221, resulting in (i) an alternative stop codon and consequently, a shortened protein by 38 amino acids and (ii) an alternative COOH terminal by 28 amino acids (Figure 17).

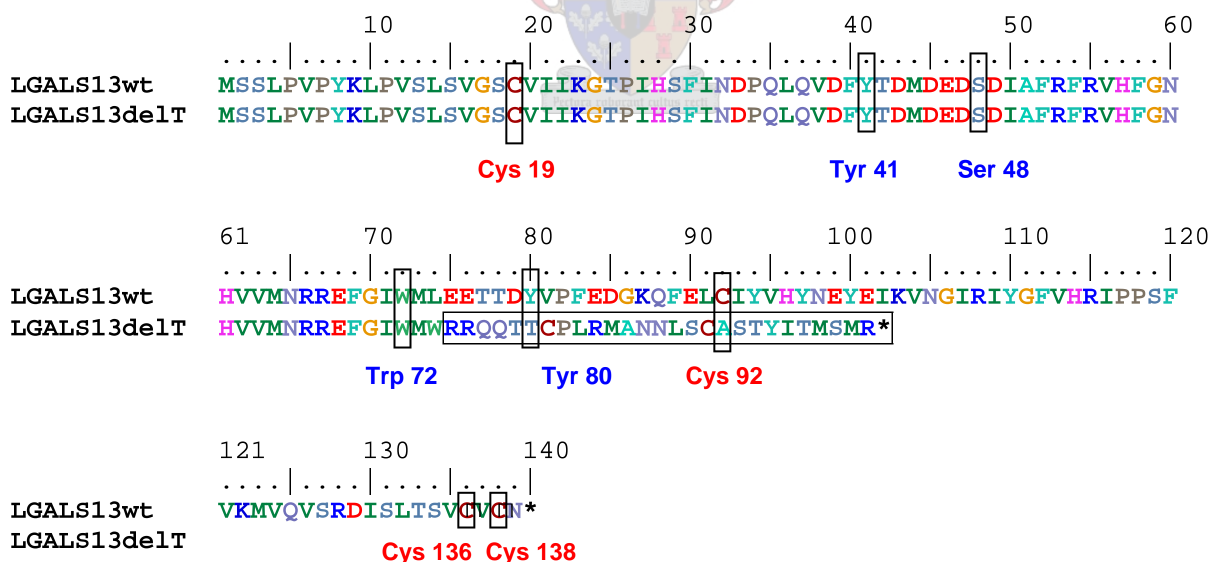


Figure 17: Sequence alignment of the wildtype and predicted 221delT (L74W) PP13 protein isoforms. Cysteine residues (shown in red) are involved in dimerisation via disulphide bonds and the serine and tyrosine residues (shown in blue) are located on the outer surface of PP13 molecule and contribute to phosphorylation (Than *et al*., 2004).

The 221delT genotypes were in Hardy Weinberg equilibrium (Table 19). There were no individuals' homozygous (delT/delT) for the variant. Adverse pregnancy outcome (*P*=0.028) and preterm labour (*P*=0.031) compared to controls differed significantly in the analysis of the total cohort. Carriers of the 221delT were more common in the adverse outcome group than in controls, with a relative risk of 2.27 (95% CI 1.20-4.29, *P*=0.027) and an odds of 3.27 (95% CI 1.18-9.11, *P*=0.030) developing preterm labour. No association was demonstrated in the overall hypertension group, but the delT/- genotype frequency was higher than controls, 5.7% vs. 2.4%, respectively.

Table 19: Genotype and allele frequencies of the **221delT** variant determined in the total **cohort**. Hardy Weinberg Equilibrium (HWE) was determined for each individual group. Statistical analysis was performed using Fisher's exact test analog to determine associations at both genotype and allele level. The corresponding *P* values are shown at the bottom of the table. Statistically significant *P* values are shown in bold.

Total Cohort (N=421)					
Genotype	**Total (N=421)**	**Controls (N=338)**	**Adverse (N=83)**	**PTL (N=33)**	**Hypertension (N=35)**
- / -	407 (96.7%)	330 (97.6%)	77 (92.8%)	30 (90.9%)	33 (94.3%)
-/del	14 (3.3%)	8 (2.4%)	6 (7.2%)	3 (9.1%)	2 (5.7%)
del/del	0	0	0	0	0
- allele	0.983	0.988	0.964	0.955	0.971
del allele	0.017	0.012	0.036	0.045	0.029
HWE	0.942	0.976	0.943	0.963	0.985

Controls vs. Adverse: ***P*=0.028** (allele); Controls vs. Preterm labour: ***P*=0.031** (allele); Controls vs. Hypertension: *P*=0.246 (allele).

Table 20 is a summary of the allele and genotype frequencies determined for 221delT in the Coloured population. The genotypes did not deviate from Hardy Weinberg equilibrium. The heterozygous status frequency in the adverse outcome group was much higher than in controls, 7.2% vs. 1.7%, respectively and demonstrated a statistically significant difference (*P*=0.017) with a 2-fold risk of developing an adverse pregnancy outcome (RR 2.58 95% CI 1.38-4.81, *P*=0.0163). No significant association was demonstrated in either the preterm labour or hypertension groups; however the frequency of the heterozygous status genotype was 7.4% and 6.9%, respectively, compared to healthy controls. This lack of association may of course reflect the difference in sample size between the control (N=237) and sub-grouped PTL (N=27) and Hypertension (N=29) groups.

Table 20: Genotype and allele frequencies of the **221delT** variant in the **Coloured** population. Hardy Weinberg Equilibrium (HWE) was determined for each individual group. Statistical analysis was performed using Fisher's exact test analog to determine associations at both genotype and allele level. The corresponding *P* values are shown at the bottom of the table. Statistically significant *P* values are shown in bold.

Coloured Females (N=306)					
Genotype	**Total (N=306)**	**Controls (N=237)**	**Adverse (N=69)**	**PTL (N=27)**	**Hypertension (N=29)**
- / -	297 (97.1%)	233 (98.3%)	64 (92.8%)	25 (92.6%)	27 (93.1%)
-/del	9 (2.9%)	4 (1.7%)	5 (7.2%)	2 (7.4%)	2 (6.9%)
del/del	0	0	0	0	0
- allele	0.985	0.992	0.964	0.963	0.966
del allele	0.015	0.008	0.036	0.037	0.034
HWE	0.966	0.992	0.952	0.9802	0.982

Controls vs. Adverse: ***P*=0.017** (allele); Controls vs. Preterm labour: *P*=0.060 (allele); Controls vs. Hypertension: *P*=0.076 (allele).

Genotype and allele frequencies in the Black participants are shown in Table 21 and no significant association could be demonstrated, possibly due to the cohort being much healthier with fewer pregnancy complications.

Table 21: Genotype and allele frequencies of the **221delT** variant in the **Black** population. Hardy Weinberg Equilibrium (HWE) was determined for each individual group.

Black Females (N=115)					
Genotype	**Total (N=115)**	**Controls (N=101)**	**Adverse (N=14)**	**PTL (N=6)**	**Hypertension (N=6)**
- / -	110 (95.7%)	97 (96%)	13 (92.9%)	5 (83.3%)	6 (100%)
-/del	5 (4.3%)	4 (4%)	1 (7.1%)	1 (16.7%)	0
del/del	0	0	0	0	0
- allele	0.978	0.980	0.964	0.917	1.000
del allele	0.022	0.020	0.036	0.083	0.000
HWE	0.972	0.979	0.990	0.976	-

Sample size insufficient to perform association analysis ($P<0.05$).

3.1.3.3. Discussion

In the analysis of our data, a novel exonic variant, 221delT, was found to be associated with adverse pregnancy outcome and preterm labour in the total cohort and subsequently, restricted to the adverse pregnancy outcome in Coloured women. Although an association was demonstrated,

the variant was only identified in eight of 338 individuals (2.4%) in the controls and six of 83 (7.2%) women in the adverse pregnancy outcome group.

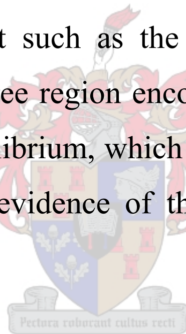
As previously mentioned, the primary strength of our study was the screening of a large cohort (N=421). Patients were recruited from a single institution, Paarl hospital, and diagnosed by a single clinician. This study is the first to report on the genetic screening of the *LGALS*13 gene. One limitation of our study includes the lack of corresponding PP13 levels measured during the course of the pregnancy. This would facilitate our understanding of whether certain variants may (i) be linked to an altered protein level and to (ii) confirm the feasibility of PP13 as an early biomarker for pregnancy-related complications (Than *et al*., 2005). The genomic regions encompassing the promoter and intronic sequences (by gene annotation) are thought to contain six SNPs, of which only three were identified in this study. The variants not identified in our study were relatively rare, with the exception of a single intronic variant found commonly in the European population (NCBI). The use of the Multiphor SSCP-HD mutation screening procedure utilised in our study has a reported sensitivity of up to 97% (Liechti-Gallati *et al*. 1999). The conventional use as SSCP-HD technique as the sole method for mutation screening could possibly have failed to detect variants due to its limited sensitivity. Furthermore, unidentified variants residing further upstream within the promoter and regulatory regions of gene, which have not yet been screened, could inevitably influence transcriptional efficiency of this gene. Consequently, all 5' and 3' regulatory regions should be included in future screening approaches.

The novel coding variant identified in exon three of the *LGALS*13 gene represents a deletion of a single thymine base at nucleotide position 221, resulting in the substitution of a leucine (L) with an tryptophan (W) residue at amino acid position 74. The result is a disruption of the open reading frame (ORF) leading to the production of an altered and truncated protein. The truncation removes 37 amino acids corresponding to *LGALS*13 exon three, which is regarded as vital for its function (Sammar *et al*., 2006).

The 221delT also removes Cys_{136} and Cys_{138} residues, important for sugar binding and dimerisation. Than *et al*., postulated if PP13 dimerises as galectin-1 and galectin-2 do, then the Cys_{136} residue may participate in dimerisation, whereas if PP13 dimerises more like galectin-7,

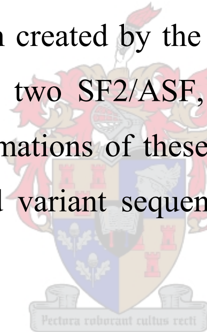
then dimerisation may involve Cys_{19}, Cys_{92} and Cys_{136}. The data described by Than *et al*., is not enough to establish the precise mechanism of dimerisation (Than *et al*., 2004a). The feature of dimerisation of disulphide bonds via cysteine residues may contribute to a change in oxygenation in the placenta (Than *et al*., 2004b). Interestingly, no homozygous variant genotypes were detected in the course of the study (involving >478 typings), possibly suggesting that delT homozygosity is incompatible with life. Further studies are required to elucidate the role of this frameshift mutation, by measuring the levels of PP13 in pregnancy of those individuals harbouring this mutation to determine whether it impacts on circulating PP13 levels. The delT homozygosity issue could be addressed by typing products of conception which can be collected following missed abortions (usually first trimester). The presence of delT homozygosity in such tissue would provide evidence for the severe impact of this variant on pregnancy maintenance and outcome.

It was interesting to identify a variant such as the delT, in a region that demonstrates (i) functional significance i.e., the exon three region encodes the carbohydrate recognition domain (CRD) of PP13 and, (ii) linkage disequilibrium, which signifies evolutionary conservation. This in itself may provide 'circumstantial' evidence of the potential negative impact of the delT variant.



Additional novel exonic variants identified include two non-synonymous variants in exon three (M44V, N=2 and K87R, N=1) and a silent mutation in exon four (P117P, N=1), all identified within individuals from the control cohort. In addition to that, Multiphor SSCP/HD analysis revealed an 11bp exonic region within which six bases are polymorphic (consequently, denoted as the 'hotspot'). Translation of the genomic region with substitutions of the 'hotspot' using BioEdit Sequence Alignment Editor (Isis Pharmaceutical, Carlsbad, California, USA), revealed three amino acids changes: T77N, T78L and D79H. No significant difference was observed in the genotype and allele frequencies in the control vs. adverse pregnancy outcome subgroups. The observed genotype frequency of the 'hotspot' in the combined Black and Coloured control group was 7.7% vs. 4.8% in the adverse outcome group. Although no association was demonstrated, the 'hotspot' appears to be more prevalent in healthy individuals, which could possibly underlie a protective effect.

SNPs occurring in the coding region of a gene resulting in amino acid substitutions may alter structure, function, receptor interaction and / or catalytic properties of a given protein. These mutations can also result in disruption of the open reading frame (ORF) causing aberrant translation that may lead to the production of short, very long or dysfunctional amino acid protein sequences (Orsi *et al*., 2007). According to SIFT (Sorting Intolerant From Tolerant, http://blocks.fhcrc.org/sift/SIFT.html) analysis on the above-mention exonic variants, all amino acid changes were tolerated and were predicted to not affect protein function. However, some mutations in the protein coding regions may inactivate an exonic splicing enhancer (ESE), resulting in an exon being skipped and consequently, having a dramatic effect on the protein product (Cartegni *et al*., 2003). All exonic variants examined by ESEfinder (http://rulai.cshl.edu/tools/ESE2/) were predicted to affect splicing by generating and / or eliminating putative ESE motifs. The M44V mutation abolished a SRp40 ESE motif, while the K87R and P117P mutations created new ESE motifs for the SF2/ASF protein, including an additional motif for the SRp40 protein created by the P117P mutation. ESE predictions for the mutated 'hotspot' sequence, removed two SF2/ASF, two SRp40, and a SRp55 motif, while creating a novel SRp40 motif. Confirmations of these predictions can be sought by testing the splicing capacity of the wildtype and variant sequences, using minigene constructs (Cooper, 2005).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Several intronic variants identified include a cluster of three variants in the 5' flanking region of exon 3 (IVS2-36 G/A (novel), IVS2-22 A/G and IVS2-15 G/A (novel)) and 1 in the 3' region (IVS3+72 T/A). Intronic variants may effect protein structure and function by (1) affecting transcription, (2) altering splicing or (3) impacting on the stability of mRNA and the relative amount and magnitude of isoforms that can possibly be pathogenic leading to physiological outcomes (Harrison and Weinberger, 2005). ASSP (Alternative Splice Site Predictor, http://es.embnet.org/~mwang/assp.html) analysis revealed that the cluster of variants identified in intron 2 did not appear to alter predictive splice sites. However, analysis of the IVS3+72 T/A showed that the acceptor site of the wildtype (T-allele) is abolished and a new site is created with the substitution of an A-allele, much earlier in the sequence. When a mutation abolishes a natural occurring splice site, the mechanism employed by the spliceosome selects the next best splice site in close proximity (Krawczak *et al*., 2007). Whether or not these intronic variants affect

splicing, should be addressed by comparing wildtype and variant mRNA transcripts using a minigene assay (Baralle *et al*., 2003).

An additional variant identified in the 5' promoter region of the *LGALS*13 gene is the -98 A/C variant [dbSNP: rs 3764843]. No association was demonstrated in the different subgroups. Although, subdivision of late-onset pre-eclamptic individuals (>37 weeks of gestation), revealed that there was a significant difference in the distribution of genotypes between controls and late onset pre-eclampsia, in both the total cohort (N=24, *P*=0.019) and Coloured individuals (N=19, *P*=0.038). However, these genotypes did not segregate under the assumptions of Hardy Weinberg equilibrium. Recruitment of additional late onset pre-eclamptic individuals are required to establish if there is a link between developing late onset pre-eclampsia and carriage of the -98 A-allele. Analysis using the Luciferase Reporter Assay is in progress to determine the effect of the -98 variant allele on promoter activity and gene expression. Furthermore, analysis should be extended to include screening sequences further upstream of exon one, to identify additional variants that may impact on transcriptional activity and gene regulation.

Most studies have relied on genetic susceptibility as the underlying cause of preterm labour. Studies focusing on the true genetic contribution to pregnancy complications are problematic, as these complex conditions involve multiple genes, non-Mendelian transmission, gene-environment and gene-gene interactions. The most frequently investigated genetic markers include point mutations in both the coding and non-coding regions (Orsi *et al*., 2007). These alterations or SNPs may help identify candidate genes or pinpoint regions that may have an important role in the etiology of preterm birth (Esplin, 2006). Candidate genes considered to influence susceptibility, with possible biological importance in pathogenesis, are of particular interest (Genc and Schantz-Dunn, 2007).

The most sensitive and accurate technique for mutation screening is direct sequencing. However, DNA sequencing applied to gene of interest is expensive, time-consuming and technically demanding (Gross *et al*., 1999). This study utilized Multiphor SSCP/HD mutation screening of promoter and intronic regions encompassing exonic sequences. This method has a reported sensitivity of 97.5% combining SSCP and HD analysis for increased detection. The method

described by Liechti-Gallati *et al*., extended the limitations of amplicons of up to 500-600bp in size, compared to conventional SSCP fragments sizes of ~300bp. The conformational structures of a given mutation are very sensitive to environmental influences such as (a) temperature, (b) gel composition, (c) ionic strength, and (d) additives. The parameters optimized in our study included resolving gels at varied temperatures (4-11°C) and keeping amplicon sizes under 300bp. The use of Multiphor SSCP/HD analysis in our study was found to be effective and fast.

An alternative method for mutation screening is Denaturing High Performance Liquid Chromatography (DHPLC). In a comparative analysis of SSCP vs. DHPLC, it was found that DHPLC was superior, with a detection rate of 92-100% (Rickard *et al*., 2001), with screening of fragment lengths up to ~1500bp (O'Donovan *et al*., 1998). Several advantages of DHPLC over conventional gel-based mutation techniques are that it is undemanding, with consistent results (Rickard *et al*., 2001), as well as simple evaluation of DHPLC results discriminating between single and multiple peaks (Gross *et al*., 1999) rather than assigning and grouping conformational patterns. One drawback is the purchase of a DHPLC apparatus, requiring low running costs and reducing sequencing preparations and reactions, making this technique more appropriate for mutation screening (Gross *et al*., 1999).

Investigation of PP13 in pregnancy pathologies has led to the identification of abnormal levels in various complications including pre-eclampsia, preterm delivery and IUGR, which contribute significantly to neonatal and maternal morbidity (Burger *et al*., 2004). It has been proposed that the PP13 molecule undergoes different cellular and molecular processing in pregnancies subsequently affected by pre-eclampsia, preterm delivery, HELLP syndrome (hemolysis, elevated liver enzymes and low platelets) and PIH (Sammar *et al*., 2005). The identification of biomarkers to predict and stratify maternal risk leading to adverse pregnancy outcome needs to be addressed by improving clinical trials for future research (Chafetz *et al*., 2007).

A variety of proteins and hormones has been studied as promising candidate markers for pregnancy outcome. These include uterine artery ultrasonography and maternal serum/urinary levels of human chorionic gonadotrophin (β-hCG), inhibin A, activin A, pregnancy-associated plasma protein A (PAPP-A), sex hormone-binding globulin, placental growth factor and soluble

fms-like tyrosine kinase 1. Nevertheless, early screening of various pregnancy complications is insufficient and disappointing at most, because these marker levels become elevated only in the second trimester, when clinical symptoms of the given disease have already manifested (Chafetz *et al*., 2007).

Burger *et al*., evaluated the use of PP13 as an initial biomarker for detecting various pregnancy complications by using a sensitive and reliable enzyme-linked immunosorbent assay (ELISA) test, with promising results (Burger *et al*., 2004). The use of this technique, being simple and non-invasive, can be routinely integrated into the clinical setting by measuring circulating levels at first booking and thereby predict via algorithms, those women at risk of developing adverse pregnancy outcomes (Chafetz *et al*., 2007).

Secretion of PP13 into maternal serum is regulated by specific binding of annexin II and β/γ actin (Than *et al*., 2004a). The binding of annexin II facilitates the formation of a so-called "molecular bridge" functioning at the endometrium at the time of implantation, while β/γ actin is involved in migration of trophoblasts to the placental interface (Chafetz *et al*., 2007). The alteration of PP13 levels could therefore be attributed to (i) dysfunctional synthesis or (ii) transport or (iii) impaired primary structure (Burger *et al*., 2004).

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Therefore, future studies should include mutation screening of annexin II and β/γ actin-encoding genes for potential genetic variants that may influence the transport and binding of microvesicles responsible for the release of PP13 into maternal circulation. Whether genetic variants in the annexin II and β/γ actin encoding genes alter their circulating protein levels that can either increase or decrease the binding of PP13 within the placenta, also needs to be addressed. Although the role of PP13 in pregnancy pathologies is not fully understood, research provides a functional basis that this molecule is involved in various biochemical and physiological processes associated with implantation, blood pressure regulation and tissue oxygenation (Burger *et al*., 2004).

In conclusion, we performed mutation detection in the *LGALS*13 gene of South African women with preterm labour and various other pregnancy complications. We identified three known and

seven novel genetic sequence variants, which will contribute to the NCBI genetic dataset, and our knowledge of PP13 as a potential biomarker for pregnancy complications.

3.1.4. Genotype: phenotype interaction

Significant association was demonstrated with three polymorphisms and gestation <37 weeks, and one variant with birth weight <2000g. The *IL*-10 -1082 A/G was significantly associated with adverse pregnancy outcome in the total cohort at both genotype and allele level, *P*=0.028 and *P*=0.008, respectively. The distribution of genotypes in the controls (≥37 weeks) was 53% (A/A), 39.7% (A/G) and 7.3% (G/G) compared to 75.8% (A/A) and 24.2% (A/G) in the adverse outcome group of the total cohort. After adjusting for ethnicity, association held in the Coloured group at allele level (*P*=0.035). The sample group for Blacks was N=6, with all individuals being homozygous for the A-allele. The homozygous (G/G) variant genotype was restricted to controls. Furthermore, the *IL*-10 -1082 A/G variant was associated (*P*=0.05) with a low birth weight (<2000g) in the Coloured group, with genotype frequency distributions of 67.9% (A/A) and 32.1% (A/G) versus 50.4% (A/A), 42.6% (A/G) and 7% (G/G) in controls (≥2000g).

The coding variant, 221delT of the *LGALS*13 gene, was association with lower gestation in both the total cohort (*P*=0.048) and Coloured patients (*P*=0.006). Additionally, the *IL*-4 -590C/T locus differed significantly (*P*=0.035) in the dominant model (heterozygous C/T and homozygous variant T/T versus homozygous wildtype C/C) between gestation <37 weeks and the total cohort.

