# MOLECULAR GENETIC ANALYSIS OF ABRUPTIO PLACENTAE

By

Marika Bosman
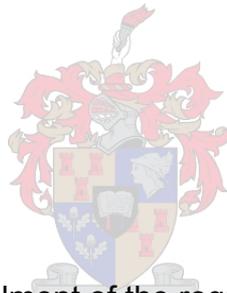
Thesis presented in partial fulfillment of the requirements for the degree of Master of Science (MSc) in Genetics at the University of Stellenbosch.

Supervisor: Dr R Hillermann-Rebello

Co-Supervisor: Dr GS Gebhardt

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DECLARATION

By submitting this thesis electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the owner of the copyright thereof (unless to the extent explicitly otherwise stated) and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

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ABSTRACT

Abruptio placentae is the premature separation of the normally implanted placenta from the uterine wall, resulting in haemorrhage before delivery of the fetus. This has serious maternal and neonatal implications, and is one of the leading causes of perinatal and maternal mortality and morbidity in South Africa. Placental vasculopathies, such as abruptio placentae, are believed to result from faults occurring in early placental development. Placental protein 13 (PP13) is a member of the pregnancy-related protein family, and is believed to function in a number of important physiological processes such as trophoblast invasion, placentation and implantation.

The aim of this study was to investigate whether DNA sequence variants in the *LGALS13* gene (encoding PP13), underlie and/or confer susceptibility to abruptio placentae. The gene was screened and genotyped in a cohort of patients whose pregnancies were complicated by abruptio placentae, as well as an ethnically matched control cohort. Statistical and *in silico* analyses were performed in order to identify potential susceptibility factors in this South African cohort and to predict whether the identified variants may impact on gene expression or the structure and function of PP13. In addition, the luciferase reporter gene assay was employed to investigate the functionality of the -98A/C variant identified in the 5' untranslated region of the *LGALS13* gene.

Statistically significant differences were observed between patient and control groups at the following loci in the Coloured population: -98A/C, IVS2 -36G/A, IVS2 -22A/G and the hotspot variant in exon 3 ($p < 0.05$). These variants could represent a susceptibility profile of this population or alternatively have implications in the pathogenesis of abruptio placentae. The deletion of a single thymine in exon 3 was shown to result in truncation of PP13 and subsequent disruption of a number of cysteine residues and putative phosphorylation sites, which could impact on dimerization and ultimately, the function of the protein. The reporter gene assay revealed a significant reduction ($p = 0.004$) in luciferase activity by the -98 C allele.

In silico analysis suggests that this reduction could be due the disruption of a NF1 or GR transcription factor binding site.

This study provides evidence that variants in the *LGALS13* gene may underlie and/or confer susceptibility to abruptio placentae by impacting on gene regulation or resulting in the expression of an aberrant form of the PP13 which could affect functionality and thereby result in the disruption of normal implantation and placentation.

OPSOMMING

Abruptio placentae is die vroeë skeiding van 'n normaal geinplanteerde plasenta van die wand van die uterus wat bloeding veroorsaak voor die verlossing van die baba. Hierdie toestand het ernstige implikasies vir moeder en baba en is een van die hoof oorsake van moederlike and fetale morbiditeit en mortaliteit in Suid Afrika. Plasentale vaskulopatie, soos abruptio placentae, se ontstaan is as die gevolg van foutiewe ontwikkeling van die plasenta in vroeër swangerskap. Plasentale proteïen 13 (PP13) is 'n lid van die swangerskap-verwante proteïenfamilie en is betrokke by 'n verskeidenheid van belangrike fisiologiese prosesse soos trofoblast infiltrasie, plasentasie en inplanting.

Die doelwit van hierdie studie was om te ondersoek of DNS variante in die *LGALS13* geen (wat PP13 kodeer) vatbaarheid vir *abruptio placentae* veroorsaak. Mutasie sifting en genotipering van 'n groep van pasiënte wie se swangerskappe gekompliseerd is deur *abruptio placentae*, asook 'n kontrole groep, is uitgevoer. Statistiese and *in silico* analises is gebruik om moontlike vatbaarheids faktore te identifiseer in die Suid-Afrikaanse populasie en om te voorspel of spesifieke variante 'n invloed het op geen uitdrukking, of die struktuur en funksie van PP13. Die lusiferase verklikkergeen sisteem is gebruik om die funksionaliteit van die -98A/C variant, wat vroeër geïdentifiseer is in die 5' ongetransleerde streek van die geen, te ondersoek.

Statisties betekenisvolle verskille tussen pasiënte en kontroles is waargeneem by die volgende lokusse in die Kleurling bevolkingsgroep: -98A/C, IVS2 -36G/A, IVS2 -22A/G en die "hotspot" mutasie (p<0.05). Hierdie variante kan moontlik 'n vatbaarheidsprofiel in die populasie verteenwoordig of alternatiewelik, kan dit implikasies voorspel by die patogenese van *abruptio placentae*. Die delesie van 'n enkel timien in ekson 3 veroorsaak die verkorting van PP13 en gevolglike versteuring van 'n paar sisteïen residue, asook veronderstelde fosforilasie setels, wat dimerisering en die struktuur en funksionering van die proteïen kan beïnvloed. Die verklikkergeen sisteem het 'n betekenisvolle afname in lusiferase aktiwiteit getoon as gevolg van die -98 C aleel. *In silico* analise impliseer dat versteuring

van 'n NF1 of GR transkripsie factor bindingsetel kan moontlik die afname in geen aktiwiteit veroorsaak.

Die studie verskaf bewyse dat variante in die *LGALS13* geen tot vatbaarheid vir *abruptio placentae* mag lei. Die variante kan moontlik geen regulasie beïnvloed, of lei tot die uitdrukking van 'n abnormale vorm van PP13 wat funksionaliteit mag affekteer en vervolgens versteuring van normale plasentasie en implantasie kan veroorsaak.

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LIST OF ABBREVIATIONS

~	approximately
µg	microgram
µl	microlitre
µM	micro molar
®	registered trademark
X^2	chi-squared
°C	degrees Celsius
5'	five prime
3'	three prime
TM	trademark
5'UTR	five prime untranslated region
A	Absorbance
A	adenine
ABI	Applied Biosystems
AFP	alpha-fetoprotein
Amp	ampicillin
AP	abruptio placentae
APH	antepartum haemorrhage
APS	ammonium persulphate $(NH_4)_2S_2O_8$
ATCC	American Type Culture Collection
ATG	transcription initiation site
BLAST	Basic Local Alignment Search Tool
*Bgl*I	*Bacillus globigii*, enzyme 2
bp	base pair
C	cytosine
cDNA	complementary DNA
CDC	Centres for Disease Control and prevention
CLC	Charcot-Leyden Crystal
CO_2	carbon dioxide
CRD	carbohydrate recognition domain
CT	Cape Town

CVS	chorionic villus sampling
Cys	cysteine
dATP	2'-deoxyadenosine-5'-triphosphate
dbSNP:rs	database single nucleotide polymorphism: reference sequence
dCTP	2'-deoxycytosine-5'-triphosphate
dH2O	distilled water
delT	deletion of a single thymine base
dGTP	2'deoxyguanine-5'-triphospate
DIC	disseminated intravascular coagulopathy
DNA	deoxyribonucleic acid
dNTP	2'-deoxy-nucleotide-5'-triphosphate
DPBS	Dulbeccos phosphate buffered saline
DRAQ5	deep red-flourescing bisalkylaminoanthraquinon number five
DTL	Diagnostic Technologies Limited
dTTP	2'-deoxythymine-5'-triphospate
E	exon
EDTA	ethylenediaminetetraacetic acid $C_{10}H_{16}N_2O_8$
ELISA	enzyme-linked immunosorbant assay
ER	endoplasmic reticulum
EtBr	ethidium bromide $C_{21}H_{20}BrN_3$
EtOH	ethanol CH_3CH_2OH
F	forward primer
factor II	factor two
factor V	factor five
FBS	fetal bovine serum
G	guanine
g	gram
gDNA	genomic deoxyribonucleic acid
hCG	human chorionic gonadotrophin
HD	heteroduplex
HEPES	N-(2-Hydroxyethyl)piperazine-N′-(2-ethanesulfonicacid)
HELLP	haemolytic anaemia, elevated liver enzymes & low platelet count
hr	hour
HS	hotspot

HWE	Hardy-Weinberg Equilibrium
IDT	Integrated DNA Technologies
IFPA	International Federation of Placenta Associations
INH-A	inhibin A
IUD	intrauterine death
IVS	intervening sequence
kb	kilo base
KCl	potassium chloride
kDA	kilo Daltons
L	litre
LB	Luria-Bertani medium
LGALS13	lectin, galactose-binding, soluble 13
Luc	luciferase
M	moles per litre / molar
MAbs	Monoclonal antibodies
MCS	multiple cloning site
mg	milligram
mg/ml	milligram per millilitre
$MgCl_2$	magnesium chloride
min	minute
ml	millilitre
mM	millimoles per litre / millimolar
mRNA	messenger ribonucleic acid
MSAFP	maternal serum alpha-fetoprotein
MTHFR	5,10-methylenetetrahydrofolate reductase
NCBI	National Centre for Biotechnology Information
NCCEMD	National Committee of Confidential Enquiries into Maternal Deaths
ng	nanogram
ng/ml	nanogram per millilitre
ng/µl	nanogram per microliter
NH_4Cl	ammonium chloride
nM	nanomoles per litre / nanomolar
nt	nucleotide
OD	optical density

p	short arm of chromosome
P1GF	pro-angiogenic growth factor
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDA	piperazine diacrylamide
PE	pre-eclampsia
pH	potential of hydrogen
PI	pulsality index
PIGF	proangiogenic placental growth factor
PIH	pregnancy-induced hypertension
PLA2	phospholipase A2
pmol	pico mole
PP13	placental protein 13
PP13-R	recombinant placental protein 13
PPH	postpartum haemorrhage
PPROM	preterm premature rupture of the membranes
PTL	preterm labour
q	long arm of chromosome
R	reverse primer
RBC	red blood cells
RE	restriction enzyme
RIA	radioimmunoassay
RNA	ribonucleic acid
rpm	revolutions per minute
RPMI	royal park memorial institute, culture medium
s	second
SA	South Africa
SDS	sodium dodecyl sulphate
sEng	soluble endoglin
Ser	serine
sFlt-1	soluble fms-like tyrosine kinase
SNP(s)	single nucleotide polymorphism(s)
SSCP	single strand conformational polymorphism