Information presented and the remaining loci screened in this study for genotype-phenotype interactions are in Appendix 11.

4. Concluding remarks

In the assessment of immunological gene variants, we found an association between the *IL*-1*RN* 86 bp repeat, the *TNF*α -308 G/A polymorphism and pregnancy complications. Additional screening of the *LGALS*13 gene revealed an association with 221delT/L74W. The question however, remains as to whether identifying these in early pregnancy would make a difference in pregnancy outcome.

Firstly, most patients 'book late' (first trimester) at obstetrics clinics. Many of them are in the second or third trimester of pregnancy and have already started developing symptoms of complications; their fetuses are growth restricted, etc. The identification of a specific genotype that places the woman in a "high-risk" category of pregnancy complication in these cases has very little value. In addition, while late booking may not impact on genotyping i.e. genotypes are invariant and not influenced by the pregnancy state; it *does influence* the issues of clinical management and intervention. Critical decisions regarding fetal management (e.g., administering corticosteroids for lung maturation) often rest on accurate gestational dates. These can be determined accurately by early (first trimester) ultrasonography. In addition, early ultrasonography can facilitate the early detection of fetal anomalies – mild and severe.

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A recommendation would be to educate women to register at obstetrics units or with their care-givers *early in pregnancy*. During this time, clinicians or nurses can inform the patient of the following:

1. Possible tests, which include, among others,
   a. Ultrasonography to establish gestation, exclude multiple and abnormal pregnancies
   b. Amniocentesis for advanced maternal age
2. The effect of possible tetratogens
   a. Such as alcohol, smoking and drug abuse.
3. Managing maternal disease during pregnancy
   a. Such as hypertension, diabetes and epilepsy, etc.

4. Self-monitoring of their pregnancy

    a. Women identified as "high-risk cases" could be provided with detailed information about the relevant condition so that they can look out for symptoms; some which they could manage themselves (e.g., bed-rest), and others, which would require immediate hospitalisation.

The feasibility of offering an effective genetic screening test to pregnant women should also be considered. Such a test would involve patient recruitment and counseling, DNA extraction, PCR amplification, REA and / or gel electrophoresis, together with manual labour cost and specialised equipment. It is likely to be quite costly, especially if every pregnant woman were to be screened. However, since primigravid status appeared to be a major risk factor for complications in our study, such a test could be implemented and provisionally limited to young, primgravid women.

The current advances in technology could contribute to the development of a hybridisation 'strip' or "personalised" chip including variants such as *IL*-1*RN*, *TNF*α –308 and 221delT, in an attempt to reduce screening costs. However, before this can be realised, the 'provisional' data presented in this thesis needs to be confirmed or refuted in a larger cohort. It is recommended that the project be continued, but with the following inclusions:

1. Measurement of levels of ILs, TNFα and PP13 by ELISA (maternal and fetal)
2. Cord blood (for fetal DNA and RNA, serum and plasma)
3. Infection 'profile' (maternal)

Regarding the size of this proposed extended study: we should aim to increase the population cohort (especially Black women) and design the study based on:

1. Genetic power calculations
    a. At 80% power with 5% significance.
2. Population stratification
    a. By matching patients to controls

b. Appropriate statistical methods to account for population structure

This is important because these two population groups are very distinct at a genomic level due to ethnic specific distribution of genotype and allele frequencies. Thus, markers demonstrating an association in one population may not be representative in another.

Another aspect to address in this study is the apparent healthier pregnancy status of Black women. The Paarl Hospital, obstetric unit, initially served largely Coloured individuals, but recently more Black women have started to use this facility. The observation in our study that Black women showed a relatively good pregnancy outcome is interesting. Why is this?

The overall incidence of adverse pregnancy outcome observed in this study is largely attributed to by the Coloured population, which raises questions as to why. Is this trend due to pure environmental and cultural factors i.e. alcohol, smoking and diet or is there an underlying genetic component together with a changing environment?

The observations that preterm labour and pre-eclampsia in Coloured women are the prevailing complications at Paarl Hospital will help determine whether the necessary facilities are in place to deal with these complications. The findings of our study will be reported back to Paarl Hospital as well as the Department of Health for comparison with previous statistics, to determine if there was an incline or decline in pregnancy complications and to establish if the current hospital care needs to be upgraded. The epidemiological information from this study should be made available to patients via clinicians or nurses, to inform them about possible risk factors, so that these women can adjust their lifestyle (and thereby improve their health and that of their baby).

The complexity of preterm labour involves the relationship between multiple etiologies and risk factors. Epidemiological studies should implement a multidisciplinary approach combining sociology and genetics to understand the complex nature of pregnancy pathologies. Approaches which involve the evaluation of single or limited candidate markers should be restricted since it

is unlikely that single a gene variant or risk factor underlies the development of preterm labour (Esplin, 2006).

Screening of multiple candidate markers with appropriate statistical methods to evaluate the underlying gene-gene and gene-environmental interactions holds great promise with increased power to detect the biological effects related to preterm labour and other pregnancy conditions. The investigation of multiple genes will help determine an underlying common pathway or function that may contribute to the development of preterm labour. PP13 for example interacts with annexin II and β/γ actin (Than *et al*., 2004a) - future investigations should include mutation screening and full characterisation of annexin II and β/γ actin. Recent research has identified a common promoter haplotype in the annexin A5 gene, which is similar to annexin II. It has been demonstrated that the annexin A5 haplotype impacts negatively on recurrent pregnancy loss (Bogdanova *et al*., 2007). Future investigations could include screening of this annexin A5 haplotype in other pregnancy complications such as preterm labour and pre-eclampsia.

An aspect not covered effectively in this project is the presence of infection. Evidence suggests that infection during pregnancy in the 1ste and 2nd trimester is commonly associated with fetal loss, chorioamnionitis, preterm labour, PPROM, prematurity, LBW infants and neonatal/maternal infectious morbidity (Genc and Schantz-Dunn, 2007). Future studies are warranted in our population to investigate the infectious status of women. This could be incorporated into the routine screenings during clinical visits. Maternal inflammatory response to microbial agents is said to influence most cases of preterm birth and future analysis should focus on the evaluating the influences of gene-environment interaction (Genc and Schantz-Dunn, 2007).

The hope of incorporating genetic marker(s) in routine screening depends on the careful design of future studies. In addition to strengthening our understanding of the pathophysiology underlying pregnancy complications such as preterm labour, much of this knowledge could be used to identify and develop effective treatment strategies and lead to a reduction in maternal and fetal morbidity and mortality.

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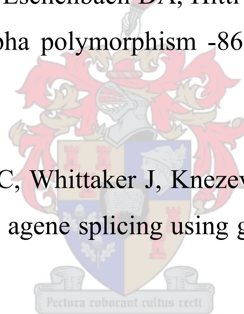

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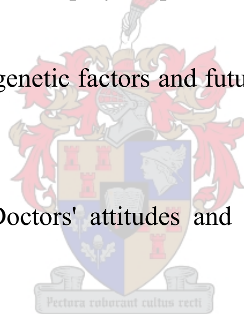

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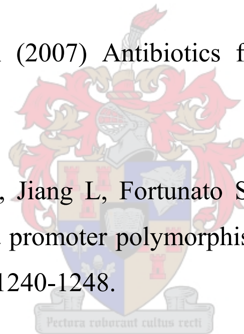

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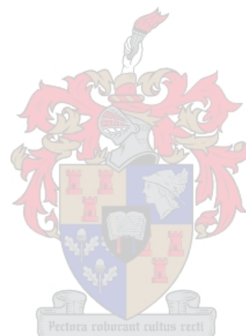

6. Appendices

Appendix 1: Ethical and Institutional consent

UNIVERSITEIT-STELLENBOSCH-UNIVERSITY
jou kennisvennoot • your knowledge partner

4 Maart 2005

Dr GS Gebhardt
Departement Verloskunde en Ginekologie

Geagte dr Gebhardt

NAVORSINGSPROJEK: "TUMOUR NECROSIS FACTOR A GENE POLYMORPHISM *TNF-1* AND PRE-TERM LABOUR: RISK ASSESSMENT IN THE WESTERN CAPE"
PROJEKNOMMER : N04/09/147

U aansoek om registrasie en goedkeuring van bogenoemde projek het op 6 Oktober 2005 voor die Komitee vir Mensnavorsing gedien. Die Komitee het in beginsel die projek goedgekeur, maar versoek dat verdere inligting voorsien word.

Hierdie inligting is verskaf en die projek is finaal goedgekeur op 2 Maart 2005. 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 PRWK nie navorsing finansieël ondersteun nie.

Die verpleegkorps van die Tygerberg-hospitaal kan ook nie omvattende verpleeghulp 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

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Afdeling Navorsingsontwikkeling en -steun • Division of Research Development and Support
Posbus/PO Box 19063 • Tygerberg 7505 • Suid-Afrika/South Africa
Tel: +27 21 938 9207 • Faks/Fax: +27 21 933 6330
E-pos/E-mail: cjvt@sun.ac.za

Appendix 2: Patient consent form

I. I HEREBY CONSENT VOLUNTARILY TO PARTICIPATE IN THIS STUDY:

Signed/*Confirmed at ………………………………… on …………………………….20……
(place) *(date)*

……………………………………………………… ……………………………………………
Signature or right thumb print 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)*
- She was encouraged and afforded adequate time to ask me any questions;
- this conversation was conducted in English ……………………………… and no translator was used
- Signed at ……………………………………………… on ………………………………20 ……
  (place) *(date)*

…………………………………………………………… ……………………………………………
Signature of investigator/representative of investigator *Signature of witness*

IMPORTANT MESSAGE TO PARTICIPANT

Dear participant

Thank you very much for your 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 Dr GS Gebhardt at 87 21 711 (all hours) or come to the labour ward on the second floor, Paarl Hospital.

***Delete where not applicable**

Appendix 3: Informed consent for DNA analysis and storage

FACULTY OF HEALTH SCIENCES

STELLENBOSCH UNIVERSITY

INFORMATION AND INFORMED CONSENT DOCUMENT FOR DNA ANALYSIS AND STORAGE

TITLE OF THE RESEARCH PROJECT: Tumour Necrosis Factor α gene polymorphism *TNF-2* and pre-term labour: risk assésssment in the Western Cape.

REFERENCE NUMBER: ……………………………………………………………………

PRINCIPAL INVESTIGATOR: Drs R Hillermann & GS Gebhardt

Address: Departments of Obstetrics and Gynaecology and Genetics, University of Stellenbosch, Tygerberg and Paarl Hospital

DECLARATION BY OR ON BEHALF OF PARTICIPANT:

I, THE UNDERSIGNED, …………………………………………………………………… *(name)*

[ID No: ……………………] the participant

of ……………………………………………………………………………………………… *(address)*.

A. HEREBY CONFIRM AS FOLLOWS:

1. I was invited to participate in the above-mentioned research project of the Departments of Obstetrics and Gynaecology and Genetics, Faculty of Health Sciences, University of Stellenbosch.

2. The following aspects have been explained to me

   Aim: Delivery before 8½ months (37 weeks)TNF can be hazardous to the baby, especially before 34 weeks (7½ months). About 20% of women with their first pregnancy will deliver before 37 weeks, due to unclear reasons. We aim with this study to see if we can identify a marker from your blood that may help us to identify this problem in time. Although this study will not help you in this pregnancy, it may be of value in your next pregnancy or in other women's pregnancies.

***Delete where not applicable**

2.12.2 **Procedures:** I will be requested to provide information about my medical history. Blood (10*ml*, about 2 teaspoonfuls) will be collected from me at booking and again at delivery.

2.3 **Genetic considerations:**

- The DNA may be stored for several years until the technology for meaningful analysis becomes available;
- The clinically relevant results of the (possible) analyses carried out on this material in the current study can be made known to me at my request and in accordance with the relevant protocol, if and when it becomes available. In addition, I authorise the investigator(s) to make the information available to ……………………………………… *(doctor's name),* the doctor involved in my care, as well as to the following family members ………………………………………… ………………………………………………………………………………… *(names);*
- The DNA will be maintained indefinitely, unless I request(s) to have it and/*or the stored clinical data destroyed by contacting the investigator conducting the present study, dr GS Gebhardt at 938 4707 or the Chairperson of the Research Subcommittee C/Ethics Committee at 9389111 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 identity will be kept confidential throughout. Information will not be associated with my 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 by name.

***Delete where not applicable**

2.5 **Voluntary participation:** Participation is voluntary and I may decline participation, or withdraw from the study at any time without any loss of benefits to which I am 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 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 participation is an indication of a higher risk of a genetic disease which could hurt my access to health or other insurance, no information about me or my 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 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 or my heirs will not receive any compensation, but profits will be reinvested into supporting the cause of further research which may bring benefits to my family and to the community, such as health screening, medical treatment, educational promotions, etc;

2.8 **Permission for further studies:** Before my genetic material is used in further projects in the future, the written approval of the Research

***Delete where not applicable**

Subcommittee C/Ethics Committee, Faculty of Health Sciences, will be obtained.

3. The information conveyed above was explained to me by .... *(name)* in Afrikaans and I am fluent in this language

4. I was afforded adequate time to pose any questions and all questions were answered to my full satisfaction.

5. I was not pressurized to participate.

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

7. I will not incur any additional costs through participation.

8. I have received a copy of this document for my 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.

***Delete where not applicable**

Appendix 4: DNA extraction method

Extraction buffers and chemicals used:

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

Proteinase K (store at -20°C)

10mg/mL dissolved in dH_2O, aliquot in 1.5mL Eppendorf tubes

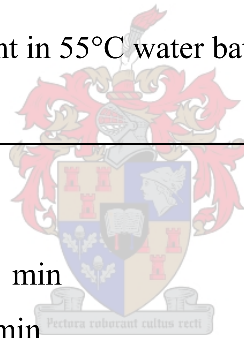
NaCl (500mL, saturated solution)

175.32g	NaCl	6M

Genomic DNA extractions from blood:

Day 1:

- Place ~10mL blood in a 50mL Falcon tube
- Add 30mL Lysis Buffer
- Place on ice for 15 min, shaking each 5 min interval
- Centrifuge @ 1500rpm for 10 min
- Poor off supernatant, keeping pellet
- Add 10mL PBS and mix
- Centrifuge @ 1500rpm for 10 min
- Pour off supernatant, keeping pellet
- Dissolve pellet in: 30mL Cell Lysis Buffer
  30µL Proteinase K (10mg/mL)
  300µL 10% SDS
- Mix well and incubate overnight in 55°C water bath


Pectora roborant cultus recti

Day 2:

- Add 1mL NaCl and shake for 1 min
- Centrifuge @ 3500rpm for 30 min
- Transfer supernatant to new tube and shake for 15sec
- Centrifuge @ 2500rpm for 15 min
- Transfer supernatant to new tube without foam or pellet
- Add volumes ice cold 100% ethanol to precipitate DNA
- Scoop DNA and place in Eppendorf tube with 500µL ethanol
- Centrifuge @ 14 000rpm for 10 min @ 4°C
- Dissolve pellet in 200-800µL ddH_2O/TE buffer depending on pellet size

Appendix 5: NCBI *LGALS*13 annotated sequence

Annotated sequence file:

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DEFINITION  Homo sapiens chromosome 19 NCBI35 partial sequence
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ACCESSION   chromosome:NCBI35:19:44780004:44794955:1
VERSION     chromosome:NCBI35:19:44780004:44794955:1
KEYWORDS    .
SOURCE      Human
  ORGANISM  Homo sapiens
            Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
            Mammalia; Eutheria; Euarchontoglires; Primates; Catarrhini;
            Hominidae; Homo.
COMMENT     This sequence was annotated by the Ensembl system. Please visit
the
            Ensembl web site, http://www.ensembl.org/ for more information.
COMMENT     All feature locations are relative to the first (5') base of the
            sequence in this file.  The sequence presented is always the
            forward strand of the assembly. Features that lie outside of the
            sequence contained in this file have clonal location coordinates
in
            the format: .:..
COMMENT     The /gene indicates a unique id for a gene,
            /note="transcript_id=..." a unique id for a transcript,
/protein_id
            a unique id for a peptide and note="exon_id=..." a unique id for
an
            exon. These ids are maintained wherever possible between
versions.
COMMENT     All the exons and transcripts in Ensembl are confirmed by
            similarity to either protein or cDNA sequences.
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Pectora roborant cultus recti

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8161-9300

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stop, (9829)(c/g)(dbSNP:rs1801654)
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10261-14952

Appendix 6: Multiphor SSCP/HD protocol

Multiphor Solutions:

40% acrylamide PDA solution: 1L

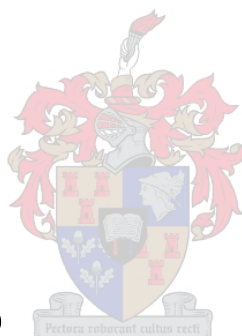
396g acrylamide
4g Piperazine diacrylamide
bring to volume (1L) with dH_2O (keep in foil in fridge)

0,75M TRIS- Formate Buffer: 1L

90.8g TRIS
600ml dH_2O (dissolve)
pH = 9.0 with formic acid
bring to volume (1L) 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)
pH = 9.0
50µL Bromophenol Blue (4% solution)
bring to volume (1L) with dH_2O

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 (50mL) with dH_2O

10% APS: 1.5mL

0.15g APS
bring to volume (1.5mL) with dH_2O

10% acetic acid (plate glue):

200µL Trimethoxysily
50 mL 100% EtOH

Gel mix: X10

53mL 40% acrylamide-PDA
85mL Tris-Formate Buffer
30mL 41% Glycerol
keep in foil in fridge

Per 1 Gel:

15ml Gel Mix
150µL 10% APS
15µL TEMED

Silver Staining solutions:

Solution I: **1L**

0.1% silver nitrate: 1g silver nitrate
1L dH_2O
Max. 2 days in the dark

15% Formaldehyde: 10mL (must be fresh each time!)

3.75mL 40% Formaldehyde stock
6.25mL dH_2O

Solution II: 1L

15g NaOH
1L dH_2O
10mL 15% Formaldehyde (FRESH!)

Multiphor Protocol:

Gel Plates preparation: do everything in quick succession

- Wash plates 4X with ethanol
- Every 4 runs clean plate with blocks with gelslick (80μL) especially over blocks
- Put 80μL plateglue and 8μL 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 (all on ice)
- 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 at 355V

Visualization of bands:

- remove gel from apparatus
- rinse X2 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 7: DNA purification protocol

Bioline SureClean ® (Bioline, UK)

Column-free protocol:

- Add an equal volume of Bioline SureClean to the amplified nucleic acid sample and incubate at room temperature for 10 min
- Centrifuge at 13 000rpm for 10 min and remove supernatant
- Add 100µL of 70% ethanol and vortex for 30sec
- Centrifuge at 13 000rpm for 10 min, remove supernatant and air-dry pellet
- Resuspend in Nuclease-free water (starting volume)

Appendix 8: The remaining immunological gene variants screened in this study

***TNF*α -238 G/A**

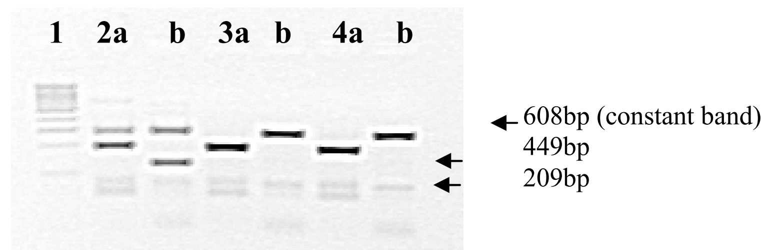


Figure 18: Agarose gel (2%) demonstrating the three genotypes of the *TNF*α -238 polymorphism detected by ARMS PCR (Yamamoto-Furusho *et al*., 2004). Lane 1 represents a 100 bp ladder (Fermentas). Lanes 2a and b represent a heterozygous genotype (209bp and 449 bp), Lanes 3a&b and 4a&b represents homozygosity for the *TNF*α-238 G-allele (449bp).

Table 22: Genotype and allele frequencies of the ***TNFα* -238 G/A** polymorphism for the total **cohort**. Hardy Weinberg equilibrium (HWE) was determined for each individual group. Statistical analysis was performed using Fisher's exact test analog to determine associations at both genotype and allele level. The corresponding *P* values are shown at the bottom of the table.

Total cohort (N=421)					
Genotype	**Total (N=421)**	**Controls (N=338)**	**Adverse (N=83)**	**PTL (N=33)**	**Hypertension (N=35)**
G/G	315 (74.8%)	258 (76.3%)	57 (68.7%)	24 (72.7%)	23 (65.7%)
G/A	106 (25.2%)	80 (23.7%)	26 (31.3%)	9 (27.3%)	12 (33.4%)
A/A	0	0	0	0	0
G-allele	0.874	0.882	0.843	0.864	0.829
A-allele	0.126	0.118	0.157	0.136	0.171
HWE	**0.0127**	**0.048**	0.436	0.663	0.473

Total cohort and controls did not reach HWE significance (*P*<0.05). Controls vs. Adverse: *P*=0.183 (allele); Controls vs. Preterm labour: *P*=0.667 (allele); Controls vs. Hypertension: *P*=0.199 (allele).

Table 23: Genotype and allele frequencies of the ***TNFα* -238 G/A** polymorphism in the **Coloured** population. Hardy Weinberg equilibrium (HWE) was determined for each individual group. Statistical analysis was performed using Fisher's exact test analog to determine associations at both genotype and allele level. The corresponding *P* values are shown at the bottom of the table.

Coloured Females (N=306)					
Genotype	**Total (N=306)**	**Controls (N=237)**	**Adverse (N=69)**	**PTL (N=27)**	**Hypertension (N=29)**
G/G	217 (70.9%)	172 (72.6%)	45 (65.2%)	19 (70.4%)	18 (62.1%)
G/A	89 (29.8%)	65 (27.4%)	24 (34.8%)	8 (29.6%)	11 (37.9%)
A/A	0	0	0	0	0
G-allele	0.855	0.863	0.826	0.852	0.810
A-allele	0.145	0.137	0.174	0.148	0.190
HWE	**0.012**	0.050	0.217	0.665	0.452

Pectora roborant cultus recti

Controls vs. Adverse: *P*=0.281 (allele); Controls vs. Preterm labour: *P*=0.824 (allele); Controls vs. Hypertension: *P*=0.281 (allele).