STBM	syncytiotrophoblast microparticle
T	thymine
Ta	annealing temperature
Taq	*Thermus aquaticus*
TBE	Tris-Borate-EDTA
T-cell	thymus cell
TE	Tris-EDTA
TEF-5	transcriptional enhancer factor 5
TEMED	N,N,N',N'-tetramethylethylenediamine
Tm	melting temperature
TRIS	trishydroxymethylaminomethane
TRIS-HCl	tris hydrochloride
Tyr	tyrosine
U	enzyme activity unit
uE3	unconjugated estriol
UCT	University of Cape Town
UK	United Kingdom
US	Stellenbosch University
USA	United States of America
UTR	untranslated region
UV	ultraviolet
V	volt
VEGF	vascular-endothelial growth factor
vs.	versus
wk(s)	week(s)
wt	wild type
w/v	weight per volume
X	times
x g	times gravity
*Xho*I	*Xanthomonas holcicola*, enzyme 1
yr(s)	year(s)

CHAPTER 1:

LITERATURE REVIEW

LITERATURE REVIEW

1.1 IMPLANTATION, PLACENTATION AND PLACENTAL DEVELOPMENT

The biological processes involved in human implantation and placentation are highly complex in nature, requiring a number of very specific, highly complicated and precisely regulated interactions of cells with the endometrial extracellular matrix. These cell interactions, particularly at the feto-maternal interface, are vital for the normal development of both fetus and placenta. Cell proliferation, migration and differentiation are important processes during placentation (Bischof & Campana, 1996). Trophoblast cells play a major role in the implantation and placentation processes by eroding the decidua and facilitating the embedding of the blastocyst in the uterine wall (De Kock & Van der Walt, 2004). Specific cell differentiation requires numerous molecular signals which all contribute towards successful implantation, placentation and the maintenance of a normal pregnancy. Placentation in itself is an intricate process which involves specific cell matrix interactions in which the role of galectins is important (Visegrady *et al.*, 2001).

These complex physiological processes begin with conception. Fertilization of the ovum occurs in the distal end of the Fallopian tube, along which the zygote advances and undergoes several mitotic divisions until it reaches a 16-cell stage and becomes known as the morula. Blastocoele or cavity formation in the morula marks its transformation into the blastocyst, a thin-walled hollow structure containing a cluster of cells, the inner cell mass or embryoblast, from which the embryo arises. The outer layers of cells, the trophoblast, give rise to the placenta and other supporting tissues needed for fetal development within the uterus (De Kock & Van der Walt, 2004).
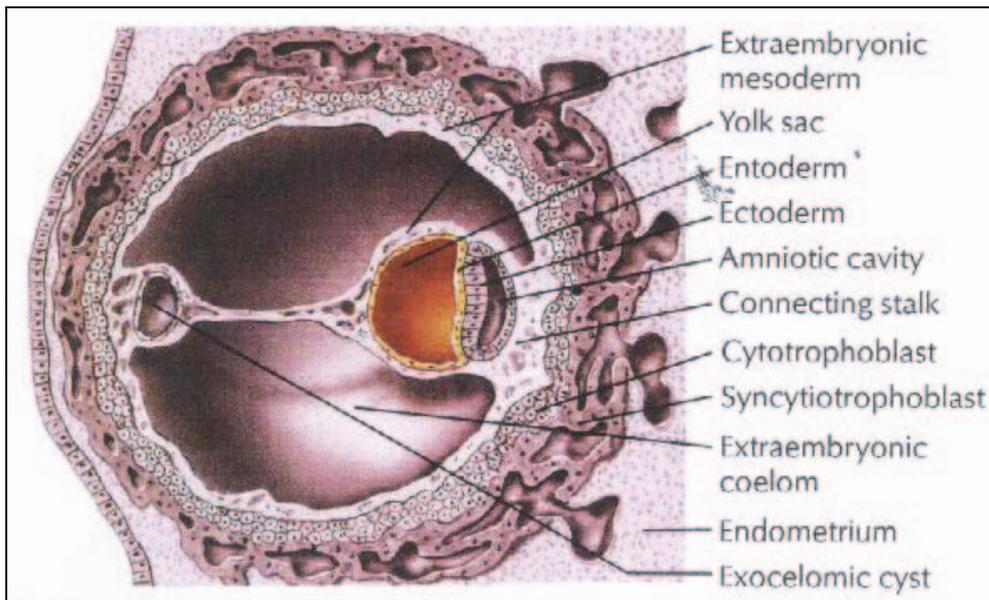
Figure 1.1 The developing embryo

Illustration by: Netter & Machado (Cochard *et al.*, 2002)

The trophoblast makes contact with, and adheres to the endometrium, which triggers differentiation of the trophoblast cells into an outer layer of cells, the syncytiotrophoblast, and an inner layer, the cytotrophoblast (Balinsky, 1981; De Kock & Van der Walt, 2004). Implantation of the blastocyst begins with the invasion of endometrial tissue by the syncytiotrophoblast. As implantation progresses, endometrial cells surrounding the implantation site swell due to the accumulation of glycogen and lipids and become known as decidual cells. From this point, the endometrium is known as the decidua, with three regions, named according to the site of blastocyst implantation: the decidua basalis is the part of the decidua underneath the embedded embryo and which forms the maternal part of the placenta, the decidua capsularis is the superficial part of the decidua which is found over the developing embryo and the remaining decidua that line the uterus is the decidua parietalis or decidua vera (Balinsky, 1981; De Kock & Van der Walt, 2004).

During the first few weeks of development, the embryo is nourished by diffusion; thereafter rapid fetal growth and development necessitate the development of the

utero-placental circulatory system. A primitive blood circulatory system is established when lacunae begin to form in the syncytiotrophoblast. The maternal uterine spiral arteries provide oxygenated blood to the lacunae, while endometrial veins remove the deoxygenated blood. Lacunae from adjacent syncytiotrophoblasts fuse to form a single connected system of lacunar networks, which are the rudimentary intervillous spaces of the placenta. Endometrial capillaries become congested, forming sinusoids which are eroded by syncytiotrophoblasts, allowing maternal blood to flow into lacunar networks. In this way, utero-placental circulation is established as maternal blood flows through the networks.
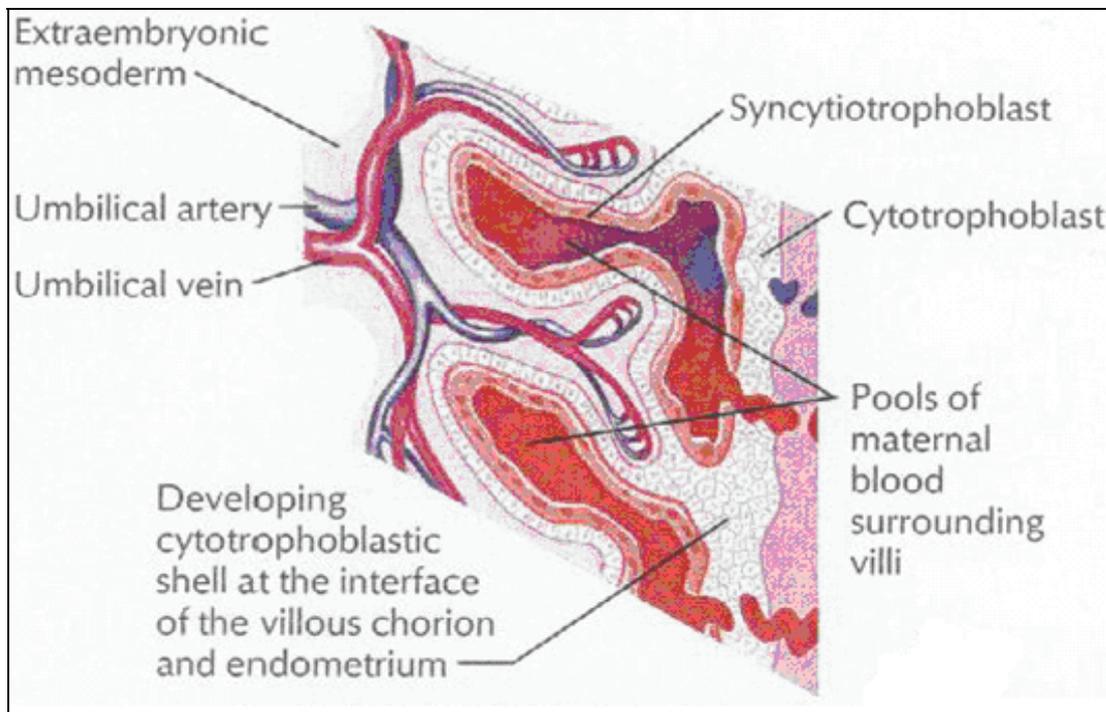
Figure 1.2 The establishment of utero-placental circulation in early embryonic development. Illustration by: Netter & Machado (Cochard *et al.*, 2002)

At this stage of development, cytotrophoblast cells begin to proliferate and produce cellular projections which grow into the overlying syncytiotrophoblast and form the primary chorionic villi (Balinsky, 1981). A villus can be defined as a branched structure that develops from the chorionic membrane as a single stem which undergoes numerous subdivisions until it ends in very fine filaments that