Table 24: Genotype and allele frequencies of the ***TNFα* -238 G/A** polymorphism in the **Black** population. Hardy Weinberg equilibrium (HWE) was determined for each individual group.

Black Females (N=115)					
Genotype	**Total (N=115)**	**Controls (N=101)**	**Adverse (N=14)**	**PTL (N=6)**	**Hypertension (N=6)**
G/G	71 (61.7%)	86 (85.1%)	12 (85.7%)	5 (83.3%)	5 (83.3%)
G/A	44 (38.3%)	37 (14.9%)	2 (14.3%)	1 (16.7%)	1 (16.7%)
A/A	0	0	0	0	0
G-allele	0.809	0.926	0.929	0.917	0.917
A-allele	0.191	0.074	0.071	0.083	0.083
HWE	**0.004**	0.723	0.959	0.976	0.976

Sample size insufficient to perform association analysis (*P*<0.05).

*TNF*α +488 G/A

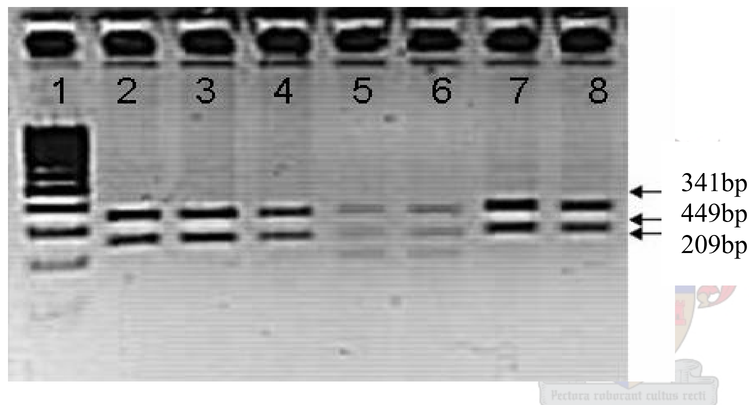


Figure 19: Agarose gel (2%) demonstrating the three genotypes of the *TNF*α +488 G/A polymorphism, detected by restriction enzyme analysis with *Nla*III (37˚C). Lane 1 is a 100 bp ladder (New England Biolabs). Lanes 2-4 and 7-8 represents the G/G genotype at 341 and 247 bp. Lanes 5-6 the heterozygous genotype (G/A) at 341, 247 and 160 bp.

Table 25: Genotype and allele frequencies of the ***TNFα* +488 G/A** polymorphism for the total **cohort**. Hardy Weinberg equilibrium (HWE) was determined for each individual group. Statistical analysis was performed using Fisher's exact test analog to determine associations at both genotype and allele level. The corresponding *P* values are shown at the bottom of the table.

Total Group (N=403)					
Genotype	**Total (N=403)**	**Controls (N=327)**	**Adverse (N=76)**	**PTL (N=30)**	**Hypertension (N=32)**
G/G	369 (91.6%)	296 (90.5%)	73 (96%)	30	29 (90.6%)
G/A	34 (8.4%)	31 (9.5%)	3 (4%)	0	3 (9.4%)
A/A	0	0	0	0	0
G-allele	0.958	0.953	0.976	1.000	0.953
A-allele	0.042	0.047	0.024	0.000	0.047
HWE	0.677	0.667	0.9814	-	0.962

Controls vs. Adverse: *P*=0.126 (allele); Controls vs. Preterm labour: *P*=0.085 (allele); Controls vs. Hypertension: *P*=0.986 (allele).

Table 26: Genotype and allele frequencies of the ***TNFα* +488 G/A** polymorphism in the **Coloured** population. Hardy Weinberg equilibrium (HWE) was determined for each individual group. Statistical analysis was performed using Fisher's exact test analog to determine associations at both genotype and allele level. The corresponding *P* values are shown at the bottom of the table.

			Coloured Females (N=306)		
Genotype	**Total (N=291)**	**Controls (N=228)**	**Adverse (N=63)**	**PTL (N=25)**	**Hypertension (N=26)**
G/G	262 (90.0%)	202 (88.6%)	60 (95.2%)	25	23 (88.5%)
G/A	29 (10.0%)	26 (11.4%)	3 (4.8%)	0	3 (11.5%)
A/A	0	0	0	0	0
G-allele	0.950	0.943	0.976	1.000	0.942
A-allele	0.050	0.057	0.024	0.000	0.058
HWE	0.6702	0.659	0.9814	-	0.952

Controls vs. Adverse: *P*=0.129 (allele); Controls vs. Preterm labour: *P*=0.083 (allele); Controls vs. Hypertension: *P*=0.984 (allele).

Table 27: Genotype and allele frequencies of the ***TNFα* +488 G/A** polymorphism in the **Black** population. Hardy Weinberg equilibrium (HWE) was determined for each individual group.

			Black Females (N=112)		
Genotype	**Total (N=112)**	**Controls (N=99)**	**Adverse (N=13)**	**PTL (N=5)**	**Hypertension (N=6)**
G/G	107 (61.7%)	94 (94.9%)	13	5	6
G/A	5 (38.3%)	5 (5.1%)	0	0	0
A/A	0	0	0	0	0
G-allele	0.978	0.975	1.000	1.000	1.000
A-allele	0.022	0.025	0.000	0.000	0.000
HWE	0.9712	0.967	-	-	-

Sample size insufficient to perform association analysis (*P*<0.05).

IL-10 -1082 A/G

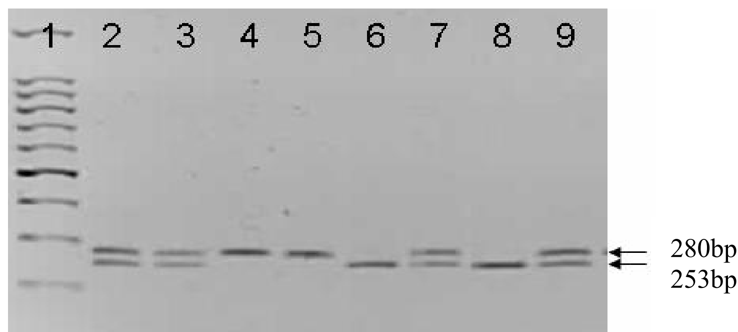


Figure 20: Agarose gel (2%) demonstrating the three genotypes of the *IL*-10 -1082 A/G polymorphism, detected by restriction enzyme analysis with *Eco*NI (37˚C). Lane 1 is a 100 bp ladder (Fermentas). Lanes

4 and 5 illustrates the A/A genotype at 280 bp and lanes 6 and 8 the G/G genotype at 253 bp. Lanes 2, 3, 7 and 9 represent the heterozygous genotype.

Table 28: Genotype and allele frequencies of the **IL-10 -1082 A/G** polymorphism for the total **cohort**. Hardy Weinberg equilibrium (HWE) was determined for each individual group. Statistical analysis was performed using Fisher's exact test analog to determine associations at both genotype and allele level. The corresponding *P* values are shown at the bottom of the table.

Total Cohort (N=421)					
Genotype	**Total (N=421)**	**Controls (N=338)**	**Adverse (N=83)**	**PTL (N=33)**	**Hypertension (N=35)**
A/A	228 (54.2%)	178 (52.7%)	50 (60.2%)	21 (63.6%)	19 (54.3%)
A/G	166 (39.4%)	135 (39.9%)	31 (37.2%)	11 (33.3%)	15 (42.9%)
G/G	27 (6.4%)	25 (7.4%)	2 (2.4%)	1 (3.0%)	1 (2.8%)
A-allele	0.739	0.726	0.789	0.803	0.757
G-allele	0.261	0.274	0.211	0.197	0.243
HWE	0.908	0.996	0.5373	0.954	0.620

Controls vs. Adverse: *P*=0.182 (genotype) and *P*=0.099 (allele); Controls vs. Preterm labour: *P*=0.401 (genotype) and *P*=0.179 (allele); Controls vs. Hypertension: *P*=0.600 (genotype) and *P*=0.581 (allele).

Table 29: Genotype and allele frequencies of the ***IL*-10 -1082 A/G** polymorphism in the **Coloured** population. Hardy Weinberg equilibrium (HWE) was determined for each individual group. Statistical analysis was performed using Fisher's exact test analog to determine associations at both genotype and allele level. The corresponding *P* values are shown at the bottom of the table.

Coloured Females (N=306)					
Genotype	**Total (N=306)**	**Controls (N=237)**	**Adverse (N=69)**	**PTL (N=27)**	**Hypertension (N=29)**
A/A	158 (51.6%)	118 (49.8%)	40 (58%)	17 (63%)	15 (51.7%)
A/G	130 (42.5%)	103 (43.4%)	27 (39.1%)	9 (33.3%)	13 (44.8%)
G/G	18 (5.9%)	16 (6.8%)	2 (2.9%)	1 (3.7%)	1 (3.5%)
A-allele	0.729	0.715	0.775	0.796	0.741
G-allele	0.271	0.285	0.225	0.204	0.259
HWE	0.4267	0.590	0.592	0.990	0.661

Controls vs. Adverse: *P*=0.320 (genotype) and *P*=0.162 (allele); Controls vs. Preterm labour: *P*=0.415 (genotype) and *P*=0.207 (allele); Controls vs. Hypertension: *P*=0.790 (genotype) and *P*=0.676 (allele).

Table 30: Genotype and allele frequencies of the ***IL*-10 -1082 A/G** polymorphism in the **Black** population. Hardy Weinberg equilibrium (HWE) was determined for each individual group.

Black Females (N=115)					
Genotype	**Total (N=115)**	**Controls (N=101)**	**Adverse (N=14)**	**PTL (N=6)**	**Hypertension (N=6)**
A/A	70 (60.9%)	60 (59.4%)	10 (71.4%)	4 (66.7%)	4 (66.7%)
A/G	36 (31.3%)	32 (31.7%)	4 (28.6%)	2 (33.3%)	2 (33.3%)
G/G	9 (7.8%)	9 (8.9%)	0	0	0
A-allele	0.765	0.752	0.857	0.833	0.833
G-allele	0.235	0.248	0.143	0.167	0.167
HWE	0.385	0.324	0.823	0.887	0.887

Sample size insufficient to perform association analysis (*P*<0.05).

***IL*-10 -819 C/T /-592 C/A (Complete linkage disequilibrium)**
Results on: *IL*-10 -592 C/A

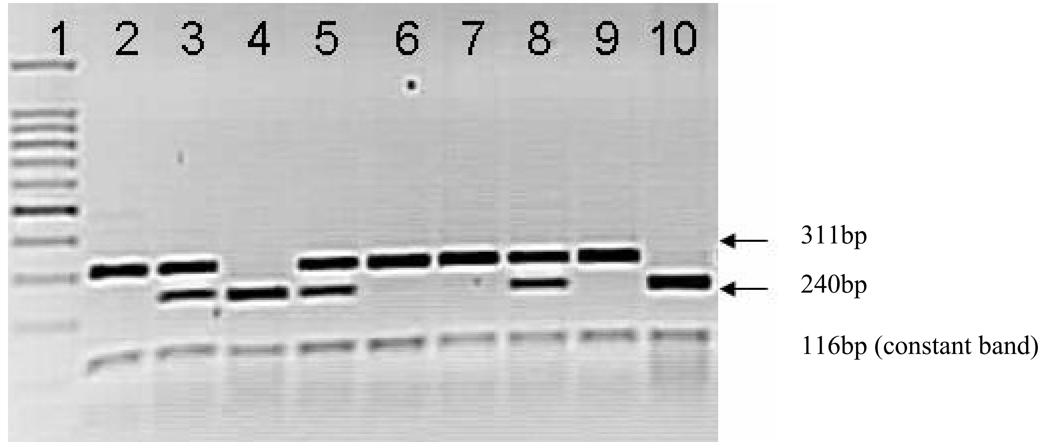


Figure 21: Agarose gel (2%) demonstrating the three genotypes of the *IL*-10 -592 C/A polymorphism, detected by restriction enzyme analysis with *Rsa*I (37˚C). Lane 1 is a 100 bp ladder (Fermentas). Lanes 2, 6, 7 and 9 illustrates the C/C genotype at 311 bp and lanes 3, 5 and 8 the A/A genotype 4 and 10 at 240 bp. Lanes 4 and 10 represent the heterozygous genotype.

Table 31: Genotype and allele frequencies of the **IL-10 -592 C/A** polymorphism for the total **cohort**. Hardy Weinberg equilibrium (HWE) was determined for each individual group. Statistical analysis was performed using Fisher's exact test analog to determine associations at both genotype and allele level. The corresponding *P* values are shown at the bottom of the table.

Total Group (N=421)					
Genotype	**Total (N=421)**	**Controls (N=338)**	**Adverse (N=83)**	**PTL (N=33)**	**Hypertension (N=35)**
C/C	151 (36.1%)	126 (37.3%)	25 (31.3%)	9 (27.3%)	11 (31%)
C/A	228 (53.9%)	176 (51.1%)	52 (61.5%)	22 (66.7%)	20 (57%)
A/A	42 (9.9%)	36 (10.6%)	6 (7.2%)	2 (6%)	4 (11%)
C-allele	0.629	0.633	0.614	0.606	0.600
A-allele	0.371	0.367	0.386	0.394	0.400
HWE	0.004	0.085	0.0134	0.075	0.530

Controls vs. Adverse: *P*=0.211 (genotype) and *P*=0.655 (allele); Controls vs. Preterm labour: *P*=0.266 (genotype) and *P*=0.664 (allele); Controls vs. Hypertension: *P*=0.791 (genotype) and *P*=0.585 (allele).

Table 32: Genotype and allele frequencies of the **IL-10 -592 C/A** polymorphism in the **Coloured** population. Hardy Weinberg Equilibrium (HWE) was determined for each individual group. Statistical analysis was performed using Fisher's exact test analog to determine associations at both genotype and allele level. The corresponding *P* values are shown at the bottom of the table.

Coloured Females (N=306)					
Genotype	**Total (N=306)**	**Controls (N=237)**	**Adverse (N=69)**	**PTL (N=27)**	**Hypertension (N=29)**
C/C	107 (35%)	87 (36.7%)	20 (29%)	7 (25.9%)	9 (31%)
C/A	168 (54.9%)	124 (52.3%)	44 (63.8%)	18 (66.7%)	17 (59%)
A/A	31 (10.1%)	26 (11%)	5 (7.2%)	2 (7.4%)	3 (10%)
C-allele	0.624	0.629	0.609	0.593	0.603
A-allele	0.376	0.371	0.391	0.407	0.397
HWE	0.012	0.178	0.019	0.141	0.480

Controls vs. Adverse: *P*=0.232 (genotype) and *P*=0.670 (allele); Controls vs. Preterm labour: *P*=0.366 (genotype) and *P*=0.604 (allele); Controls vs. Hypertension: *P*=0.805 (genotype) and *P*=0.708 (allele).

Table 33: Genotype and allele frequencies of the ***IL*-10 -592 C/A** polymorphism in the **Black** population. Hardy Weinberg equilibrium (HWE) was determined for each individual group

Black Females (N=115)					
Genotype	**Total (N=115)**	**Controls (N=101)**	**Adverse (N=14)**	**PTL (N=6)**	**Hypertension (N=6)**
C/C	44 (38.3%)	39 (38.6%)	5 (36%)	2 (33.3%)	2 (33.3%)
C/A	60 (52.2%)	52 (51.5%)	8 (57%)	4 (66.7%)	3 (50%)
A/A	11 (9.6%)	10 (9.9%)	1 (7%)	0	1 (16.7%)
C-allele	0.643	0.644	0.643	0.667	0.582
A-allele	0.357	0.356	0.357	0.333	0.417
HWE	0.339	0.470	0.658	0.472	0.998

Sample size insufficient to perform association analysis (*P*<0.05).

Pectora roborant cultus recti

IL-1β +3954 C/T

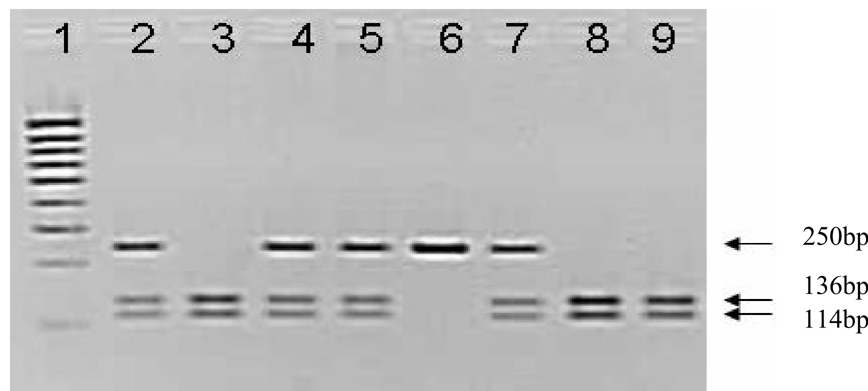


Figure 22: Agarose gel (2%) demonstrating the three genotypes of the *IL*-1β +3954 C/T polymorphism, detected by restriction enzyme analysis with *Taq*αI (37˚C). Lane 1 is a 100 bp ladder (Fermentas). Lanes 3, 8 and 9 illustrates the C/C genotype at 114 and 136 bp and lane 6 the T/T genotype at 250 bp. Lanes 2, 4, 5 and 7 represent the heterozygous genotype.

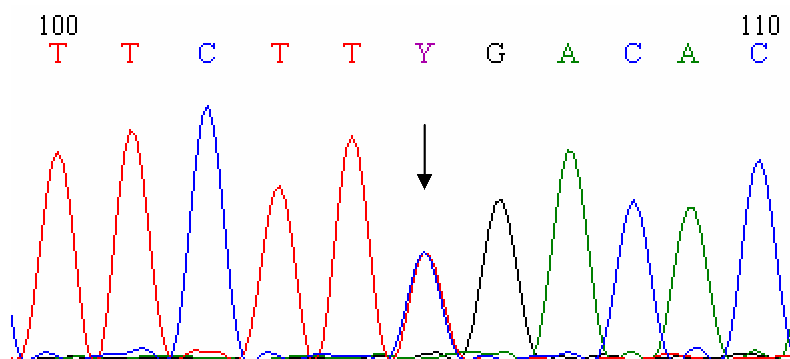


Figure 23: Electropherogram demonstrating the heterozygous form (arrowed) of the +3594 C/T sequenced in the forward (5'→3') direction.

Table 34: Genotype and allele frequencies of the ***IL*-1β +3954 C/T** polymorphism in the total **cohort**. Hardy Weinberg equilibrium (HWE) was determined for each individual group. Statistical analysis was performed using Fisher's exact test analog to determine associations at both genotype and allele level. The corresponding *P* values are shown at the bottom of the table.

Total Group (N=421)					
Genotype	**Total (N=421)**	**Controls (N=338)**	**Adverse (N=83)**	**PTL (N=33)**	**Hypertension (N=35)**
C/C	316 (75.1%)	253 (74.8%)	63 (75.9%)	23 (69.7%)	31 (88.6%)
C/T	98 (22.3%)	80 (23.7%)	18 (21.7%)	8 (24.2%)	4 (11.4%)
T/T	7 (1.7%)	5 (1.5%)	2 (2.4%)	2 (6.1%)	0
C-allele	0.867	0.867	0.867	0.818	0.943
T-allele	0.133	0.133	0.133	0.182	0.057
HWE	0.9822	0.897	0.875	0.568	0.938

Controls vs. Adverse: *P*=0.791 (genotype) and *P*=0.984 (allele); Controls vs. Preterm labour: *P*=0.178 (genotype) and *P*=0.273 (allele); Controls vs. Hypertension: *P*=0.182 (genotype) and *P*=0.068 (allele).

Pectora roborant cultus recti

Table 35: Genotype and allele frequencies of the ***IL*-1β +3954 C/T** polymorphism in the **Coloured** population. Hardy Weinberg equilibrium (HWE) was determined for each individual group. Statistical analysis was performed using Fisher's exact test analog to determine associations at both genotype and allele level. The corresponding *P* values are shown at the bottom of the table.

Coloured Females (N=306)					
Genotype	**Total (N=306)**	**Controls (N=237)**	**Adverse (N=69)**	**PTL (N=27)**	**Hypertension (N=29)**
C/C	228 (74.5%)	174 (73.4%)	54 (78.3%)	19 (70.4%)	26 (89.7%)
C/T	74 (24.2%)	59 (24.9%)	15 (21.7%)	8 (29.6%)	3 (10.3%)
T/T	4(1.3%)	4 (1.7%)	0	0	0
C-allele	0.866	0.859	0.891	0.852	0.948
T-allele	0.134	0.141	0.109	0.148	0.052
HWE	0.763	0.926	0.599	0.665	0.958

Controls vs. Adverse: *P*=0.460 (genotype) and *P*=0.322 (allele); Controls vs. Preterm labour: *P*=0.704 (genotype) and *P*=0.892 (allele); Controls vs. Hypertension: *P*=0.154 (genotype) and *P*=0.056 (allele).

Table 36: Genotype and allele frequencies of the ***IL*-1β +3954 C/T** polymorphism in the **Black** population. Hardy Weinberg equilibrium (HWE) was determined for each individual group

Black Females (N=115)					
Genotype	**Total (N=115)**	**Controls (N=101)**	**Adverse (N=14)**	**PTL (N=6)**	**Hypertension (N=6)**
C/C	88 (76.5%)	79 (78.2%)	9 (64.3%)	4 (66.7%)	5 (83.3%)
C/T	24 (20.9%)	21 (20.8%)	3 (21.4%)	0	1 (16.7%)
T/T	3 (2.6%)	1 (1%)	2 (14.3%)	2 (33.3%)	0
C-allele	0.870	0.886	0.750	0.667	0.917
T-allele	0.130	0.114	0.250	0.333	0.083
HWE	0.692	0.955	0.277	**0.049**	0.976

Sample size insufficient to perform association analysis (*P*<0.05).