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attach themselves to the decidua basalis. These primary villi have a cytotrophoblast core with an outer covering of syncytiotrophoblast and contain no blood vessels (De Kock & Van der Walt, 2004). The developing mesoblast grows into the primary villi, at which stage they become known as the secondary villi. These villi differentiate into connective tissue and blood vessels and, through their selectivity, chorionic villi will be able to absorb from the maternal blood all the substances required by the developing embryo. The secondary villi connect up with the embryonic blood vessels and become tertiary villi containing both the differentiated blood vessels and a mesodermal centre (Balinsky, 1981; De Kock & Van der Walt, 2004). From this stage onwards gases, nutrients and waste will diffuse from the maternal and fetal blood and pass through four layers i.e. capillary endothelium of the villi, surrounding connective tissue, cytotrophoblast and syncytiotrophoblast. These four elements form what is known as the placental barrier.
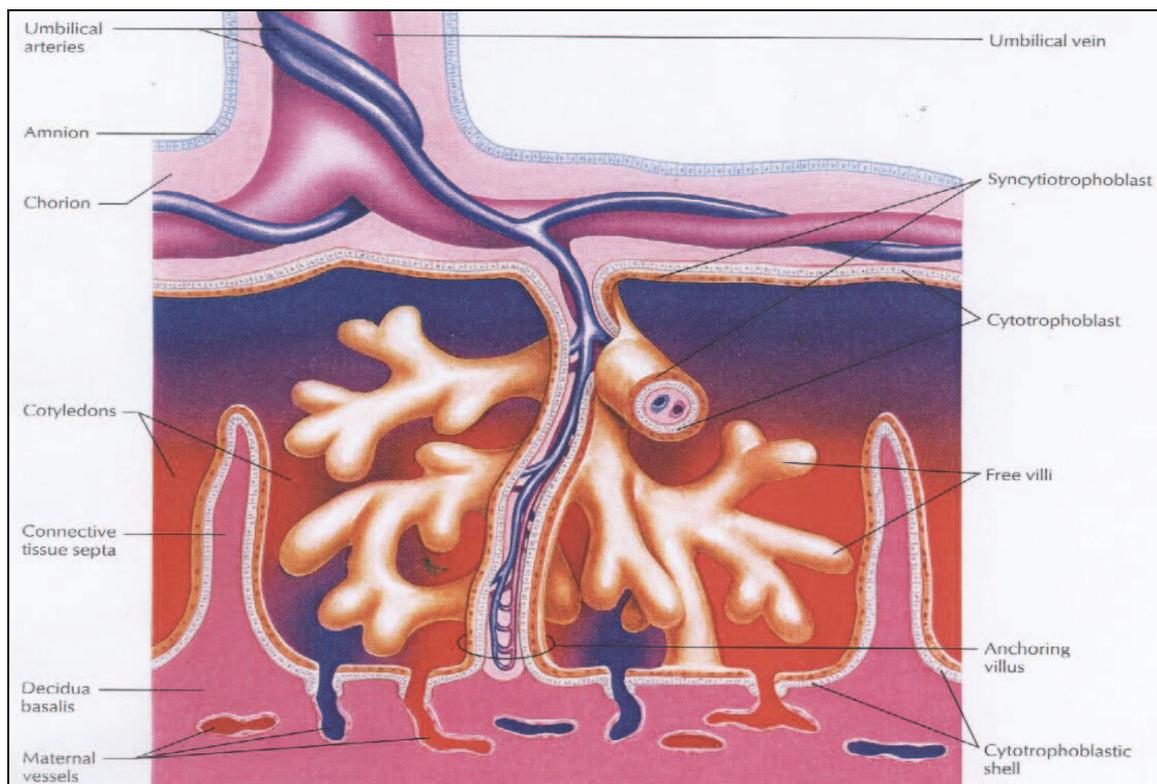
Figure 1.3 A cross-section of the fully developed placenta

Illustration by: Machado (Cochard *et al.*, 2002)

Trophoblast proliferation and the development of the chorionic sac and villi are characteristic of early placental development. In the early stages of development, chorionic villi surround the outer surface of the chorionic plate. Chorionic villi grow abundantly on the embryonic pole, associated with the decidua basalis, and are known as the chorion frondosum (also known as the villous chorion), which ultimately forms the placenta. These villi penetrate maternal blood vessels of the decidua basalis and become surrounded by maternal blood. These blood filled spaces are known as the intervillous spaces. Some chorionic villi attach to the decidua and are called anchoring villi. The villi opposite the embryonic pole associated with the decidua capsularis, degenerate and produce the smooth chorion (De Kock & Van der Walt, 2004).

The placenta comprises a fetal component, which develops from the chorion frondosum, and a maternal component which is formed by the decidua basalis. These two placental components are connected by the cytotrophoblastic shell, which is an external layer of trophoblast cells found on the maternal surface of the placenta (Balinsky, 1981). The placenta is fixed to the uterine wall by the formation of cytotrophoblastic columns from the anchoring villi. Fibronectin molecules connect the extravillous trophoblast and trophoblastic columns to the decidua at implantation sites (Eskes, 1997).

During placenta formation, the intervillous spaces are enlarged by further infiltration of the decidua basalis by chorionic villi and subsequent erosion of the decidual tissue. Erosion of the decidua produces placental septa which divide the fetal part of the placenta into cotyledons, each one consisting of a number of stem and branch villi.

Maternal spiral arteries in the decidua basalis pass through the cytotrophoblastic shell and provide the intervillous spaces with maternal blood. Blood drains via endometrial veins which also pass through the cytotrophoblastic shell. Many branch villi from stem chorionic villi are in contact with maternal blood in the intervillous space, which carries oxygen and nutritional substances for fetal growth and development. The umbilical arteries transport poorly oxygenated blood from the fetus to the placenta. At the site of umbilical cord attachment, the arteries

divide into chorionic arteries that branch in the chorionic plate before entering the chorionic villi. Fetal capillaries carry well-oxygenated blood to the site of attachment at the umbilical cord where they unite as the umbilical vein, which carries the oxygenated blood to the fetus (Balinsky, 1981).

1.2 MORPHOLOGY AND FUNCTIONS OF THE PLACENTA

The placenta is represented by two components: the maternal and the fetal component. The maternal component, also known as the basal plate, is in contact with the decidua and the fetal component, or chorionic plate, is the site of insertion of the umbilical cord.
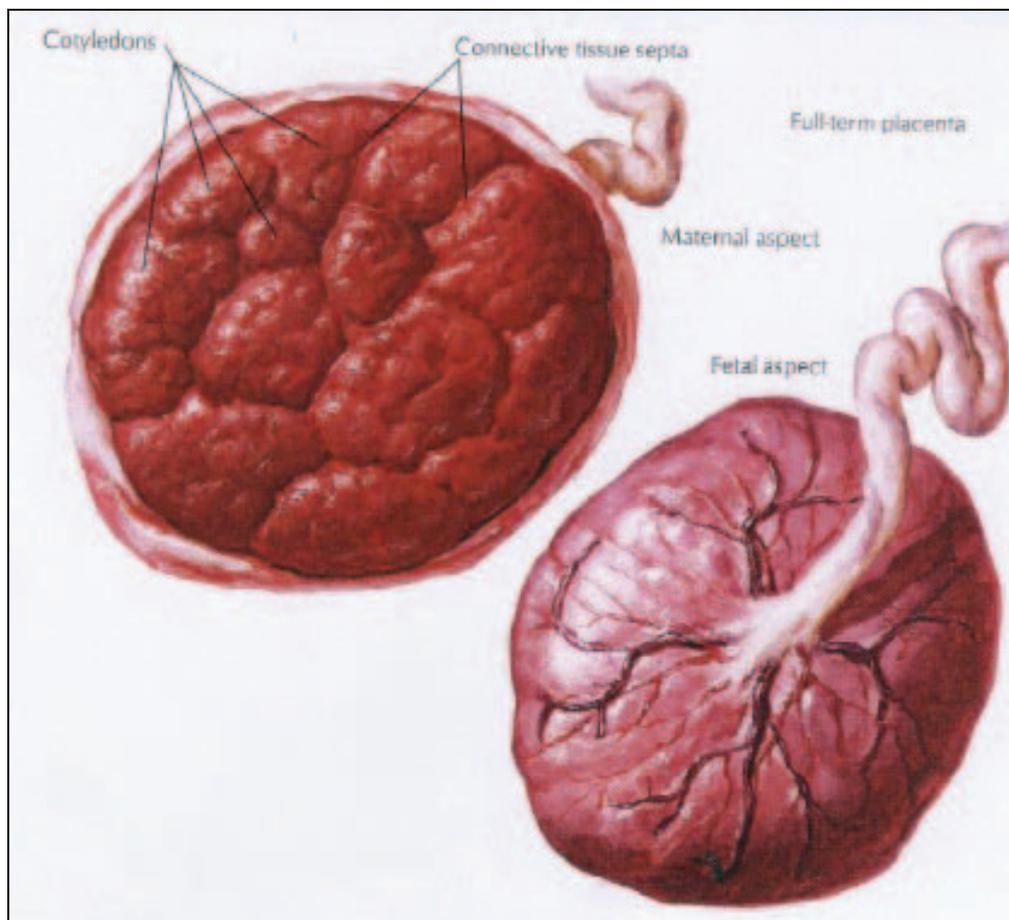
Figure 1.4 External morphology of the placenta at term

Illustration by: Netter (Cochard *et al.,* 2002)

The placenta is responsible for the exchange of nutrients and protection of the fetus. Essentially, its main function is to provide everything that the developing fetus requires. During this process, the placenta selects and transports the substances necessary for fetal life and growth from the mother's blood; it also converts some of these substances so that they can be fully utilized by the fetus. The efficiency of the placenta in this function is dependent on the adequacy of uterine blood flow. The placental barrier is formed by structures which represent both maternal and fetal blood, this barrier allows for selective permeability by the placenta. Water, oxygen, nutrients and hormones are allowed through the placental barrier from the mother to the fetus, whilst waste products are allowed from fetus to mother. Placental functions can be classified as nutritional, respiratory, excretory, endocrine and protective with the transport of gases, nutritional substances, hormones, electrolytes, maternal antibodies, waste products, drugs and even infectious agents (Balinsky, 1981; De Kock & Van der Walt, 2004).