IL-4 –590 C/T

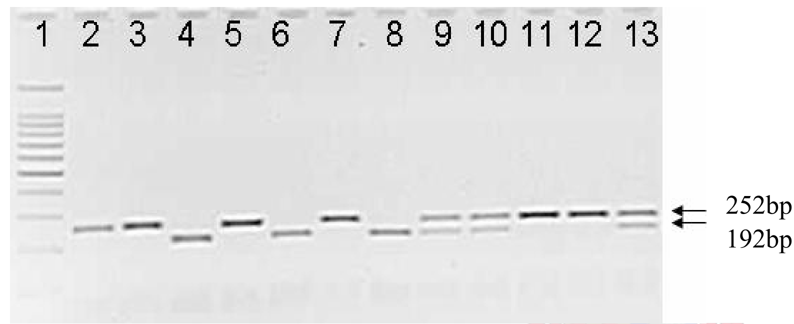


Figure 24: Agarose gel (2%) demonstrating the three genotypes of the *IL*-4 -590 C/T polymorphism, detected by restriction enzyme analysis with *Bsmf*I (37˚C). Lane 1 is a 100 bp ladder (Fermentas). Lanes 4, 6 and 8 illustrates the C/C genotype at 192 bp and lane 2, 3, 5, 7, 11 and 12 the T/T genotype at 252 bp. Lanes 9 and 10 represent the heterozygous genotype.

Table 37: Genotype and allele frequencies of the ***IL*-4 -590 C/T** polymorphism for the total **cohort**. Hardy Weinberg equilibrium (HWE) was determined for each individual group. Statistical analysis was performed using Fisher's exact test analog to determine associations at both genotype and allele level. The corresponding *P* values are shown at the bottom of the table.

Total Group (N=420)					
Genotype	**Total (N=420)**	**Controls (N=338)**	**Adverse (N=82)**	**PTL (N=33)**	**Hypertension (N=34)**
C/C	67 (15.9%)	58 (17.2%)	9 (11%)	2 (6.1%)	6 (17.6%)
C/T	182 (43.2%)	141 (41.7%)	41 (50%)	17 (51.5%)	15 (44.1%)
T/T	172 (40.9%)	139 (41.1%)	32 (39%)	14 (42.4%)	13 (38.2%)
C-allele	0.375	0.380	0.360	0.318	0.397
T-allele	0.625	0.620	0.640	0.682	0.603
HWE	0.278	0.108	0.742	0.561	0.900

Controls vs. Adverse: *P*=0.259 (genotype) and *P*=0.628 (allele); Controls vs. Preterm labour: *P*=0.225 (genotype) and *P*=0.321 (allele); Controls vs. Hypertension: *P*=0.947 (genotype) and *P*=0.785 (allele).

Table 38: Genotype and allele frequencies of the ***IL*-4 -590 C/T** polymorphism in the **Coloured** population. Hardy Weinberg equilibrium (HWE) was determined for each individual group. Statistical analysis was performed using Fisher's exact test analog to determine associations at both genotype and allele level. The corresponding *P* values are shown at the bottom of the table.

Coloured Females (N=305)					
Genotype	**Total (N=305)**	**Controls (N=237)**	**Adverse (N=68)**	**PTL (N=27)**	**Hypertension (N=28)**
C/C	59 (19.3%)	51 (21.5%)	8 (11.8%)	2 (7.4%)	5 (17.9%)
C/T	138 (45.2%)	103 (43.5%)	35 (51.5%)	14 (51.9%)	13 (46.4%)
T/T	108 (35.4%)	83 (35%)	25 (36.8%)	11 (40.7%)	10 (35.7%)
C-allele	0.420	0.432	0.375	0.333	0.411
T-allele	0.580	0.568	0.625	0.667	0.589
HWE	0.463	0.211	0.721	0.687	0.977

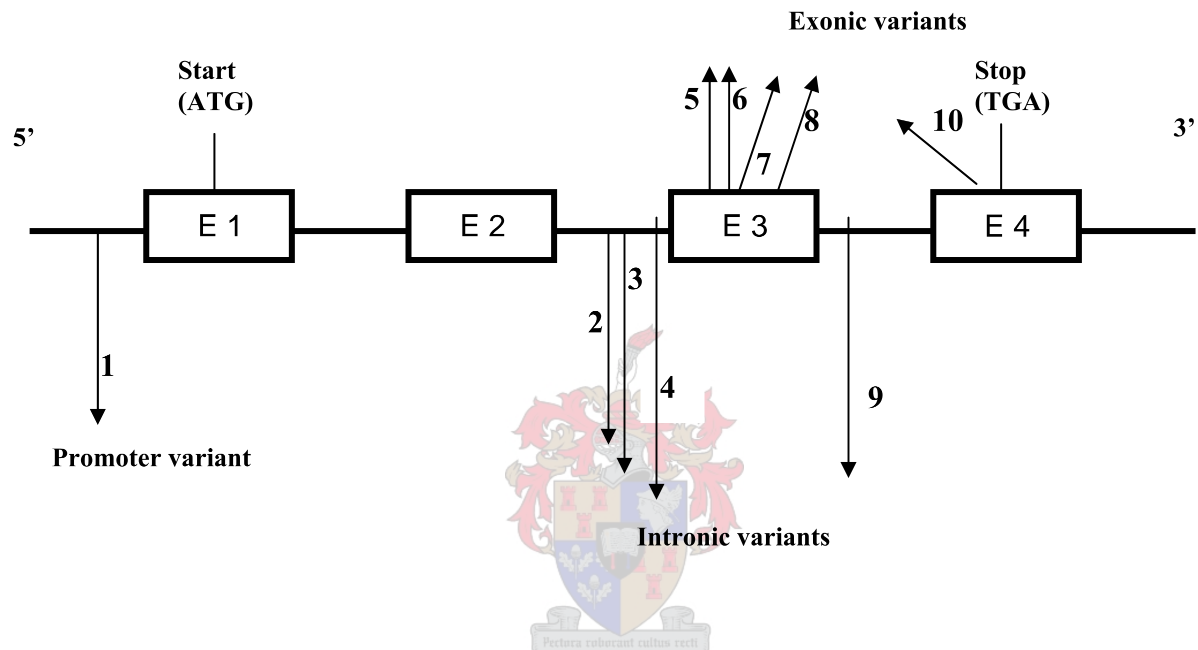
Controls vs. Adverse: P=0.183 (genotype) and P=0.231 (allele); Controls vs. Preterm labour: P=0.222 (genotype) and P=0.162 (allele); Controls vs. Hypertension: P=0.899 (genotype) and P=0.756 (allele).

Table 39: Genotype and allele frequencies of the ***IL*-4 -590 C/T** polymorphism in the **Black** population. Hardy Weinberg equilibrium (HWE) was determined for each individual group.

Black Females (N=115)					
Genotype	**Total (N=115)**	**Controls (N=101)**	**Adverse (N=14)**	**PTL (N=6)**	**Hypertension (N=6)**
C/C	8 (6.9%)	7 (6.9%)	1 (7.1%)	0	1 (16.7%)
C/T	44 (38.3%)	38 (37.6%)	6 (42.9%)	3 (50%)	2 (33.3%)
T/T	63 (54.8%)	56 (55.4%)	7 (50%)	3 (50%)	3 (50%)
C-allele	0.261	0.257	0.286	0.250	0.333
T-allele	0.739	0.743	0.714	0.750	0.667
HWE	0.997	0.987	0.983	0.717	0.829

Sample size insufficient to perform association analysis (P<0.05).

Appendix 9: *LGALS*13 gene variants diagrammatically illustrated



1. -98A/C (dbSNP rs: 3764843)
2. IVS2-36 G/A (novel)
3. IVS2-22 A/G (dbSNP rs: 2233706)
4. IVS2-15 G/A (novel)
5. 130 A→G / M44V (novel)
6. 221delT / L74W (novel)
7. Hotspot / T77N, T78L, D79H (novel)
8. 260 A→G / K87R (novel)
9. IVS3+72 T/A (dbSNP rs: 2233708)
10. 351 G→A / P117P (novel)

Appendix 10: The remaining *LGALS*13 gene variants screened in this study

-98 A/C (dbSNP rs: 3764843)

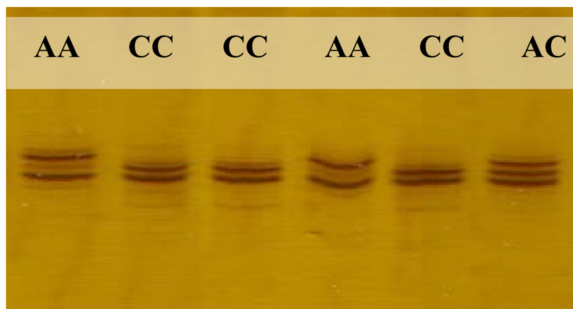


Figure 25: Three conformational banding patterns for the -98 A/C 'promoter' variant detected by Multiphor SSCP/HD analysis, demonstrated only in the SSCP component of the analysis.

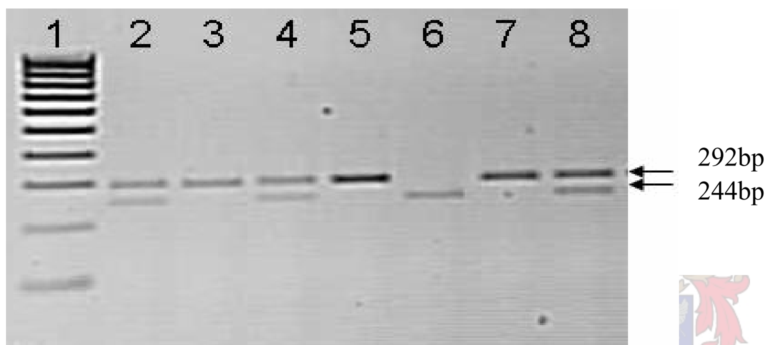


Figure 26: This documented variant was screened using restriction enzyme analysis. Agarose gel (2%) demonstrating the different genotypes of the -98 A/C promoter variant, detected by restriction enzyme analysis with *Ava*I (37˚C). Lane 1 is a 100 bp ladder. Lanes 3, 5 and 7 corresponds to the A/A genotype (292 bp), Lanes 2, 4 and 8 the heterozygous genotype (A/C) and Lane 6 represents the homozygous C/C genotype (244 bp).

Table 40: Genotype and allele frequencies of the **-98 A/C** variant for the total **cohort**. Hardy Weinberg equilibrium (HWE) was determined for each individual group. Statistical analysis was performed using Fisher's exact test analog to determine associations at both genotype and allele level. The corresponding *P* values are shown at the bottom of the table.

Total Cohort (N=421)					
Genotype	**Total (N=421)**	**Controls (N=338)**	**Adverse (N=83)**	**PTL (N=33)**	**Hypertension (N=35)**
A/A	220 (52.3%)	176 (52.1%)	44 (53.7%)	16 (48.5%)	23 (65.7%)
A/C	171 (40.6%)	139 (41.1%)	32 (37.8%)	15 (45.5%)	8 (22.9%)
C/C	30 (7.1%)	23 (6.8%)	7 (8.5%)	2 (6%)	4 (11.4%)
A-allele	0.726	0.726	0.723	0.712	0.771
C-allele	0.274	0.274	0.277	0.288	0.224
HWE	0.918	0.818	0.943	0.823	0.257

Controls vs. Adverse: *P*=0.832 (genotype) and *P*=0.929 (allele); Controls vs. Preterm labour: *P*=0.889 (genotype) and *P*=0.805 (allele); Controls vs. Hypertension: *P*=0.094 (genotype) and *P*=0.418 (allele).

Table 41: Genotype and allele frequencies of the **-98 A/C** variant in the **Coloured** population. Hardy Weinberg equilibrium (HWE) was determined for each individual group. Statistical analysis was performed using Fisher's exact test analog to determine associations at both genotype and allele level. The corresponding *P* values are shown at the bottom of the table.

Coloured Females (n=306)					
Genotype	**Total (N=306)**	**Controls (N=237)**	**Adverse (N=69)**	**PTL (N=27)**	**Hypertension (N=29)**
A/A	160 (52.3%)	125 (53%)	35 (50.7%)	11 (40.7%)	19 (66%)
A/C	122 (39.9%)	93 (39%)	29 (42%)	14 (51.9%)	7 (24%)
C/C	24 (7.8%)	19 (8%)	5 (7.2%)	2 (7.4%)	3 (10%)
A-allele	0.722	0.724	0.717	0.667	0.776
C-allele	0.278	0.276	0.283	0.333	0.224
HWE	0.994	0.958	0.983	0.687	0.257

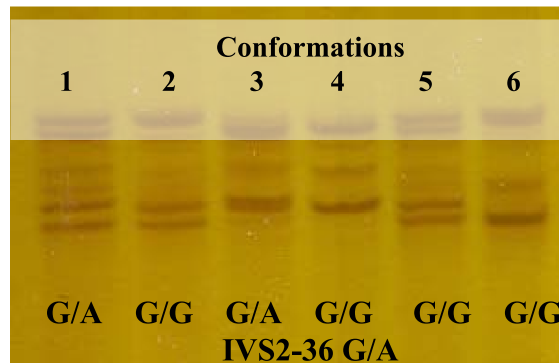
Controls vs. Adverse: *P*=0.911 (genotype) and *P*=0.886 (allele); Controls vs. Preterm labour: *P*=0.440 (genotype) and *P*=0.378 (allele); Controls vs. Hypertension: *P*=0.284 (genotype) and *P*=0.398 (allele).

Table 42: Genotype and allele frequencies of the **-98 A/C** variant in the **Black** population. Hardy Weinberg equilibrium (HWE) was determined for each individual group.

Black Females (n=115)					
Genotype	**Total (N=115)**	**Controls (N=101)**	**Adverse (N=14)**	**PTL (N=6)**	**Hypertension (N=6)**
A/A	60 (52.2%)	51 (50.5%)	9 (64.3%)	58 (83.3%)	4 (66.7%)
A/C	49 (42.6%)	46 (45.5%)	3 (21.4%)	1 (16.7%)	1 (16.7%)
C/C	6 (5.2%)	4 (4%)	2 (12.3%)	0	1 (16.7%)
A-allele	0.735	0.733	0.750	0.771	0.750
C-allele	0.265	0.267	0.250	0.29	0.250
HWE	0.607	0.263	0.277	0.115	0.396

Sample size insufficient to perform association analysis (*P*<0.05).

IVS2-36 G/A (NOVEL)



Conformation	IVS2-15 G/A	IVS2-22 A/G	IVS2-36 G/A
1	G/G	A/A	G/A
2	G/A	A/A	G/G
3	G/G	A/G	G/A
4	G/G	G/G	G/G
5	G/G	A/G	G/G
6	G/G	A/A	G/G

Figure 27: Multiphor SSCP/HD gel illustrating the different conformations for the IVS2-36 G/A variant detected in only the SSCP component of the analysis. The included table illustrates the cluster of variants in the 5' intronic region flanking exon three.

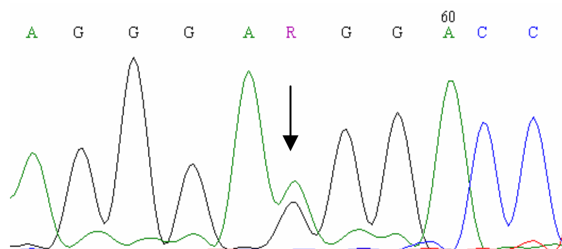


Figure 28: Electropherogram depicting the heterozygous form (arrowed) of the IVS2-36 locus, sequenced in the forward (5'→3') direction.

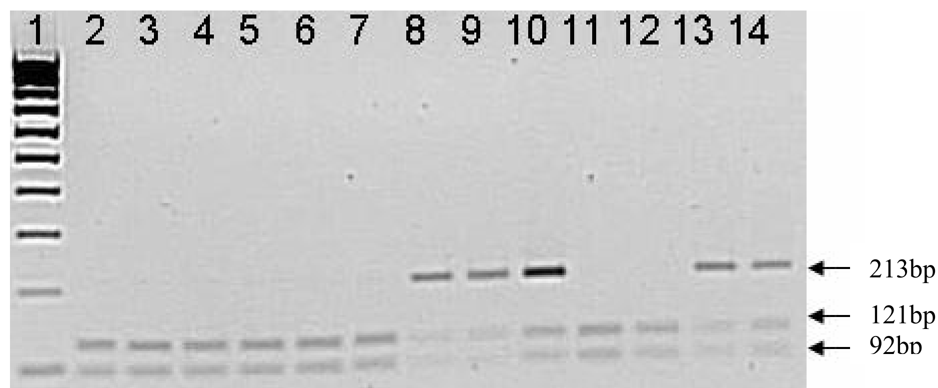


Figure 29: Agarose gel (2.5%) demonstrating the two genotypes of the IVS2-36 G/A variant, detected by restriction enzyme analysis with *Nla*IV (37˚C). Lane 1 is a 100 bp ladder (Fermentas). Lanes 2-7, 11 and 12 represents the G/G genotype (121 and 92 bp). Lanes 8-10, 13 and 14 the heterozygous genotypes G/A (213, 121 and 92 bp).

Table 43: Genotype and allele frequencies of the **IVS2-36 G/A** variant for the total **cohort**. Hardy Weinberg equilibrium (HWE) was determined for each individual group. Statistical analysis was performed using Fisher's exact test analog to determine associations at both genotype and allele level. The corresponding *P* values are shown at the bottom of the table.

Total Cohort (N=421)					
Genotype	**Total (N=421)**	**Controls (N=338)**	**Adverse (N=83)**	**PTL (N=33)**	**Hypertension (N=35)**
G//G	390 (92.6%)	311 (92%)	79 (95.2%)	33	34 (97.1%)
GA	31 (7.4%)	27 (8%)	4 (4.8%)	0	1 (2.9%)
A/A	0	0	0	0	0
G-allele	0.963	0.960	0.976	1.000	0.986
A-allele	0.037	0.040	0.024	0.000	0.014
HWE	0.735	0.746	0.975	-	0.996

Controls vs. Adverse: *P*=0.331 (allele); Controls vs. Preterm labour: *P*=0.098 (allele); Controls vs. Hypertension: *P*=0.282 (allele).

Table 44: Genotype and allele frequencies of the **IVS2-36 G/A** variant in the **Coloured** population. Hardy Weinberg equilibrium (HWE) was determined for each individual group. Statistical analysis was performed using Fisher's exact test analog to determine associations at both genotype and allele level. The corresponding *P* values are shown at the bottom of the table.

Coloured Females (N=306)					
Genotype	**Total (N=306)**	**Controls (N=237)**	**Adverse (N=69)**	**PTL (N=27)**	**Hypertension (N=29)**
G/G	288 (94.1%)	222 (93.7%)	66 (95.7%)	27	28 (96.6%)
G/A	18 (5.9%)	15 (6.3%)	3 (4.3%)	0	1 (3.4%)
A/A	0	0	0	0	0
G-allele	0.971	0.968	0.978	1.000	0.983
A-allele	0.029	0.032	0.022	0.000	0.017
HWE	0.869	0.881	0.983	-	0.996

Controls vs. Adverse: *P*=0.544 (allele); Controls vs. Preterm labour: *P*=0.458 (allele); Controls vs. Hypertension: *P*=0.841 (allele).

Table 45: Genotype and allele frequencies of the **IVS2-36 G/A** variant in the **Black** population. Hardy Weinberg equilibrium (HWE) was determined for each individual group.

Black Females (N=115)					
Genotype	**Total (N=115)**	**Controls (N=101)**	**Adverse (N=14)**	**PTL (N=6)**	**Hypertension (N=6)**
G/G	102 (88.7%)	89 (88.1%)	13 (92.9%)	6	6
G/A	13 (11.3%)	12 (11.9%)	1 (7.1%)	0	0
A/A	0	0	0	0	0
G-allele	0.943	0.941	0.964	1.000	1.000
A-allele	0.057	0.059	0.036	0.000	0.000
HWE	**0.814**	0.818	0.990	-	-

Sample size insufficient to perform association analysis ($P<0.05$).

IVS2-22 A/G (dbSNP rs: 2233706)

Table 46: Genotype and allele frequencies of the **IVS2-22 G/A** variant in the **Coloured** population. Hardy Weinberg equilibrium (HWE) was determined for each individual group. Statistical analysis was performed using Fisher's exact test analog to determine associations at both genotype and allele level. The corresponding *P* values are shown at the bottom of the table.
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Coloured Females (N=306)					
Genotype	**Total (N=306)**	**Good (N=237)**	**Poor (N=69)**	**PTL (N=27)**	**Hypertension (N=29)**
A/A	231 (75.4%)	174 (73.4%)	57 (82.6%)	24 (88.9%)	22 (75.9%)
A/G	71 (23.3%)	60 (25.3%)	11 (15.9%)	3 (11.1%)	6 (20.7%)
G/G	4 (1.3%)	3 (1.3%)	1 (1.5%)	0	1 (3.4%)
A-allele	0.871	0.861	0.906	0.944	0.862
G-allele	0.129	0.139	0.094	0.056	0.138
***P* value**	0.856	0.688	0.861	0.954	0.783

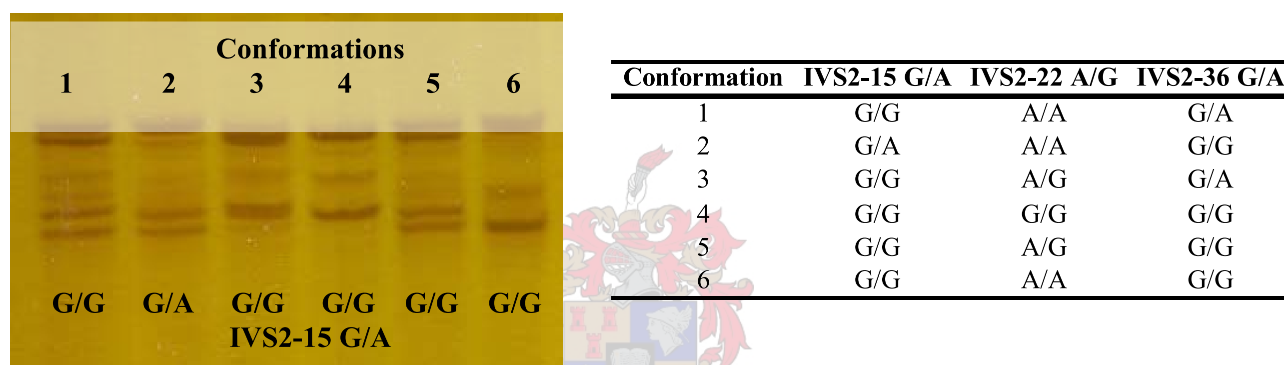
Controls vs. Adverse: *P*=0.268 (genotype) and *P*=0.165 (allele); Controls vs. Preterm labour: *P*=0.162 (genotype) and *P*=0.299 (allele); Controls vs. Hypertension: *P*=0.588 (genotype) and *P*=0.978 (allele).