1.3 HAEMORRHAGE IN PREGNANCY

Haemorrhage in pregnancy is one of the major causes of maternal mortality and morbidity in developing countries and is one of the top five obstetric causes of death in South Africa. Haemorrhage can occur before or after viability of the fetus, with or without pain and may occur before, during or after delivery of the baby (De Kock & Van der Walt, 2004). In South Africa, the National Committee on Confidential Enquiries into Maternal Deaths (NCCEMD) report (www.doh.gov.za) for the period 2002 to 2004 showed that obstetric haemorrhage accounted for 13.4% of all maternal deaths and is the third most common cause of maternal death in South Africa.

1.3.1 Antepartum haemorrhage

Antepartum haemorrhage (APH) is defined as vaginal bleeding that occurs during pregnancy any time between potential viability of the fetus to delivery of the baby (Morgan & Arulkumaran, 2003). APH may have many causes, including abruptio placentae, placenta previa, vasa previa, bleeding from lower genital tract and

bleeding of unknown origin. Abruptio placentae and placenta previa account for almost half the cases of APH (Morgan & Arulkumaran, 2003; Ngeh & Bhide, 2006). According to the NCCEMD report, abruptio placentae (with and without hypertension) was responsible for 70.4% of maternal deaths related to APH in South Africa.

1.3.2 Postpartum haemorrhage

Postpartum haemorrhage (PPH) is defined as vaginal bleeding that occurs within the first 24 hours of delivery (De Kock & Van der Walt, 2004). After placental detachment, the uterus retracts, which cuts off the blood supply to the placental implantation site. In the case of PPH, uterine muscle fibres do not retract, blood vessels remain open and bleeding will occur. Factors which contribute to the occurrence of PPH include uterine atony, trauma to the genital tract and coagulation defects which may result from amniotic fluid embolism, sepsis, eclampsia and abruptio placentae (De Kock & Van der Walt, 2004).

1.4 ABRUPTIO PLACENTAE

Abruptio placentae is the premature separation of the normally implanted placenta from the wall of the uterus, which results in haemorrhage before the fetus is delivered. This has serious implications for both mother and fetus, and may cause compromised fetal blood supply and fetal distress. It is a sudden and devastating condition and is one of the leading causes of perinatal and maternal mortality and morbidity in South Africa.

1.4.1 Underlying pathology

Placental abruption is caused by haemorrhage into the decidua basalis, leading to the formation of a haematoma and a subsequent increase in hydrostatic pressure, which results in separation of the placenta from the uterine wall (Ngeh & Bhide, 2006). If the haematoma does not reach the margin of the placenta and the cervix, bleeding may be concealed and the extent of haemorrhage may not truly reflect the amount of blood loss. Increased fragility of vessels, vascular malformations or

abnormalities in placentation and other vascular or placental abnormalities have been implicated in hypertension in pregnancy and abruptio placentae (Ngeh & Bhide, 2006).

In normal pregnancy, the endothelium of the spiral arteries is replaced by trophoblast cells. The distal tips of the spiral arteries are covered with a layer of cytotrophoblasts which are continuous with the proliferating tips of the anchoring villi. After trophoblast invasion of the decidual parts of the spiral artery, the cells are incorporated into the artery walls and the blood vessel undergoes physiological changes which allow a greater volume of blood to enter and leave the intervillous spaces, and subsequently creates a low- resistance placental vascular bed (Eskes, 1997).

In pregnancies complicated with abruptio placentae, these histological and physiological changes do not occur and there is no transformation of the utero-placental arteries. Additionally, there may be signs of vasculopathy such as atherosis, necrosis and thrombosis. Circulating vasoactive substances may worsen endothelial damage, which may ultimately result in disturbed coagulation, maternal vasoconstriction and reduced organ perfusion (Eskes, 2001). These vasculopathies may in turn lead to formation of placental infarcts and vessel rupture which cause retroplacental haemorrhage (Eskes 1997; Ngeh & Bhide, 2006).

1.4.2 Clinical presentation and diagnosis

Abruptio placentae presents clinically with the sudden onset of severe abdominal pain, uterine contractions, abdominal tenderness and vaginal bleeding. Diagnosis of placental abruption is made clinically and confirmed at postpartum inspection of the placenta, which may reveal clots and/or depressions covering >15% of the maternal surface of the placenta (Odendaal *et al.*, 2000).

1.4.3 Maternal and fetal outcomes

1.4.3.1 Maternal implications

In severe cases of abruptio placentae, complications such as disseminated intravascular coagulopathy (DIC), infection, postpartum haemorrhage, renal failure, congestive heart failure and hypovolaemia may occur (Ngeh & Bhide, 2006). Hypovolaemia or hypovolaemic shock is caused by a large reduction in blood volume and decrease in red blood cells. If not managed correctly, it may result in organ dysfunction, multiple organ failure and eventually death (De Kock & Van der Walt, 2004). Should bleeding infiltrate the myometrium, a condition known as Couvelaire uterus may result, which may require a hysterectomy to correct (Eskes, 1997; Ngeh & Bhide, 2006).