Table 47: Genotype and allele frequencies of the **IVS2-36 G/A** variant in the **Black** population. Hardy Weinberg equilibrium (HWE) was determined for each individual group.

	Black Females (N=115)				
Genotype	**Total (N=115)**	**Good (N=101)**	**Poor (N=14)**	**PTL (N=6)**	**Hypertension (N=6)**
A/A	69 (60%)	59 (58.4%)	10 (71.4%)	4 (66.7%)	4 (66.7%)
A/G	40 (34.8%)	37 (36.6%)	3 (21.4%)	1 (16.7%)	2 (33.3%)
G/G	6 (5.2%)	5 (5%)	1 (7.1%)	1 (16.7%)	0
A-allele	0.774	0.767	0.821	0.750	0.833
G-allele	0.226	0.233	0.179	0.250	0.167
***P* value**	0.998	0.966	0.601	0.396	0.887

Sample size insufficient to perform association analysis ($P<0.05$).

IVS2-15 G/A (NOVEL)



Conformation	IVS2-15 G/A	IVS2-22 A/G	IVS2-36 G/A
1	G/G	A/A	G/A
2	G/A	A/A	G/G
3	G/G	A/G	G/A
4	G/G	G/G	G/G
5	G/G	A/G	G/G
6	G/G	A/A	G/G

Figure 30: Multiphor SSCP/HD demonstrating the different conformations of the IVS2-15 G/A detected in the SSCP component of the gel. The included table illustrates the cluster of variants in the 5' intronic region flanking exon three.

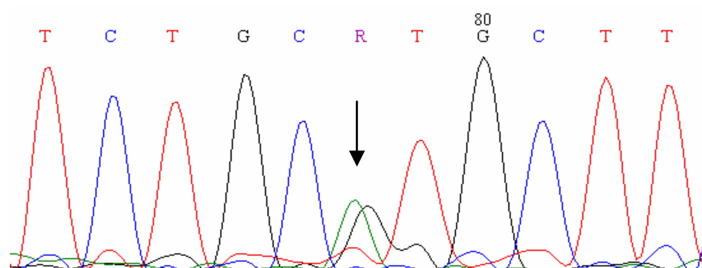


Figure 31: Electropherogram depicting the heterozygous form (arrowed) of the IVS2-15 G/A variant, sequenced in the forward (5'→3') direction.

Table 48: Genotype and allele frequencies of the **IVS2-15 G/A** variant for the total **cohort**. Hardy Weinberg equilibrium (HWE) was determined for each individual group. Statistical analysis was performed using Fisher's exact test analog to determine associations at both genotype and allele level. The corresponding *P* values are shown at the bottom of the table.

Total Group (n=421)					
Genotype	**Total (N=421)**	**Controls (N=338)**	**Adverse (N=83)**	**PTL (N=33)**	**Hypertension (N=35)**
G/G	413 (74.8%)	332 (98.2%)	81 (97.6%)	32 (97%)	34 (97.1%)
G/A	8 (25.2%)	6 (1.8%)	2 (2.4%)	1 (3%)	1 (2.9%)
A/A	0	0	0	0	0
G-allele	0.990	0.991	0.988	0.985	0.986
A-allele	0.010	0.009	0.012	0.015	0.014
HWE	0.981	0.987	0.994	0.996	0.996

Controls vs. Adverse: *P*=0.706 (allele); Controls vs. Preterm labour: *P*=0.615 (allele); Controls vs. Hypertension: *P*=0.655 (allele).

Table 49: Genotype and allele frequencies of the **IVS2-22 G/A** variant in the **Coloured** population. Hardy Weinberg equilibrium (HWE) was determined for each individual group. Statistical analysis was performed using Fisher's exact test analog to determine associations at both genotype and allele level. The corresponding *P* values are shown at the bottom of the table.

Coloured Females (n=306)					
Genotype	**Total (N=306)**	**Controls (N=237)**	**Adverse (N=69)**	**PTL (N=27)**	**Hypertension (N=29)**
G/G	300 (70.9%)	233 (98.3%)	67 (97.1%)	26 (96.3%)	28 (96.6%)
G/A	6 (29.8%)	4 (1.7%)	2 (2.9%)	1 (3.7%)	1 (3.4%)
A/A	0	0	0	0	0
G-allele	0.990	0.992	0.986	0.981	0.983
A-allele	0.010	0.008	0.014	0.019	0.017
HWE	0.985	0.992	0.993	0.995	0.996

Controls vs. Adverse: *P*=0.525 (allele); Controls vs. Preterm labour: *P*=0.469 (allele); Controls vs. Hypertension: *P*=0.512 (allele).

Table 50: Genotype and allele frequencies of the **IVS2-36 G/A** variant in the **Black** population. Hardy Weinberg equilibrium (HWE) was determined for each individual group.

Black Females (n=115)					
Genotype	**Total (N=115)**	**Controls (N=101)**	**Adverse (N=14)**	**PTL (N=6)**	**Hypertension (N=6)**
G/G	113 (61.7%)	99 (98%)	14	6	6
G/A	2 (38.3%)	2 (2%)	0	0	0
A/A	0	0	0	0	0
G-allele	0.991	0.990	1.000	1.000	1.000
A-allele	0.009	0.010	0.000	0.000	0.000
HWE	0.996	0.995	-	-	-

Sample size insufficient to perform association analysis (*P*<0.05).

Pectora roborant cultus recti

"Hotspot" (HS) (NOVEL)

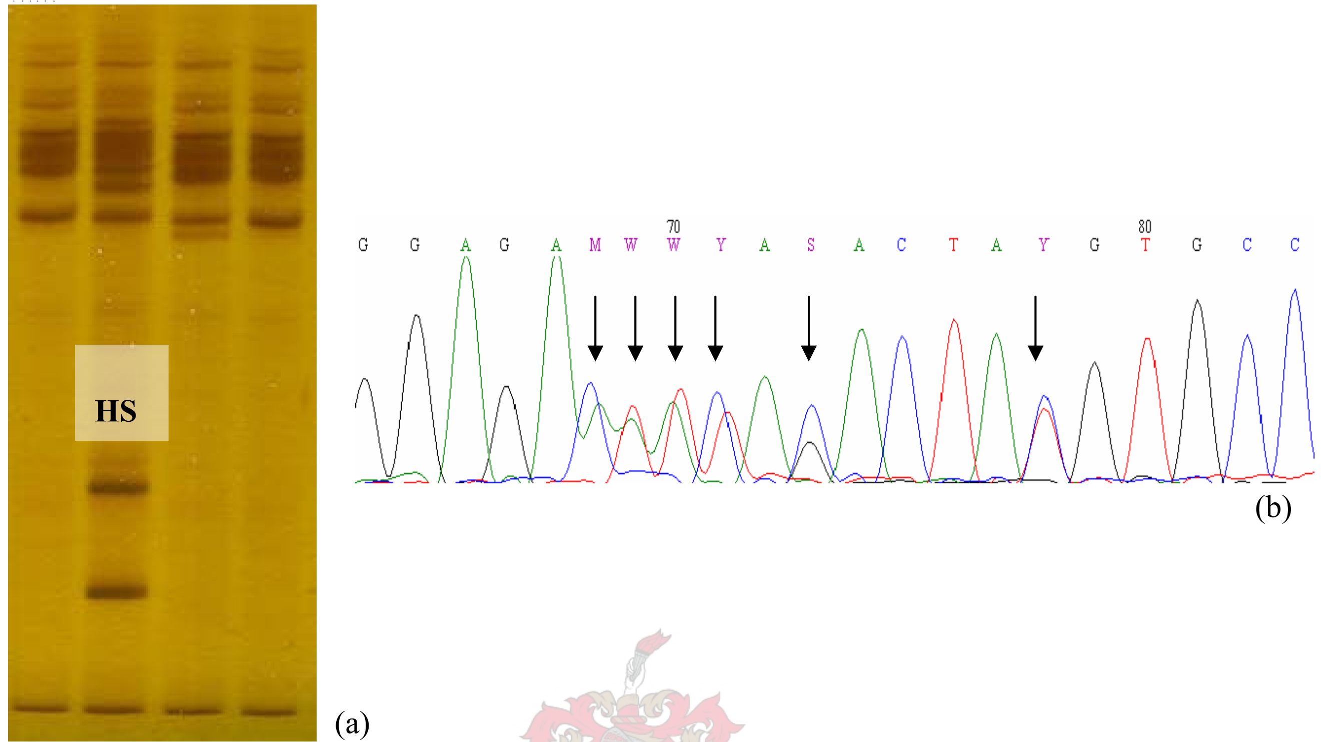


Figure 32: (a) Multiphor SSCP/HD conformation demonstrating the conformation of the "hotspot" detected in both the SSCP and HD component of the gel. (b) The corresponding electropherogram demonstrating the heterozygous form, sequenced in the forward (5'→3') direction. Sequence characterisation revealed an 11bp region of which six bases was mutated (arrowed).

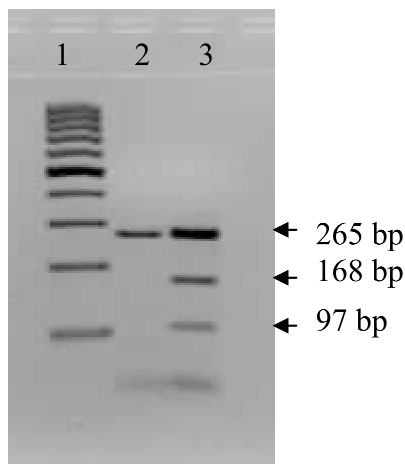


Figure 33: Agarose gel (2.5%) demonstrating the 2 genotypes of the 'hotspot' sequence variant, detected by restriction enzyme analysis with *Apo*I (50˚C). Lane 1 is a 100 bp ladder (Fermentas). Lane represents the wildtype sequence and lane 3 the 11 bp region containing six mutated bases (265bp, 168bp and 97bp).

Table 51: Genotype and allele frequencies of the **'hotspot'** sequence variant for the total **cohort**. Hardy Weinberg equilibrium (HWE) was determined for each individual group. Statistical analysis was performed using Fisher's exact test analog to determine associations at both genotype and allele level. The corresponding *P* values are shown at the bottom of the table.

Total Group (n=421)					
Genotype	**Total (N=421)**	**Controls (N=338)**	**Adverse (N=83)**	**PTL (N=33)**	**Hypertension (N=35)**
- / -	391 (92.9%)	312 (92.3%)	79 (95.2%)	33	34 (97.1%)
- / HS	30 (7.1%)	26 (7.7%)	4 (4.8%)	0	1 (2.9%)
HS / Hs	0	0	0	0	0
- allele	0.964	0.981	0.976	1.000	0.986
HS allele	0.036	0.019	0.024	0.000	0.014
HWE	0.7503	0.944	0.975	-	0.996

Controls vs. Adverse: *P*=0.371 (allele); Controls vs. Preterm labour: *P*=0.105 (allele); Controls vs. Hypertension: *P*=0.303 (allele).

Table 52: Genotype and allele frequencies of the **'hotspot'** sequence variant in the **Coloured** population. Hardy Weinberg equilibrium (HWE) was determined for each individual group. Statistical analysis was performed using Fisher's exact test analog to determine associations at both genotype and allele level. The corresponding *P* values are shown at the bottom of the table.

Coloured Females (n=306)					
Genotype	**Total (N=306)**	**Controls (N=237)**	**Adverse (N=69)**	**PTL (N=27)**	**Hypertension (N=29)**
- / -	288 (94.1%)	222 (93.7%)	66 (95.7%)	27	28 (96.6%)
- / HS	18 (5.9%)	15 (6.3%)	3 (4.3%)	0	1 (3.4%)
HS / Hs	0	0	0	0	0
- allele	0.971	0.968	0.978	1.000	0.983
HS allele	0.029	0.032	0.022	0.000	0.017
HWE	0.869	0.881	0.983	-	0.996

Controls vs. Adverse: *P*=0.544 (allele); Controls vs. Preterm labour: *P*=0.185 (allele); Controls vs. Hypertension: *P*=0.544 (allele).

Table 53: Genotype and allele frequencies of the **'hotspot'** sequence variant in the **Black** population. Hardy Weinberg equilibrium (HWE) was determined for each individual group.

Black Females (n=115)					
Genotype	**Total (N=115)**	**Controls (N=101)**	**Adverse (N=14)**	**PTL (N=6)**	**Hypertension (N=6)**
- / -	103 (88.8%)	90 (90.1%)	13 (92.9%)	6	6
- / HS	13 (11.2%)	11 (10.9%)	1 (7.1%)	0	0
HS / Hs	0	0	0	0	0
- allele	0.944	0.946	0.964	1.000	1.000
HS allele	0.056	0.054	0.036	0.000	0.000
HWE	0.815	0.846	0.990	-	-

Sample size insufficient to perform association analysis (*P*<0.05).

IVS3+72 T/A (dbSNP rs: 2233708)

Table 54: Genotype and allele frequencies of the **IVS3+72 T/A** variant in the **Coloured** population. Hardy Weinberg equilibrium (HWE) was determined for each individual group. Statistical analysis was performed using Fisher's exact test analog to determine associations at both genotype and allele level. The corresponding *P* values are shown at the bottom of the table.

	Coloured Females (n=306)				
Genotype	**Total (N=306)**	**Good (N=237)**	**Poor (N=69)**	**PTL (N=27)**	**Hypertension (N=29)**
T/T	229 (74.8%)	172 (72.6%)	57 (82.6%)	23 (85.2%)	22 (75.9%)
T/A	74 (24.3%)	63 (26.6%)	11 (15.9%)	4 (14.8%)	6 (20.7%)
A/A	3 (0.9%)	2 (0.8%)	1 (1.5%)	0	1 (3.4%)
T-allele	0.869	0.859	0.906	0.96	0.862
A-allele	0.131	0.141	0.094	0.074	0.138
***P* value**	0.529	0.343	0.861	0.917	0.783

Controls vs. Adverse: *P*=0.181 (genotype) and *P*=0.148 (allele); Controls vs. Preterm labour: *P*=0.178 (genotype) and *P*=0.482 (allele); Controls vs. Hypertension: *P*=0.379 (genotype) and *P*=0.944 (allele).

Table 55: Genotype and allele frequencies of the **IVS3+72 T/A** variant in the **Black** population. Hardy Weinberg equilibrium (HWE) was determined for each individual group.

	Black Females (n=115)				
Genotype	**Total (N=115)**	**Good (N=91)**	**Poor (N=14)**	**PTL (N=6)**	**Hypertension (N=6)**
T/T	71 (61.7%)	62 (61.4%)	9 (64.3%)	3 (50%)	4 (66.7%)
T/A	39 (33.9%)	35 (34.7%)	4 (28.6%)	2 (33.7%)	2 (33.3%)
A/A	5 (4.4%)	4 (3.9%)	1 (7.1%)	1 (16.7%)	0
T-allele	0.787	0.787	0.786	0.667	0.833
A-allele	0.213	0.213	0.214	0.333	0.16
***P* value**	0.993	0.943	0.852	0.89	0.887

Sample size insufficient to perform association analysis (*P*<0.05).

The following variants were only identified in the control group:

130 A→G /M44V (NOVEL)

- **2 individuals**

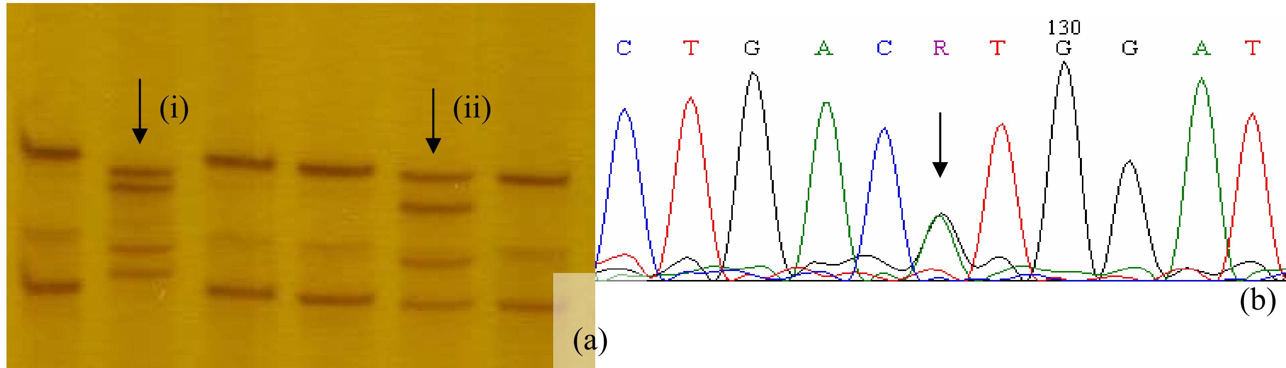


Figure 34: (a) Multiphor SSCP/HD analysis of exon three revealed two distinct conformations in the SSCP component of the gel (arrowed), identified in the control cohort. (b) Sequencing analysis in the forward direction (5'→3') demonstrated a heterozygous from in these individuals, (130A→G /M44V). Individual (i) was heterozygous at both IVS2-36 and IVS2-22 loci, whereas individual (ii) was heterozygous at IVS2-36 and homozygous (A/A) at IVS2-22 loci.

260 A→G /K87R (NOVEL)

- **1 individual**

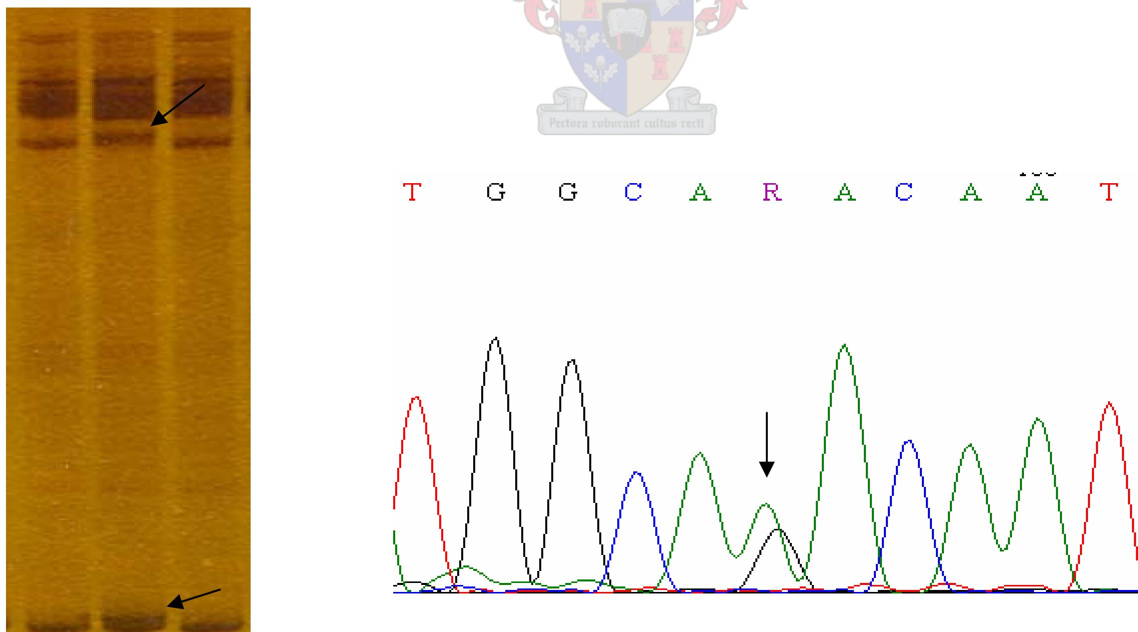


Figure 35: (a) Multiphor SSCP/HD analysis of exon three identified a variant in both the SSCP and HD component of the gel (arrowed), identified in the control cohort. (b) Sequencing analysis in the forward direction (5'→3') demonstrated a heterozygous from of an exonic mutation, 260 A→G /K87R.

351 G→A / P117P (NOVEL)

- **1 individual**

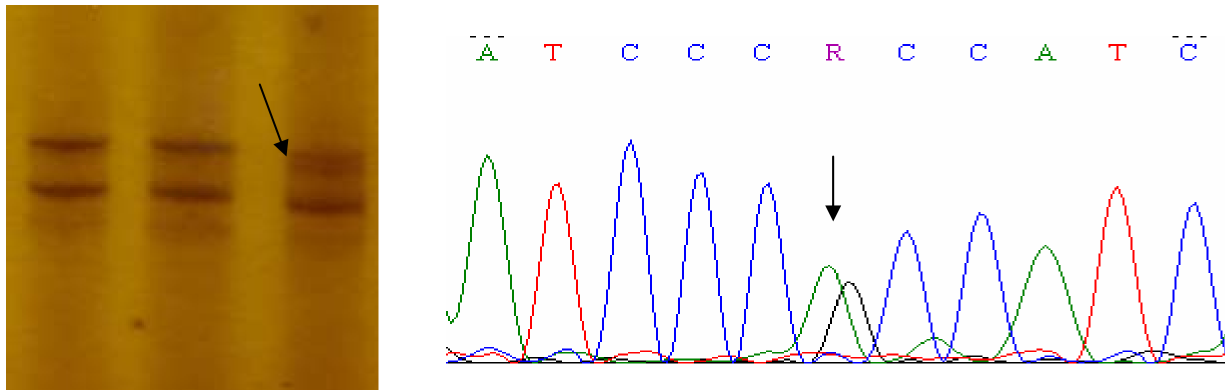


Figure 36: (a) Multiphor SSCP/HD analysis of exon four identified a variant in only the SSCP component of the gel (arrowed), identified in the control cohort. (b) Sequencing analysis in the forward direction (5'→3') demonstrated a heterozygous from of a silent exonic mutation, 351 G→A / P117P.

Appendix 11: Genotype-phenotype interactions

Immunological gene variants

TNFα -308 G/A

Table 56: Genotype and allele frequencies of the ***TNFα*-308 G/A** polymorphism in the total **cohort**, **Coloured**, **Black** population and gestation. Hardy Weinberg Equilibrium (HWE) was determined for each individual group. Statistical analysis was performed using Fisher's exact test analog to determine associations at both genotype and allele level. The corresponding *P* values are shown at the bottom of the table.

	≥37 (Controls N=370)			**<37 (Adverse N=33)**		
Genotype	**Total (N=370)**	**Coloured (N=261)**	**Black (N=109)**	**Total (N=33)**	**Coloured (N=27)**	**Black (N=6)**
G/G	275 (74.3%)	206 (78.9%)	69 (63.3%)	20 (60.6%)	18 (66.7%)	2 (33.3%)
G/A	94 (25.4%)	54 (20.7%)	40 (36.7%)	13 (39.4%)	9 (33.3%)	4 (66.7%)
A/A	1 (0.3%)	1 (0.4%)	0	0	0	0
G-allele	0.870	0.893	0.817	0.803	0.833	0.667
A-allele	0.130	0.107	0.183	0.197	0.167	0.333
HWE	0.055	0.432	0.064	0.371	0.583	0.472

≥37 vs. <37weeks (group): *P*=0.212 (genotype); *P*=0.126 (allele), ≥37 vs. <37weeks (CF): *P*=0.307 (genotype); *P*=0.1892 (allele), ≥37 vs. <37weeks (BF): Sample size insufficient

Table 57: Genotype and allele frequencies of the ***TNFα*-308 G/A** polymorphism in the total **cohort**, **Coloured**, **Black** population birth weight. Hardy Weinberg Equilibrium (HWE) was determined for each individual group. Statistical analysis was performed using Fisher's exact test analog to determine associations at both genotype and allele level. The corresponding *P* values are shown at the bottom of the table.

	≥2000g (Controls N=367)			**<2000g(Adverse N=31)**		
Genotype	**Total N=367**	**Coloured N=256**	**Black N=111**	**Total N=31**	**Coloured (N=28)**	**Black (N=3)**
G/G	270 (73.5%)	201 (78.5%)	69 (62.2%)	20 (64.5%)	19 (67.9%)	1 (33.3%)
G/A	96 (26.2%)	54 (21.1%)	42 (37.8%)	11 (35.5%)	9 (32.1%)	2 (66.7%)
A/A	1 (0.3%)	1 (0.4%)	0	0	0	0
G-allele	0.866	0.819	0.804	0.823	0.839	0.667
A-allele	0.134	0.109	0.196	0.177	0.161	0.333
HWE	**0.044**	0.417	0.139	0.486	0.599	0.687

≥2000g vs. <2000g (group): **not HWE**, ≥2000g vs. <2000g (CF): *P*=0.6969 (allele), ≥2000g vs. <2000g (BF): Sample size insufficient

TNFα **-238 G/A**

Table 58: Genotype and allele frequencies of the ***TNFα*-238 G/A** polymorphism in the total **cohort**, **Coloured**, **Black** population and gestation. Hardy Weinberg Equilibrium (HWE) was determined for each individual group. Statistical analysis was performed using Fisher's exact test analog to determine associations at both genotype and allele level. The corresponding *P* values are shown at the bottom of the table.

	≥37 (Controls N=370)			**<37 (Adverse N=33)**		
Genotype	**Total (N=370**	**Coloured (N=216)**	**Black (N=109)**	**Total (N=33)**	**Coloured (N=27)**	**Black (N=6)**
G/G	286 (77.3%)	193 (73.9%)	93 (85.3%)	22 (66.7%)	17 (63%)	5 (83.3%)
G/A	84 (22.7%)	68 (26.1%)	16 (14.7%)	11 (33.3%)	10 (37%)	1 (16.7%)
A/A	0	0	0	0	0	0
G-allele	0.886	0.870	0.927	0.833	0.815	0.917
A-allele	0.114	0.130	0.073	0.167	0.185	0.083
HWE	**0.048**	0.054	0.710	0.517	0.498	0.976

≥37 vs. <37weeks (group): *P*=0.199 (allele), ≥37 vs. <37weeks (CF): *P*=0.262 (allele), ≥37 vs. <37weeks (BF): Sample size insufficient

Table 59: Genotype and allele frequencies of the ***TNFα*-238 G/A** polymorphism in the total **cohort**, **Coloured**, **Black** population and birth weight. Hardy Weinberg Equilibrium (HWE) was determined for each individual group. Statistical analysis was performed using Fisher's exact test analog to determine associations at both genotype and allele level. The corresponding *P* values are shown at the bottom of the table.

	≥2000g (Controls N=367)			**<2000g(Adverse N=31)**		
Genotype	**Total N=367**	**Coloured N=256**	**Black N=111**	**Total N=31**	**Coloured (N=28)**	**Black (N=3)**
G/G	283 (77.1%)	189 (73.8%)	94 (84.7%)	22 (71%)	19 (67.9%)	3
G/A	84 (22.9%)	67 (26.2%)	17 (15.35%)	9 (29%)	9 (32.1%)	0
A/A	0	0	0	0	0	0
G-allele	0.886	0.869	0.923	0.855	0.839	1.000
A-allele	0.114	0.131	0.077	0.145	0.161	0.000
HWE	**0.047**	0.055	0.6827	0.639	0.599	-

≥2000g vs. <2000g (group): *P*=0.469, ≥2000g vs. <2000g (CF): *P*=0.055 (allele), ≥2000g vs. <2000g (BF): Sample size insufficient

Pectura roborant cultus recti

***TNFα* +488 G/A**

Table 60: Genotype and allele frequencies of the ***TNFα*+488G/A** polymorphism in the total **cohort**, **Coloured**, **Black** population and gestation. Hardy Weinberg Equilibrium (HWE) was determined for each individual group. Statistical analysis was performed using Fisher's exact test analog to determine associations at both genotype and allele level. The corresponding *P* values are shown at the bottom of the table.

	≥37 (Controls N=356)			**<37 (Adverse N=30)**		
Genotype	**Total (N=356)**	**Coloured (N=249)**	**Black (N=107)**	**Total (N=30)**	**Coloured (N=25)**	**Black (N=5)**
G/G	324 (91%)	222 (89.2%)	102 (95.3%)	29 (96.7%)	24 (96%)	5
G/A	32 (9%)	27 (10.8%)	5 (4.7%)	1 (3.3%)	1 (4%)	0
A/A	0	0	0	0	0	0
G-allele	0.955	0.946	0.977	0.983	0.980	1.000
A-allele	0.045	0.054	0.023	0.017	0.020	0.000
HWE	0.674	0.664	0.969	0.996	0.995	-

≥37 vs. <37weeks (group): *P*=0.298 (allele), ≥37 vs. <37weeks (CF): *P*=0.295 (allele), ≥37 vs. <37weeks (BF): Sample size insufficient

Table 61: Genotype and allele frequencies of the ***TNFα*+488G/A** polymorphism in the total **cohort**, **Coloured**, **Black** population and birth weight. Hardy Weinberg Equilibrium (HWE) was determined for each individual group. Statistical analysis was performed using Fisher's exact test analog to determine associations at both genotype and allele level. The corresponding *P* values are shown at the bottom of the table.

	≥2000g (Controls)			**<2000g(Adverse)**		
Genotype	**Total (N=353)**	**Coloured (n=245)**	**Black (N=108)**	**Total (N=30)**	**Coloured (N=27)**	**Black (N=3)**
G/G	321 (90.9%)	218 (89%)	103 (95.4%)	29 (96.7%)	26 (96.3%)	3
G/A	32 (9.1%)	27 (11%)	5 (4.6%)	1 (3.3%)	1 (3.7%)	0
A/A	0	0	0	0	0	0
G-allele	0.955	0.945	0.977	0.983	0.981	1.000
A-allele	0.045	0.055	0.023	0.017	0.019	0.000
HWE	0.672	0.659	0.970	0.996	0.995	-

≥2000g vs. <2000g (group): *P*=0.294, ≥2000g vs. <2000g (CF): *P*=0.248 (allele), ≥2000g vs. <2000g (BF): Sample size insufficient

IL-10 -1082 A/G

Table 62: Genotype and allele frequencies of the ***IL*-10 -1082 A/G** polymorphism in the total **cohort**, **Coloured**, **Black** population and gestation. Hardy Weinberg Equilibrium (HWE) was determined for each individual group. Statistical analysis was performed using Fisher's exact test analog to determine associations at both genotype and allele level. The corresponding *P* values are shown at the bottom of the table. Statistically significant *P* values are shown in bold.

	≥37 (Controls N=370)			**<37 (Adverse N=33)**		
Genotype	**Total (N=370)**	**Coloured (N=261)**	**Black (N=109)**	**Total (N=33)**	**Coloured (N=27)**	**Black (N=6)**
A/A	196 (53%)	132 (50.6%)	64 (58.7%)	25 (75.8%)	19 (70.4%)	6
A/G	147 (39.7%)	111 (42.5%)	36 (33%)	8 (24.2%)	8 (29.6%)	0
G/G	27 (97.3%)	18 (6.9%)	9 (8.3%)	0	0	0
A-allele	0.728	0.718	0.752	0.879	0.852	1.000
G-allele	0.272	0.282	0.248	0.121	0.148	0.000
HWE	0.997	0.711	0.494	0.731	0.665	-

≥37 vs. <37weeks (group): ***P*=0.028** (genotype); ***P*=0.008** (allele), ≥37 vs. <37weeks (CF): ***P*=0.097** (genotype); *P*=0.035 (allele), ≥37 vs. <37weeks (BF): Sample size insufficient

Table 63: Genotype and allele frequencies of the ***IL*-10 -1082 A/G** polymorphism in the total **cohort**, **Coloured**, **Black** population and birth weight. Hardy Weinberg Equilibrium (HWE) was determined for each individual group. Statistical analysis was performed using Fisher's exact test analog to determine associations at both genotype and allele level. The corresponding *P* values are shown at the bottom of the table. Statistically significant *P* values are shown in bold.

	≥2000g (Controls N=367)			**<2000g(Adverse N=31)**		
Genotype	**Total N=367**	**Coloured N=256**	**Black N=111**	**Total N=31**	**Coloured (N=28)**	**Black (N=3)**
A/A	196 (53.4%)	129 (50.4%)	67 (60.4%	21 (67.7%)	19 (67.9%)	2 (66.7%)
A/G	144 (39.2%)	109 (42.6%)	35 (31.5%)	10 (32.3%)	9 (32.1%)	1 (33.3%)
G/G	27 (7.4%)	18 (7%)	9 (8.15%)	0	0	0
A-allele	0.730	0.692	0.761	0.839	0.839	0.833
G-allele	0.270	0.308	0.239	0.161	0.161	0.167
HWE	0.997	0.856	0.377	0.564	0.599	0.942

≥2000g vs. <2000g (group): *P*=0.156 (genotype); *P*=0.062 (allele), ≥2000g vs. <2000g (CF): *P*=0.128 (genotype); ***P*=0.050** (allele), ≥2000g vs. <2000g (BF): Sample size insufficient

IL-10 -819 C/T /-592 C/A

Table 64: Genotype and allele frequencies of the ***IL*-10 -592 C/A** polymorphism in the total **cohort**, **Coloured**, **Black** population and gestation. Hardy Weinberg Equilibrium (HWE) was determined for each individual group. Statistical analysis was performed using Fisher's exact test analog to determine associations at both genotype and allele level.

	≥37 (Controls N=370)			**<37 (Adverse N=33)**		
Genotype	**Total (N=370)**	**Coloured (N=261)**	**Black (N=109)**	**Total (N=33)**	**Coloured (N=27)**	**Black (N=6)**
C/C	135 (36.5%)	94 (36%)	41 (37.6%)	9 (27.3%)	6 (22.2%)	3 (50%)
C/A	198 (53.5%)	141 (54%)	57 (52.3%)	20 (60.6%)	17 (63%)	3 (50%)
A/A	37 (10%)	26 (10%)	11 (10.1%)	4 (12.1%)	4 (14.85)	0
C-allele	0.632	0.630	0.638	0.576	0.537	0.750
A-allele	0.368	0.370	0.362	0.424	0.463	0.250
HWE	**0.015**	**0.037**	0.389	0.385	0.384	0.7165

≥37 vs. <37weeks (group): **not HWE**, ≥37 vs. <37weeks (CF): **not HWE**, ≥37 vs. <37weeks (BF): Sample size insufficient

Table 65: Genotype and allele frequencies of the ***IL*-10 -592 C/A** polymorphism in the total **cohort**, **Coloured**, **Black** population and birth weight. Hardy Weinberg Equilibrium (HWE) was determined for each individual group. Statistical analysis was performed using Fisher's exact test analog to determine associations at both genotype and allele level.

	≥2000g (Controls N=367)			**<2000g(Adverse N=31)**		
Genotype	**Total N=367**	**Coloured N=256**	**Black N=111**	**Total N=31**	**Coloured (N=28)**	**Black (N=3)**
C/C	136 (37.1%)	93 (36.3%)	43 (38.7%)	6 (19.4%)	5 (17.9%)	1 (33.3%)
C/A	195 (53.1%)	138 (53.9%)	57 (51.4%)	21 (67.7%)	19 (67.9%)	2 (66.7%)
A/A	36 (9.8%)	25 (9.8%)	11 (9.9%)	4 (12.9%)	4 (14.2%)	0
C-allele	0.636	0.633	0.644	0.532	0.518	0.667
A-allele	0.364	0.367	0.356	0.468	0.482	0.333
HWE	**0.018**	**0.038**	0.449	0.133	0.165	0.687

≥2000g vs. <2000g (group): **not HWE**, ≥2000g vs. <2000g (CF): **not HWE**, ≥2000g vs. <2000g (BF): Sample size insufficient

***IL*-1β+3954C/T**

Table 66: Genotype and allele frequencies of the ***IL*-1β +3954C/T** polymorphism in the total **cohort**, **Coloured**, **Black** population and gestation. Hardy Weinberg Equilibrium (HWE) was determined for each individual group. Statistical analysis was performed using Fisher's exact test analog to determine associations at both genotype and allele level. The corresponding *P* values are shown at the bottom of the table.

	≥37 (Controls N=370)			**<37 (Adverse N=33)**		
Genotype	**Total (N=370)**	**Coloured (N=261)**	**Black (N=109)**	**Total (N=33)**	**Coloured (N=27)**	**Black (N=6)**
C/C	280 (75.7%)	195 (74.7%	85 (78%)	22 (66.7%)	19 (70.4%)	3 (50%)
C/T	84 (22.7%)	62 (23.8%)	22 (20.2%)	10 (30.3%)	8 (29.6%)	2 (33.3%)
T/T	6 (1.6%)	4 (1.5%)	2 (1.8%)	1 (3%)	0	1 (16.7%)
C-allele	0.870	0.866	0.881	0.818	0.852	0.667
T-allele	0.130	0.134	0.119	0.182	0.148	0.333
HWE	0.995	0.934	0.919	0.994	0.665	0.829

≥37 vs. <37weeks (group): P=0.491 (genotype); P=0.234 (allele), ≥37 vs. <37weeks (CF): P=0.663 (genotype); P=0.774 (allele), ≥37 vs. <37weeks (BF): Sample size insufficient

Table 67: Genotype and allele frequencies of the ***IL*-1β +3954C/T** polymorphism in the total **cohort**, **Coloured**, **Black** population and birth weight. Hardy Weinberg Equilibrium (HWE) was determined for each individual group. Statistical analysis was performed using Fisher's exact test analog to determine associations at both genotype and allele level. The corresponding *P* values are shown at the bottom of the table.

	≥2000g (Controls N=367)			**<2000g(Adverse N=31)**		
Genotype	**Total N=367**	**Coloured N=256**	**Black N=111**	**Total N=31**	**Coloured (N=28)**	**Black (N=3)**
C/C	277 (75.5%)	191 (74.6%)	86 (77.5%)	23 (74.2%)	22 (78.6%)	1 (33.3%)
C/T	83 (22.6%)	61 (23.8%)	22 (19.8%)	8 (25.8%)	6 (21.4%)	2 (66.7%)
T/T	7 (1.9%)	4 (1.6%)	3 (2.7%)	0	0	0
C-allele	0.868	0.865	0.874	0.871	0.893	0.667
T-allele	0.132	0.135	0.126	0.129	0.107	0.333
HWE	0.965	0.941	0.568	0.712	0.817	0.687

≥2000g vs. <2000g (group): P=0.696 (genotype); P=0.944 (allele), ≥2000g vs. <2000g (CF): P=0.759 (genotype); P=0.5621 (allele), ≥2000g vs. <2000g (BF): Sample size insufficient

IL-1*RN*

Table 68: Genotype and allele frequencies of the ***IL*-1*RN*** polymorphism in the total **cohort**, **Coloured**, **Black** population and gestation. Hardy Weinberg Equilibrium (HWE) was determined for each individual group. Statistical analysis was performed using Fisher's exact test analog to determine associations at both genotype and allele level. The corresponding *P* values are shown at the bottom of the table.

	≥37 (Controls N=333)			**<37 (Adverse N=31)**		
Genotype	**Total (N=333)**	**Coloured (N=236)**	**Black (N=97)**	**Total (N=31)**	**Coloured (N=26)**	**Black (N=5)**
1/1	227 (68.2%)	148 (62.7%)	79 (81%)	18 (58.1%)	15 (57.7%)	3 (60%)
1/2	90 (27%)	73 (30.9%)	17 (18%)	12 (38.7%)	10 (38.5 %)	2 (40%)
2/2	16 (4.8%)	15 (6.4%)	1 (1%)	1 (3.2%)	1 (3.8%)	0
1-allele	0.817	0.782	0.902	0.774	0.769	0.667
2-allele	0.183	0.218	0.098	0.226	0.231	0.333
HWE	0.210	0.357	0.997	0.837	0.914	0.829

≥37 vs. <37weeks (group): *P*=0.375 (genotype); *P*=0.410 (allele), ≥37 vs. <37weeks (CF): *P*=0.685 (genotype); *P*=0.836 (allele), ≥37 vs. <37weeks (BF): Sample size insufficient

Table 69: Genotype and allele frequencies of the ***IL*-1*RN*** polymorphism in the total **cohort**, **Coloured**, **Black** population and birth weight. Hardy Weinberg Equilibrium (HWE) was determined for each individual group. Statistical analysis was performed using Fisher's exact test analog to determine associations at both genotype and allele level. The corresponding *P* values are shown at the bottom of the table.

	≥2000g (Controls N=331)			**<2000g(Adverse N=28)**		
Genotype	**Total N=331**	**Coloured N=231**	**Black N=100**	**Total N=28**	**Coloured (N=27)**	**Black (N=1)**
1/1	222 (67.1%)	141 (61%)	81 (81%)	18 (64.3%)	17 (63%)	1
1/2	94 (28.4%)	76 (32.9%)	18 (18%)	9 (32.1%)	9 (33.3%)	0
2/2	15 (4.5%)	14 (6.1%)	1 (1%)	1 (3.6%)	1 (3.7%)	0
1-allele	0.813	0.772	0.900	0.804	0.796	1.000
2-allele	0.187	0.228	0.100	0.196	0.204	0.000
HWE	0.474	0.560	1.000	0.995	0.990	-

≥2000g vs. <2000g (group): *P*=0.901 (genotype); *P*=0.867 (allele), ≥2000g vs. <2000g (CF): *P*=0.884 (genotype); *P*=0.721 (allele), ≥2000g vs. <2000g (BF): Sample size insufficient

IL-4 -590 C/T

Table 70: Genotype and allele frequencies of the ***IL*-4 -590 C/T** polymorphism in the total **cohort**, **Coloured**, **Black** population and gestation. Hardy Weinberg Equilibrium (HWE) was determined for each individual group. Statistical analysis was performed using Fisher's exact test analog to determine associations at both genotype and allele level. The corresponding *P* values are shown at the bottom of the table.

	≥37 (Controls N=369)			<37 (Adverse N=33)		
Genotype	**Total (N=369)**	**Coloured (N=260)**	**Black (N=109)**	**Total (N=33)**	**Coloured (N=27)**	**Black (N=6)**
C/C	63 (17.1%)	55 (21.2%)	8 (7.3%)	1 (3%)	1 (3.7%)	0
C/T	154 (41.7%)	113 (43.3%)	41 (37.6%)	17 (51.5%)	14 (51.9%)	3 (50%)
T/T	152 (41.2%)	92 (35.4%)	60 (55.1%)	15 (45.5%)	12 (44.4%)	3 (50%)
C-allele	0.379	0.429	0.261	0.288	0.296	0.250
T-allele	0.621	0.571	0.739	0.712	0.704	0.750
HWE	0.092	0.191	0.964	0.338	0.449	0.717

≥37 vs. <37weeks (group): P=0.102 (genotype); P=0.141 (allele), ≥37 vs. <37weeks (CF): P=0.093 (genotype); P=0.060 (allele), ≥37 vs. <37weeks (BF): Sample size insufficient

Table 71: Genotype and allele frequencies of the ***IL*-4 -590 C/T** polymorphism in the total **cohort**, **Coloured**, **Black** population and birth weight. Hardy Weinberg Equilibrium (HWE) was determined for each individual group. Statistical analysis was performed using Fisher's exact test analog to determine associations at both genotype and allele level. The corresponding *P* values are shown at the bottom of the table.

	≥2000g (Controls N=367)			<2000g(Adverse N=31)		
Genotype	**Total N=367**	**Coloured N=256**	**Black N=111**	**Total N=31**	**Coloured (N=28)**	**Black (N=3)**
C/C	61 (16.6%)	53 (20.7%)	8 (7.2%)	31 (10%)	3 (11.1%)	0
C/T	154 (42%)	113 (44.1%)	41 (36.9%)	16 (53.3%)	14 (51.9%)	2 (66.7%)
T/T	152 (41.4%)	90 (35.2%)	62 (55.9%)	11 (36.7%)	10 (37%)	1 (33.3%)
C-allele	0.376	0.428	0.257	0.367	0.370	0.333
T-allele	0.624	0.572	0.743	0.633	0.630	0.667
HWE	0.128	0.289	0.944	0.719	0.845	0.687

≥2000g vs. <2000g (group): *P*=0.418 (genotype); *P*=0.886 (allele), ≥2000g vs. <2000g (CF): *P*=0476 (genotype); *P*=0.417 (allele), ≥2000g vs. <2000g (BF): Sample size insufficient

*LGALS*13 gene variants

-98 A/C

Table 72: Genotype and allele frequencies of the **-98 A/C** variant in the total **cohort**, **Coloured**, **Black** population and birth weight. Hardy Weinberg Equilibrium (HWE) was determined for each individual group. Statistical analysis was performed using Fisher's exact test analog to determine associations at both genotype and allele level. The corresponding *P* values are shown at the bottom of the table.

	≥37 (Controls N=370)			**<37 (Adverse N=33)**		
Genotype	**Total (N=370)**	**Coloured (N=261)**	**Black (N=109)**	**Total (N=33)**	**Coloured (N=27)**	**Black (N=6)**
A/A	194 (52.4%)	137 (52.5%)	57 (52.3%)	16 (48.5%)	14 (50%)	3 (50%)
A//C	148 (40%)	100 (38.3%)	48 (44%)	15 (45.5%)	14 (50%)	1 (16.7%)
C/C	28 (7.6%)	24 (9.2%)	4 (3.7%)	2 (6%)	0	2 (33.3%)
A-allele	0.724	0.716	0.743	0.712	0.750	0.583
C-allele	0.276	0.284	0.257	0.288	0.250	0.417
HWE	0.999	0.655	0.277	0.823	0.211	0.274

≥37 vs. <37weeks (group): *P*=0.816 (genotype); *P*=0.832 (allele), ≥37 vs. <37weeks (CF): *P*=0.176 (genotype); *P*=0.596 (allele), ≥37 vs. <37weeks (BF): Sample size insufficient

Table 73: Genotype and allele frequencies of the **-98 A/C** variant in the total **cohort**, **Coloured, Black** population and birth weight. Hardy Weinberg Equilibrium (HWE) was determined for each individual group. Statistical analysis was performed using Fisher's exact test analog to determine associations at both genotype and allele level. The corresponding *P* values are shown at the bottom of the table.

	≥2000g (Controls N=367)			**<2000g(Adverse N=31)**		
Genotype	**Total N=367**	**Coloured N=256**	**Black N=111**	**Total N=31**	**Coloured (N=28)**	**Black (N=3)**
A/A	191 (52%)	133 (52%)	58 (52.3%)	15 (48.4%)	14 (50%)	1 (33.3%)
A/C	147 (40.1%)	99 (38.7%)	48 (43.2%)	15 (48.4%)	14 (50%)	1 (33.3%)
C/C	29 (7.9%)	24 (9.3%)	5 (4.5%)	1 (3.2%)	0	1 (33.3%)
A-allele	0.721	0.713	0.739	0.726	0.750	0.5
C-allele	0.279	0.287	0.261	0.274	0.250	0.5
HWE	0.995	0.676	0.448	0.4862	0.211	0.847

≥2000g vs. <2000g (group): *P*=0.499 (genotype); *P*=0.932 (allele), ≥2000g vs. <2000g (CF): *P*=0.177 (genotype); *P*=0.559 (allele), ≥2000g vs. <2000g (BF): Sample size insufficient

IVS2-36 G/A

Table 74: Genotype and allele frequencies of the **IVS2-36 G/A** variant in the total **cohort**, **Coloured, Black** population and gestation. Hardy Weinberg Equilibrium (HWE) was determined for each individual group. Statistical analysis was performed using Fisher's exact test analog to determine associations at both genotype and allele level. The corresponding *P* values are shown at the bottom of the table.

	≥37 (Controls N=370)			**<37 (Adverse N=33)**		
Genotype	**Total (N=370)**	**Coloured (N=261)**	**Black (N=109)**	**Total (N=33)**	**Coloured (N=27)**	**Black (N=6)**
G/G	343 (92.7%)	246 (94.3%)	97 (89%)	31 (93.9%)	26 (96.3%)	5 (83.3%)
G/A	27 (7.3%)	15 (5.7%)	12 (11%)	2 (6.1%)	1 (3.7%)	1 (16.7%)
A/A	0	0	0	0	0	0
G-allele	0.964	0.971	0.945	0.970	0.981	0.917
A-allele	0.036	0.029	0.055	0.030	0.019	0.083
HWE	0.767	0.892	0.831	0.984	0.995	0.976

≥37 vs. <37weeks (group): *P*=0.796 (allele), ≥37 vs. <37weeks (CF): *P*=0.082 (allele), ≥37 vs. <37weeks (BF): Sample size insufficient

Table 75: Genotype and allele frequencies of the **IVS2-36 G/A** variant in the total **cohort**, **Coloured, Black** population and birth weight. Hardy Weinberg Equilibrium (HWE) was determined for each individual group. Statistical analysis was performed using Fisher's exact test analog to determine associations at both genotype and allele level. The corresponding *P* values are shown at the bottom of the table.

	≥2000g (Controls N=367)			**<2000g(Adverse N=31)**		
Genotype	**Total N=367**	**Coloured N=256**	**Black N=111**	**Total N=31**	**Coloured (N=28)**	**Black (N=3)**
G/G	340 (92.6%)	241 (94.1%)	99 (89.2%)	29 (93.5%)	27 (96.4%)	2 (66.7%)
G/A	27 (7.4%)	15 (5.9%)	12 (10.8%)	2 (6.5%)	1 (3.6%)	1 (33.3%)
A/A	0	0	0	0	0	0
G-allele	0.963	0.971	0.946	0.968	0.982	0.833
A-allele	0.037	0.029	0.054	0.032	0.018	0.167
HWE	0.765	0.890	0.834	0.983	0.995	0.942

≥37 vs. <37weeks (group): *P*=0.855 (allele), ≥37 vs. <37weeks (CF): *P*=0.623 (allele), ≥37 vs. <37weeks (BF): Sample size insufficient

IVS2-22 A/G

Table 76: Genotype and allele frequencies of the **IVS2-22 G/A** variant in the total **cohort**, **Coloured, Black** population and gestation. Hardy Weinberg Equilibrium (HWE) was determined for each individual group. Statistical analysis was performed using Fisher's exact test analog to determine associations at both genotype and allele level. The corresponding *P* values are shown at the bottom of the table.

	≥37 (Controls N=370)			**<37 (Adverse N=33)**		
Genotype	**Total (N=370)**	**Coloured (N=261)**	**Black (N=109)**	**Total (N=33)**	**Coloured (N=27)**	**Black (N=6)**
A/A	258 (69.7%)	194 (74.3%)	64 (58.7%)	28 (84.8%)	23 (85.2%)	5 (83.3%)
A/G	102 (27.6%)	63 (24.1%)	39 (35.8%)	5 (15.2%)	4 (14.8%)	1 (16.7%)
G/G	10 (2.7%)	4 (1.5%)	6 (5.5%)	0	0	0
A-allele	0.835	0.864	0.766	0.924	0.926	0.917
G-allele	0.165	0.136	0.234	0.076	0.074	0.083
HWE	0.9998	0.909	0.9998	0.896	0.917	0.976

≥37 vs. <37weeks (group): *P*=0.163 (genotype); *P*=0.057 (allele), ≥37 vs. <37weeks (CF): *P*=0.425 (genotype); *P*=0.198 (allele), ≥37 vs. <37weeks (BF): Sample size insufficient

Table 77: Genotype and allele frequencies of the **IVS2-22 G/A** variant in the total **cohort**, **Coloured, Black** population and birth weight. Hardy Weinberg Equilibrium (HWE) was determined for each individual group. Statistical analysis was performed using Fisher's exact test analog to determine associations at both genotype and allele level. The corresponding *P* values are shown at the bottom of the table.

	≥2000g (Controls N=367)			**<2000g(Adverse N=31)**		
Genotype	**Total N=367**	**Coloured N=256**	**Black N=111**	**Total N=31**	**Coloured (N=28)**	**Black (N=3)**
A/A	259 (70.6%)	194 (75.8%)	65 (58.6%)	24 (77.4%)	21 (75%)	3
A/G	99 (27%)	59 (23%)	40 (36%)	6 (19.4%)	6 (21.4%)	0
G/G	9 (2.4%)	3 (1.2%)	6 (5.4%)	1 (3.2%)	1 (3.6%)	0
A-allele	0.841	0.873	0.766	0.871	0.857	1.000
G-allele	0.159	0.127	0.234	0.129	0.143	0.000
HWE	0.992	0.817	0.999	0.742	0.804	-

≥2000g vs. <2000g (group): *P*=0.642 (genotype); *P*=0.528 (allele), ≥2000g vs. <2000g (CF): *P*=0.588 (genotype); *P*=0.736 (allele), ≥2000g vs. <2000g (BF): Sample size insufficient

IVS2-15 G/A

Table 78: Genotype and allele frequencies of the **IVS2-15 G/A** variant in the total **cohort**, **Coloured, Black** population and gestation. Hardy Weinberg Equilibrium (HWE) was determined for each individual group. Statistical analysis was performed using Fisher's exact test analog to determine associations at both genotype and allele level. The corresponding *P* values are shown at the bottom of the table.

	≥37 (Controls N=370)			**<37 (Adverse N=33)**		
Genotype	**Total (N=370)**	**Coloured (N=261)**	**Black (N=109)**	**Total (N=33)**	**Coloured (N=27)**	**Black (N=6)**
G/G	36 (97.8%)	255 (97.7%)	107 (98.2%)	33	27	6
G/A	8 (2.2%)	6 (2.3%)	2 (1.8%)	0	0	0
A/A	0	0	0	0	0	0
G-allele	0.989	0.989	0.991	1.000	1.000	1.000
A-allele	0.011	0.011	0.009	0.000	0.000	0.000
HWE	0.978	0.953	0.995	-	-	-

≥37 vs. <37weeks (group): *P*=0.396 (allele), ≥37 vs. <37weeks (CF): *P*=0.428 (allele), ≥37 vs. <37weeks (BF): Sample size insufficient

Table 79: Genotype and allele frequencies of the **IVS2-15 G/A** variant in the total **cohort**, **Coloured, Black** population and birth weight. Hardy Weinberg Equilibrium (HWE) was determined for each individual group. Statistical analysis was performed using Fisher's exact test analog to determine associations at both genotype and allele level. The corresponding *P* values are shown at the bottom of the table.

	≥2000g (Controls N=367)			**<2000g(Adverse N=31)**		
Genotype	**Total N=367**	**Coloured N=256**	**Black N=111**	**Total N=31**	**Coloured (N=28)**	**Black (N=3)**
G/G	360 (98.1%)	251 (98%)	109 (98.2%)	30 (96.8%)	27 (96.4%)	3
G/A	7 (0.9%)	5 (2%)	2 (1.8%)	1 (3.2%)	1 (3.6%)	0
A/A	0	0	0	0	0	0
G-allele	0.990	0.990	0.990	0.984	0.982	1.000
A-allele	0.010	0.010	0.010	0.016	0.018	0.000
HWE	0.983	0.988	0.995	0.996	0.995	-

≥2000g vs. <2000g (group): *P*=0.617 (allele), ≥2000g vs. <2000g (CF): *P*=0.574 (allele), ≥2000g vs. <2000g (BF): Sample size insufficient

‘Hotspot’

Table 80: Genotype and allele frequencies of the **‘hotspot’** variant in the total **cohort**, **Coloured, Black** population and gestation. Hardy Weinberg Equilibrium (HWE) was determined for each individual group. Statistical analysis was performed using Fisher's exact test analog to determine associations at both genotype and allele level. The corresponding *P* values are shown at the bottom of the table.

	≥37 (Controls N=370)			**<37 (Adverse N=33)**		
Genotype	**Total (N=370)**	**Coloured (N=261)**	**Black (N=109)**	**Total (N=33)**	**Coloured (N=27)**	**Black (N=6)**
- / -	344 (93%)	246 (94.3%)	98 (89.9%)	31 (93.9%)	26 (96.3%)	5 (83.3%)
- / HS	26 (7%)	15 (5.7%)	11 (10.1%)	2 (6.1%)	1 (3.7%)	1 (16.7%)
HS / HS	0	0	0	0	0	0
- allele	0.965	0.971	0.950	0.970	0.981	0.917
HS allele	0.035	0.029	0.050	0.030	0.019	0.083
HWE	0.783	0.892	0.857	0.984	0.995	0.976

≥37 vs. <37weeks (group): *P*=0.837 (allele), ≥37 vs. <37weeks (CF): *P*=0.664 (allele), ≥37 vs. <37weeks (BF): Sample size insufficient

Table 81: Genotype and allele frequencies of the **‘hotspot’** variant in the total **cohort**, **Coloured, Black** population and birth weight. Hardy Weinberg Equilibrium (HWE) was determined for each individual group. Statistical analysis was performed using Fisher's exact test analog to determine associations at both genotype and allele level. The corresponding *P* values are shown at the bottom of the table.

	≥2000g (Controls N=367)			**<2000g(Adverse N=31)**		
Genotype	**Total N=367**	**Coloured N=256**	**Black N=111**	**Total N=31**	**Coloured (N=28)**	**Black (N=3)**
- / -	341 (92.9%)	241 (94.1%)	100 (90.1%)	29 (93.5%)	27 (96.4%)	2 (66.7%)
- / HS	26 (7.01%)	15 (5.9%)	11 (9.9%)	2 (6.5%)	1 (3.6%)	1 (33.3%)
HS / HS	0	0	0	0	0	0
- allele	0.965	0.971	0.950	0.968	0.982	0.833
HS allele	0.035	0.029	0.050	0.032	0.018	0.167
HWE	0.781	0.890	0.860	0.983	0.995	0.942

≥2000g vs. <2000g (group): *P*=0.897 (allele), ≥2000g vs. <2000g (CF): *P*=0.623 (allele), ≥2000g vs. <2000g (BF): Sample size insufficient

221delT/ L74W

Table 82: Genotype and allele frequencies of the **221delT** in the total **cohort**, **Coloured**, **Black** population and gestation. Hardy Weinberg Equilibrium (HWE) was determined for each individual group. Statistical analysis was performed using Fisher's exact test analog to determine associations at both genotype and allele level. The corresponding *P* values are shown at the bottom of the table. Statistically significant *P* values are shown in bold.

	≥37 (Controls N=370)			**<37 (Adverse N=33)**		
Genotype	**Total (N=370)**	**Coloured (N=261)**	**Black (N=109)**	**Total (N=33)**	**Coloured (N=27)**	**Black (N=6)**
- / -	360 (97.3%)	256 (98.1%)	104 (95.4%)	30 (90.9%)	24 (88.9%)	6
- / del	10 (2.7%)	5 (1.95%)	5 (4.6%)	3 (9.1%)	3 (11.1%)	0
del / del	0	0	0	0	0	0
- allele	0.986	0.990	0.977	0.955	0.944	1.000
del allele	0.014	0.010	0.023	0.045	0.056	0.000
HWE	0.966	0988	0.970	0.963	0.954	-

≥37 vs. <37weeks (group): ***P*=0.048** (allele), ≥37 vs. <37weeks (CF): ***P*=0.006** (allele), ≥37 vs. <37weeks (BF): Sample size insufficient

Table 83: Genotype and allele frequencies of the **221delT** in the total **cohort**, **Coloured**, **Black** population and birth weight. Hardy Weinberg Equilibrium (HWE) was determined for each individual group. Statistical analysis was performed using Fisher's exact test analog to determine associations at both genotype and allele level. The corresponding *P* values are shown at the bottom of the table.

	≥2000g (Controls N=367)			**<2000g(Adverse N=31)**		
Genotype	**Total N=367**	**Coloured N=256**	**Black N=111**	**Total N=31**	**Coloured (N=28)**	**Black (N=3)**
- / -	357 (9.3%)	250 (97.7%)	107 (96.4%)	29 (93.5%)	27 (96.4%)	2 (33.7%)
- / del	10 (2.7%)	6 (2.3%)	4 (3.6%)	2 (6.5%)	1 (3.6%)	1 (33.3%)
del / del	0	0	0	0	0	0
- allele	0.986	0.988	0.982	0.968	0.982	0.833
del allele	0.014	0.012	0.018	0.032	0.018	0.167
HWE	0.966	0.982	0.982	0.983	0.995	0.942

≥2000g vs. <2000g (group): *P*=0.248 (allele), ≥2000g vs. <2000g (CF): *P*=0.693 (allele), ≥2000g vs. <2000g (BF): Sample size insufficient

IVS3+72 T/A

Table 84: Genotype and allele frequencies of the **IVS3+72 T/A** in the total **cohort**, **Coloured**, **Black** population and gestation. Hardy Weinberg Equilibrium (HWE) was determined for each individual group. Statistical analysis was performed using Fisher's exact test analog to determine associations at both genotype and allele level. The corresponding *P* values are shown at the bottom of the table.

	≥37 (Controls N=370)			**<37 (Adverse N=33)**		
Genotype	**Total (N=370)**	**Coloured (N=261)**	**Black (N=109)**	**Total (N=33)**	**Coloured (N=27)**	**Black (N=6)**
T/T	260 (70.3%)	193 (73.9%)	67 (61.5%)	27 (81.8%)	23 (85.2%)	4 (66.7%)
T/A	102 (27.6%)	65 (24.9%)	37 (33.9%)	6 (18.2%)	4 (14.8%)	2 (33.3%)
A/A	8 (2.1%)	3 (1.2%)	5 (4.6%)	0	0	0
T-allele	0.841	0.864	0.784	0.909	0.926	0.833
A-allele	0.159	0.136	0.216	0.091	0.074	0.167
HWE	0.861	0.629	0.999	0.848	0.917	0.887

≥37 vs. <37weeks (group): P=0.320 (genotype); P=0.139 (allele), ≥37 vs. <37weeks (CF): P=0.414 (genotype); P=0.198 (allele), ≥37 vs. <37weeks (BF): Sample size insufficient

Table 85: Genotype and allele frequencies of the **IVS3+72 T/A** in the total **cohort**, **Coloured**, **Black** population and birth weight. Hardy Weinberg Equilibrium (HWE) was determined for each individual group. Statistical analysis was performed using Fisher's exact test analog to determine associations at both genotype and allele level. The corresponding *P* values are shown at the bottom of the table.

	≥2000g (Controls N=367)			**<2000g(Adverse N=31)**		
Genotype	**Total N=367**	**Coloured N=256**	**Black N=111**	**Total N=31**	**Coloured (N=28)**	**Black (N=3)**
T/T	260 (70.8%)	193 (75.4%)	67 (60.4%)	24 (77.4%)	21 (75%)	3
T/A	100 (27.2%)	61 (23.8%)	39 (35.1%)	6 (19.4%)	6 (21.4%)	0
A/A	7 (2%)	2 (0.8%)	5 (4.5%)	1 (3.2%)	1 (3.6%)	0
T-allele	0.845	0.873	0.779	0.871	0.857	1.000
A-allele	0.155	0.127	0.221	0.129	0.143	0.000
HWE	0.762	0.487	0.975	0.742	0.804	-

≥2000g vs. <2000g (group): *P*=0.580 (genotype); *P*=0.581 (allele), ≥2000g vs. <2000g (CF): *P*=0.382 (genotype); *P*=0.736 (allele), ≥2000g vs. <2000g (BF): Sample size insufficient

Appendix 12: 2X2 contingency analyses of associations demonstrated in this study

CF-Coloured females; BF-Black females

Genotype analysis

IL-1RA (raw analysis)

Hardy-Weinberg

	Controls			Poor outcome			PTL			Hypertension		
	Cohort	CF	BF	Cohort	CF	BF	Cohort	CF	BF	Cohort	CF	BF
1/1	212 70.2%	138 65.1%	74 82.2%	42 (53.2%)	34 (50.7%)	8 66.7%	15 48.4%	12 46.2%	3 60%	15 44.1%	11 39.3%	4 66.7%
1/2	77 25.5%	62 29.2%	15 16.7%	32 (40.5%)	28 (41.8%)	4 33.3%	15 48.4%	13 50%	2 40%	15 44.1%	13 46.4%	2 33.3%
2/2	13 4.3%	12 5.7%	1 1.1%	5 (6.3%)	5 (7.5%)	0	1 3.2%	1 3.8%	0	4 11.8%	4 14.3%	0
1	0.829	0.797	0.906	0.734	0.716	0.833	0.726	0.712	0.800	0.662	0.625	0.833
2	0.171	0.203	0.094	0.266	0.284	0.167	0.274	0.288	0.200	0.338	0.375	0.167
P	0.229	0.379	0.971	0.945	0.973	0.787	0.486	0.539	0.855	0.996	0.999	0.887

Fishers Exact Test

	Control vs. Poor (Cohort)		Control vs. Poor (CF)		Control vs. Poor (BF)	
	Genotype	Allele	Genotype	Allele	Genotype	Allele
P	**0.017**	**0.0066**	0.108	**0.050**	0.362	0.2741

	Control vs. PTL (Cohort)		Control vs. PTL (CF)		Control vs. PTL (BF)	
	Genotype	Allele	Genotype	Allele	Genotype	Allele
P	**0.025**	**0.0431**	0.099	0.154	0.410	0.2788

	Control vs. Hyper (Cohort)		Control vs. Hyper (CF)		Control vs. Hyper (BF)	
	Genotype	Allele	Genotype	Allele	Genotype	Allele
P	**0.006**	**0.0008**	**0.021**	**0.0036**	0.572	0.4172

2X2 Tables

Good vs. Poor (Cohort)

1/1&1/2 vs. 2/2

5	13
74	289

OR	1.50 (0.41-4.66)
RR	1.36 (0.63-2.95)
Uncorrected	0.4502
Mantel-Haenszel	0.4508
Yates corrected	0.6475
1-tailed	0.3092
2-tailed	0.5494

1/1 vs. 1/2&2/2

37	90
42	212

OR	**2.08 (1.20-3.55)**
RR	**1.76 (1.20-2.60)**
Uncorrected	**0.0042**
Mantel-Haenszel	**0.0043**
Yates corrected	**0.0064**

Good vs. Poor (CF)

1/1&1/2 vs. 2/2

5	12
62	200

OR	1.34 (0.36-4.29)
RR	1.24 (0.58-2.68)
Uncorrected	0.5909
Mantel-Haenszel	0.5915
Yates corrected	0.8067
1-tailed	0.3873
2-tailed	0.5660

1/1 vs. 1/2&2/2

33	74
34	138

OR	**1.86 (1.03-3.39)**
RR	**1.60 (1.05-2.43)**
Uncorrected	**0.0277**
Mantel-Haenszel	**0.0280**
Yates corrected	**0.0398**

Good vs. Poor (BF)

1/1&1/2 vs. 2/2

0	1
12	89

OR	0.00 (0.00-292.50)
RR	-
Uncorrected	0.7137
Mantel-Haenszel	0.7150
Yates corrected	0.2330
1-tailed	0.8824
2-tailed	1.0000

1/1 vs. 1/2&2/2

4	16
8	74

OR	**2.31 (0.45-9.90)**
RR	**2.05 (0.68-6.14)**
Uncorrected	0.2023
Mantel-Haenszel	0.2046
Yates corrected	0.3746
1-tailed	0.1832
2-tailed	0.2446

Good vs. PTL (Cohort)
1/1&1/2 vs. 2/2

1	13
30	289

OR	0.74 (0.02-5.28)
RR	0.76 (0.11-5.18)
Uncorrected	0.7756
Mantel-Haenszel	0.7759
Yates corrected	0.8533
1-tailed	0.6188
2-tailed	1.000

1/1 vs. 1/2&2/2

16	90
15	212

OR	**2.51 (1.12-5.64)**
RR	**2.28 (1.17-4.44)**
Uncorrected	**0.0130**
Mantel-Haenszel	**0.0132**
Yates corrected	**0.0226**

Good vs. PTL (CF)
1/1&1/2 vs. 2/2

1	12
25	200

OR	0.67 (0.02-4.89)
RR	0.69 (0.10-4.72)
Uncorrected	0.7008
Mantel-Haenszel	0.7014
Yates corrected	0.9418
1-tailed	0.5734
2-tailed	1.0000

1/1 vs. 1/2&2/2

14	74
12	138

OR	**2.18 (0.89-5.33)**
RR	**1.99 (0.96-4.10)**
Uncorrected	0.0589
Mantel-Haenszel	0.0595
Yates corrected	0.0943

Good vs. PTL (BF)
1/1&1/2 vs. 2/2

0	1
5	89

OR	0.00 (0.00-702.00)
RR	-
Uncorrected	0.8127
Mantel-Haenszel	0.8137
Yates corrected	0.0439
1-tailed	0.9474
2-tailed	1.0000

1/1 vs. 1/2&2/2

2	16
3	74

OR	3.08 (0.24-28.84)
RR	2.85 (0.51-15.83)
Uncorrected	0.2171
Mantel-Haenszel	0.2196
Yates corrected	0.5170
1-tailed	0.2386
2-tailed	0.2386

Good vs. Hypertension (Cohort)
1/1&1/2 vs. 2/2

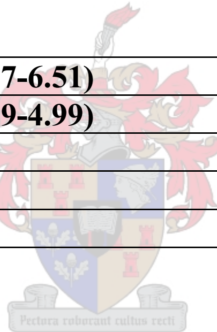
4	13
30	289

OR	2.96 (0.66-10.37)
RR	2.50 (0.99-6.29)
Uncorrected	0.0599
Mantel-Haenszel	0.0603
Yates corrected	0.1418
1-tailed	0.0803
2-tailed	0.0803

1/1 vs. 1/2&2/2

19	90
15	212

OR	**2.98 (1.37-6.51)**
RR	**2.64 (1.39-4.99)**
Uncorrected	**0.0021**
Mantel-Haenszel	**0.0021**
Yates corrected	**0.0039**



Good vs. Hypertension (CF)
1/1&1/2 vs. 2/2

4	12
24	200

OR	2.78 (0.60-10.10)
RR	2.33 (0.92-5.91)
Uncorrected	0.0855
Mantel-Haenszel	0.0861
Yates corrected	0.1879
1-tailed	0.1002
2-tailed	0.1002

1/1 vs. 1/2&2/2

17	74
11	138

OR	**2.88 (1.20-6.99)**
RR	**2.53 (1.24-5.16)**
Uncorrected	**0.0082**
Mantel-Haenszel	**0.0083**
Yates corrected	**0.0148**

Good vs. Hypertension (BF)
1/1&1/2 vs. 2/2

0	1
6	89

OR	0.00 (0.00-585.00)
RR	-
Uncorrected	0.7952
Mantel-Haenszel	0.7963
Yates corrected	0.0692
1-tailed	0.9375
2-tailed	1.0000

1/1 vs. 1/2&2/2

2	16
4	74

OR	**2.31 (0.27**
RR	**2.17 (0.43-10.93)**
Uncorrected	0.3445
Mantel-Haenszel	0.3471
Yates corrected	0.6854
1-tailed	0.3130
2-tailed	0.3130

TNF-308 G/A
Hardy-Weinberg

	Controls			Poor outcome			PTL			Hypertension		
	Cohort	CF	BF	Cohort	CF	BF	Cohort	CF	BF	Cohort	CF	BF
G/G	253 74.9%	189 79.7%	64 63.4%	54 (65.1%)	47 (68.1%)	7 50%	20 60.6%	17 63%	3 50%	24 68.6%	20 69%	4 66.7%
G/A	85 25.1%	48 20.3%	37 36.6%	28 (33.7%)	21 (30.4%)	7 50%	12 36.4%	9 33.3%	3 50%	11 31.4%	9 31%	2 33.3%
A/A	0	0	0	1 (1.2%)	1 (1.5%)	0	1 3%	1 3.7%	0	0	0	0
G	0.874	0.899	0.817	0.819	0.833	0.75	0.788	0.796	0.75	0.843	0.845	0.833
A	0.124	0.101	0.183	0.183	0.167	0.25	0.212	0.204	0.25	0.157	0.155	0.167
P	**0.030**	0.222	0.079	0.436	0.729	0.459	0.880	0.99	0.717	0.544	0.613	0.887

Fishers Exact Test

	Control vs. Poor (Cohort)		Control vs. Poor (CF)		Control vs. Poor (BF)	
	Genotype	Allele	Genotype	Allele	Genotype	Allele
P	**Not HWE**	**Not HWE**	**0.033**	**0.0347**	-	0.3995

	Control vs. PTL (Cohort)		Control vs. PTL (CF)		Control vs. PTL (BF)	
	Genotype	Allele	Genotype	Allele	Genotype	Allele
P	**Not HWE**	**Not HWE**	**0.003**	**0.0236**	-	0.564

	Control vs. Hyper (Cohort)		Control vs. Hyper (CF)		Control vs. Hyper (BF)	
	Genotype	Allele	Genotype	Allele	Genotype	Allele
P	**Not HWE**	**Not HWE**	-	0.2102	-	0.8856

2X2 Tables

Good vs. Poor (Cohort) **Not HWE**
G/G&G/A vs. A/A
G/G vs. G/A&A/A

Good vs. Poor (CF)
G/G&G/A vs. A/A

1	0
68	237

OR	Undefined
RR	4.49 (3.64-5.53)
Uncorrected	0.0630
Mantel-Haenszel	0.0638
Yates corrected	0.5106
1-tailed	0.2255
2-tailed	0.2255

G/G vs. G/A&A/A

22	48
47	189

OR	**1.84 (0.96-3.47)**
RR	**1.58 (1.03-2.43)**
Uncorrected	**0.0429**
Mantel-Haenszel	**0.0433**
Yates corrected	0.0627

Good vs. Poor (BF)
G/G&G/A vs. A/A

7	37
7	64

OR	1.73 (0.50-6.05)
RR	1.61 (0.61-4.29)
Uncorrected	0.3349
Mantel-Haenszel	0.3369
Yates corrected	0.5022

Good vs. PTL (CF)
G/G&G/A vs. A/A

1	0
26	237

OR	Undefined
RR	10.12 (7.02-14.57)
Uncorrected	0.0029
Mantel-Haenszel	0.0030
Yates corrected	0.1885
1-tailed	0.1022
2-tailed	0.1022

G/G vs. G/A&A/A

10	48
17	189

OR	**2.32 (0.92-5.78)**
RR	**2.09 (1.01-4.31)**
Uncorrected	**0.0459**
Mantel-Haenszel	**0.0464**
Yates corrected	0.0800

Good vs. PTL (BF)
G/G&G/A vs. A/A

7	37
7	64

OR	1.73 (0.50-6.05)
RR	1.61 (0.61-4.29)
Uncorrected	0.3349
Mantel-Haenszel	0.337
Yates corrected	0.5022

Good vs. Hypertension (CF)
G/G&G/A vs. A/A

9	48
20	189

OR	1.77 (0.70-4.43)
RR	0.65 (0.80-3.42)
Uncorrected	0.0792
Mantel-Haenszel	0.0799
Yates corrected	0.1327

Good vs. Hypertension (BF)
G/G&G/A vs. A/A

2	37
4	64

OR	0.86 (0.19-3.35)
RR	0.87 (0.17-4.54)
Uncorrected	0.8704
Mantel-Haenszel	0.871
Yates corrected	0.7846
1-tailed	0.6194
2-tailed	1.0000

*LGALS*13 221delT

Hardy-Weinberg

	Controls			Poor outcome			PTL			Hypertension		
	Cohort	CF	BF	Cohort	CF	BF	Cohort	CF	BF	Cohort	CF	BF
- / -	330 97.6%	233 98.3%	97 96%	77 (92.8%)	64 (92.8%)	13 92.9%	30 90.9%	25 92.6%	5 83.3%	33 94.3%	27 93.1%	6
- / del	8 2.4%	4 1.7%	4 4%	6 (7.2%)	5 (7.2%)	1 7.1%	3 9.1%	2 7.4%	1 16.7%	2 5.7%	2 6.9%	0
del/del	0	0	0	0	0	0	0	0	0	0	0	0
-	0.988	0.992	0.980	0.964	0.964	0.964	0.955	0.963	0.917	0.971	0.966	1.000
del	0.012	0.008	0.020	0.036	0.036	0.036	0.045	0.037	0.083	0.029	0.034	0.000
P	0.976	0.992	0.979	0.943	0.952	0.990	0.963	0.9802	0.976	0.985	0.982	-

Fishers Exact Test

	Control vs. Poor (Cohort)		Control vs. Poor (CF)		Control vs. Poor (BF)	
	Genotype	Allele	Genotype	Allele	Genotype	Allele
P	-	**0.0282**	-	**0.017**	-	0.5884

	Control vs. PTL (Cohort)		Control vs. PTL (CF)		Control vs. PTL (BF)	
	Genotype	Allele	Genotype	Allele	Genotype	Allele
P	-	**0.031**	-	0.0603	-	0.1569

	Control vs. Hyper (Cohort)		Control vs. Hyper (CF)		Control vs. Hyper (BF)	
	Genotype	Allele	Genotype	Allele	Genotype	Allele
P	-	0.2464	-	0.0762	-	0.6227

2X2 Tables

Good vs. Poor (Cohort)

- -vs. - /del&del/del

6	8
77	330

OR	**3.21 (0.89-10.08)**
RR	**2.27 (1.20-4.29)**
Uncorrected	**0.0269**
Mantel-Haenszel	**0.0270**
Yates corrected	0.0612
1-tailed	**0.0385**
2-tailed	**0.0385**

Good vs. Poor (CF)

- -vs. - /del&del/del

5	4
64	233

OR	**4.55 (0.94-23.48)**
RR	**2.58 (1.38-4.81)**
Uncorrected	**0.0162**
Mantel-Haenszel	**0.0163**
Yates corrected	**0.0455**
1-tailed	**0.0301**
2-tailed	**0.0301**

Good vs. Poor (BF)

- -vs. - /del&del/del

1	4
13	97

OR	1.87 (0.04-20.74)
RR	1.69 (0.27-10.51)
Uncorrected	0.5842
Mantel-Haenszel	0.5859
Yates corrected	0.8791
1-tailed	0.4839
2-tailed	0.4839

Good vs. PTL (Cohort)
- -vs. - /del&del/del

3	8
30	330

OR	**4.13 (0.67-18.26)**
RR	**3.27 (1.18-9.11)**
Uncorrected	**0.0297**
Mantel-Haenszel	**0.0299**
Yates corrected	0.1018
1-tailed	0.0647
2-tailed	0.0647

Good vs. PTL (CF)
- -vs. - /del&del/del

2	4
25	233

OR	**4.66 (0.40-34.09)**
RR	**3.44 (1.05-11.32)**
Uncorrected	0.0588
Mantel-Haenszel	0.0593
Yates corrected	0.2270
1-tailed	0.1169
2-tailed	0.1169

Good vs. PTL (BF)
- -vs. - /del&del/del

1	4
5	97

OR	4.85 (0.08-62.31)
RR	4.08 (0.58-28.69)
Uncorrected	0.1519
Mantel-Haenszel	0.1539
Yates corrected	0.6619
1-tailed	0.2549
2-tailed	0.2549

Good vs. Hypertension (Cohort)
- -vs. - /del&del/del

2	8
33	330

OR	**2.50 (0.25-13.22)**
RR	**2.20 (0.61-7.93)**
Uncorrected	0.2432
Mantel-Haenszel	0.2438
Yates corrected	0.5369
1-tailed	0.2392
2-tailed	0.2392

Good vs. Hypertension (CF)
- -vs. - /del&del/del

2	4
27	233

OR	**4.31 (0.37-31.47)**
RR	**3.21 (0.98-10.52)**
Uncorrected	0.0746
Mantel-Haenszel	0.0751
Yates corrected	0.2624
1-tailed	0.1306
2-tailed	0.1306

Good vs. Hypertension (BF)
- -vs. - /del&del/del

0	4
6	97

OR	0.00 (0.00-29.55)
RR	-
Uncorrected	0.6193
Mantel-Haenszel	0.6209
Yates corrected	0.5414
1-tailed	0.7911
2-tailed	1.0000

Genotype-phenotype interactions

***IL*-4 -590 C/T (gestation)**

Hardy-Weinberg

	≥37			<37		
	Cohort	CF	BF	Cohort	CF	BF
C/C	63 17.1%	55 35%	8 7.3%	1 3%	1 3.7%	0
C/T	154 41.7%	10 6.4%	41 37.6%	17 51.5%	14 51.9%	3 50%
T/T	152 41.2%	92 58.6%	60 55.1%	15 45.5%	12 44.4%	3 50%
C	0.379	0.382	0.261	0.288	0.296	0.250
T	0.621	0.618	0.739	0.712	0.704	0.750
P	0.092	**0.000**	0.964	0.338	0.449	0.717

Fishers Exact Test

	≥37 vs. <37 weeks (cohort)		≥37 vs. <37 weeks (CF)		≥37 vs. <37 weeks (BF)	
	Genotype	Allele	Genotype	Allele	Genotype	Allele
P	0.102	0.1405	**NOT HWE**	**NOT HWE**	0.707	0.9298

2X2 Tables

≥37 vs. <37 weeks (cohort)

C/C&C/T vs. T/T

15	152
18	217

OR	1.19 (0.55-2.57)
RR	1.17 (0.61-2.26)
Uncorrected	0.6341
Mantel-Haenszel	0.6345
Yates corrected	0.7705

C/C vs. C/T&T/T

32	306
1	63

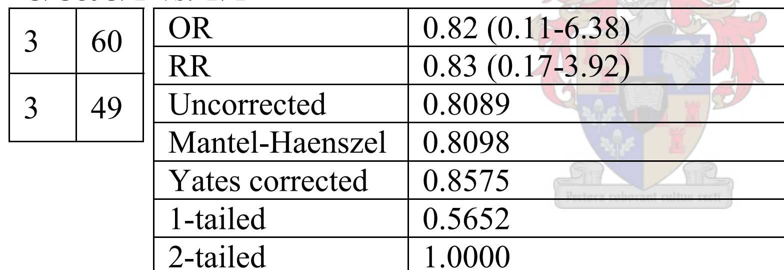
OR	**6.59 (1.06-272.45)**
RR	**6.06 (0.84-43.55)**
Uncorrected	**0.0346**
Mantel-Haenszel	**0.0349**
Yates corrected	**0.0623**

≥37 vs. <37 weeks (CF)

Not HWE

≥37 vs. <37 weeks (BF)

C/C&C/T vs. T/T



3	60
3	49

OR	0.82 (0.11-6.38)
RR	0.83 (0.17-3.92)
Uncorrected	0.8089
Mantel-Haenszel	0.8098
Yates corrected	0.8575
1-tailed	0.5652
2-tailed	1.0000

C/C vs. C/T&T/T

0	8
6	101

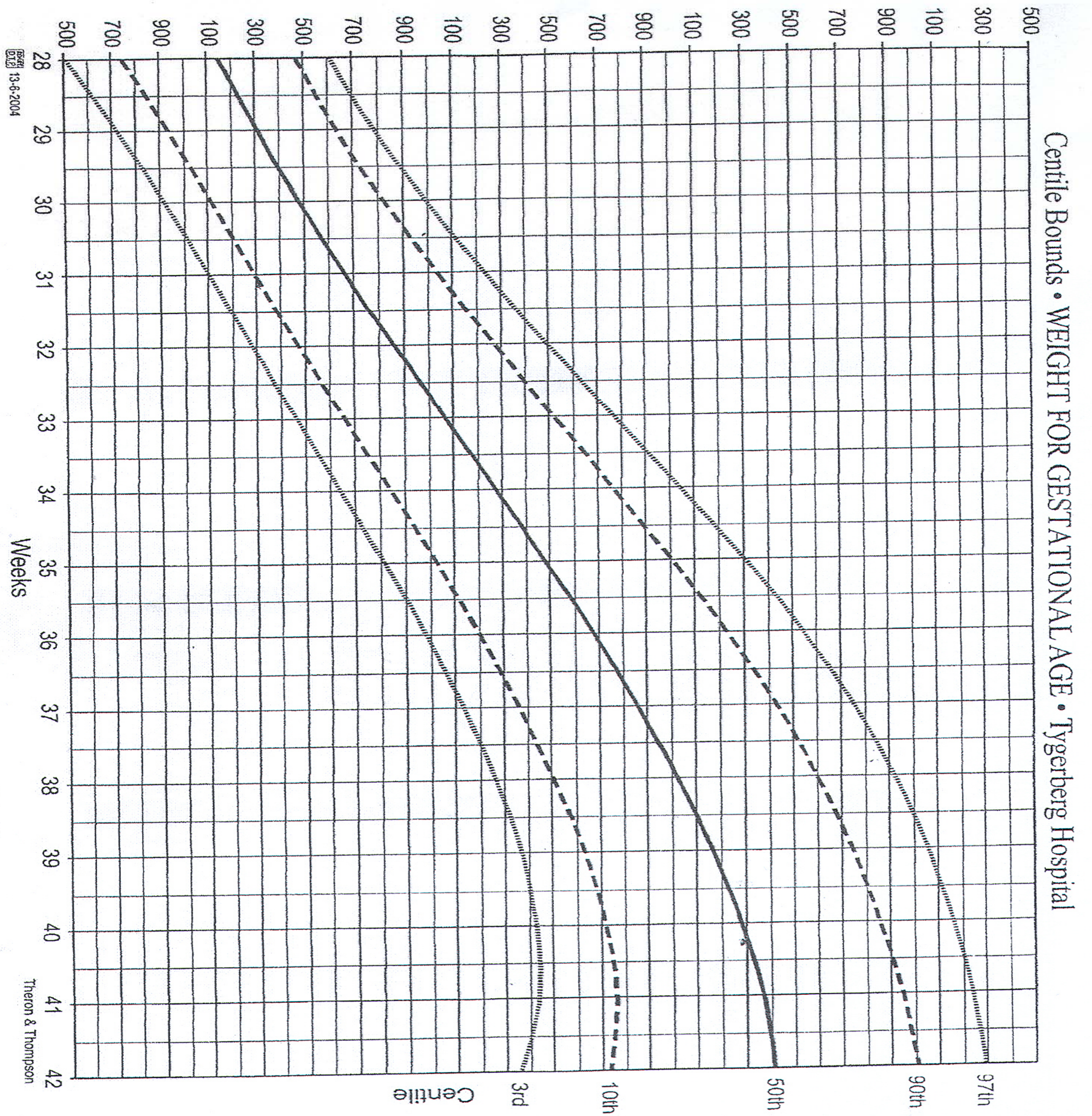
OR	0.00 (0.00-12.98)
RR	-
Uncorrected	0.4915
Mantel-Haenszel	0.4934
Yates corrected	0.8917
1-tailed	0.6423
2-tailed	1.0000

Appendix 13: Nomenclature

- Primer sequences and restriction enzyme conditions obtained from Genc *et al.*, 2002 for the screening of *IL*-1β +3953 C/G in this study were actually the +3954 C/T, determined upon sequencing, after dbSNP number could not be obtained.

- Discrepancies in the nature of the polymorphism at position -1082 of the *IL*-10 gene.
    - Makris *et al*., 2005 [*IL*-10 -1082 G→A]
    - dbSNP rs:1800896 [*IL*-10 -1082 A→G]

Appendix 14: Fetal growth chart



Appendix 15: Congress outputs

Presented at the 12th South African Society for Human Genetics (SASHG), Bloemfontein, Golden Gate Mountain Resort, March 2007.

INVESTIGATION OF THE *LGALS* - 13 GENE VARIANTS AS MARKERS FOR POOR PREGNANCY OUTCOME: A PROSPECTIVE STUDY

N Bruiners[1], GS Gebhardt[2], G Rebello[3], R Hillermann[1]

[1]Department of Genetics and [2]Department of Obstetrics and Gynaecology, Tygerberg Campus, Stellenbosch University, [3]Division of Human Genetics, University of Cape Town, South Africa.

Introduction: Placental insufficiency is responsible for among others, low birth weight, gestational hypertension and proteinuria, newborn mortality and life-long morbidity. Although the function of placental protein 13 (PP-13) in pregnancy remains unknown, several studies provide evidence for its effect on the trophoblast membrane in terms of implantation, blood pressure regulation and tissue oxygenation. The aim of the study was to investigate whether variants in the encoding gene, *LGALS*13, contribute to poor pregnancy outcome which includes preterm labour, pre-eclampsia, abruptio placentae and intra-uterine death.

Methods: We performed a prospective study involving 275 Coloured participants recruited at first booking at Paarl hospital. Variants in the coding region of the *LGALS*13 gene were detected by a combination of heteroduplex-SSCP gel electrophoresis, automated sequencing and restriction enzyme analysis, where applicable.

Results and Conclusion: To date, 164 women have delivered. Of these deliveries, 125 (0.76) have normal clinical outcomes, and 39 (0.24) are complicated. Significant associations have been demonstrated between poor pregnancy outcome and variant alleles at the following known loci: -98 A/C [dbSNP:rs3764843], IVS2-36 G/A [dbSNP:rs2233706] and IVS3+72 T/A [dbSNP:rs2233708]. A similar trend was observed at novel loci IVS2-22 A/G and IVS2-15 G/A. Statistical analysis and clinical correlation should be performed again upon completion of this pilot study, when the sample sizes should be more adequate and all patients delivered. Future analysis should involve characterisation of the promoter variant and intronic variants (novel and previously documented) identified in this study.