1.4.3.2 Fetal implications

When abruption is severe and the placenta is completely separated, fetal death is highly likely (Ananth *et al.*, 1999). In less severe cases, when the placenta is only partially separated, there is an increased risk of fetal brain damage (Eskes, 2001). The main adverse perinatal outcomes and major causes of fetal morbidity are prematurity, anaemia, hyperbilirubinaemia (Ananth *et al.*, 1999; De Kock & Van der Walt, 2004). Several studies have shown an association between cerebral palsy and other infant neurodevelopmental disorders, and abruptio placentae (Matsuda *et al.*, 2003).

1.4.4 Risk factors

1.4.4.1 Clinical and environmental factors

As with many complex multifactorial diseases, the etiology of abruptio placentae is largely unknown. The underlying cause of this condition is likely to be a combination of genetic and environmental factors. Advanced maternal age, grand multiparity, multiple pregnancies, alcohol consumption, cigarette smoking, cocaine use during pregnancy (Eskes, 1997), male fetal gender (Ananth *et al.*, 1996,

Kramer *et al.*, 1997, Odendaal *et al.*, 2000), hypertension, preterm premature rupture of the membranes (PPROM), oligohydramnios, intrauterine infections, abdominal trauma and thrombophilias (Ananth *et al.*, 2004, Ngeh & Bhide, 2006) have been implicated as risk factors for abruptio placentae.

Associations have been found with pregnancy-induced hypertension, preterm delivery, chronic hypertension and diabetes. It is possible that these conditions may share common pathophysiological mechanisms (Rasmussen *et al.*, 1999). There are conflicting reports on the validity of these factors and to date, there is no definitive set of etiological factors that may be associated with abruptio placentae with absolute certainty. However, two factors in particular seem to be reiterated in countless studies: hypertension in pregnancy and/or abruptio placentae in a prior pregnancy (Rasmussen *et al.*, 2000; Ananth *et al.*, 2006) and maternal cigarette smoking (Ananth *et al.*, 1996, 1999a and 1999b; Kramer *et al.*, 1997; Andres & Day, 2000; Kyrkland-Blomberg *et al.*, 2001; Odendaal *et al.*, 2001; Zdravkovic *et al.*, 2005).

The recurrence rate of abruptio placentae is considerable, making a prior history of this condition a notable risk factor in the current pregnancy. A study by Toivenen *et al.* in 2004 reported a recurrence rate of 11.9% in women with a history of placental abruption, which was significantly higher than the 0.7% occurrence rate of women with no prior history of such complications. It has been suggested that damage to the endometrium which underlies the implantation site, resulting from prior placental dysfunction, could lead to an increased incidence of abruption in subsequent pregnancies (Ananth *et al.* 1996).

Cigarette smoking during pregnancy has been associated with a large number of complications and a vast amount of literature exists confirming it as a risk factor for abruptio placentae (Ananth *et al.*, 1996, 1999a and 1999b; Kramer *et al.*, 1997; Andres & Day, 2000; Kyrkland-Blomberg *et al.*, 2001; Odendaal *et al.*, 2001; Zdravkovic *et al.*, 2005). In a study by Ananth *et al.* (1999), smoking was associated with a 90% increased risk of abruptio placentae. A proposed mechanism for this increased risk is that smoking affects capillary fragility and may cause changes in the endothelial cells, leading to vasoconstriction and subsequent

placental under perfusion, ischemia of the decidua basalis, decidual necrosis and haemorrhage (Ananth *et al.*, 1996, 1999b). Interestingly, smoking appears to reduce the frequency of pre-eclampsia (Salafia & Shiverick, 1999). Despite this protective effect, if pre-eclampsia does develop in smokers, it leads to an increase in vascular resistance, resulting in chronic hypoxia and consequently, an increased risk of developing abruptio placentae (Ananth *et al.*, 1999b; Salafia & Shiverick, 1999).

In a study by Ananth *et al.* in 2006, vaginal bleeding in early pregnancy, which may be indicative of disrupted placentation, was found to be strongly associated with abruptio placentae in advanced gestation. The same study demonstrated a significant association between placental lesions and an increased risk of placental abruption. These chronic inflammatory lesions are believed to be due to prolonged inflammation (Ananth *et al.*, 2006). Severe chorioamnionitis was found to be strongly associated with the progression of abruptio placentae in a recent study (Nath *et al.*, 2007). These studies provide insight and speculation into the contribution of an inflammatory-mediated etiology for abruptio placentae.

1.4.4.2 Genetic factors

The underlying genetic components of abruptio placentae are, to date, unclear. Numerous studies have aimed to identify risk factors for the condition, with varying results. The recurrence rate of this condition is approximately 9 - 11% (Ward, 2008), which cannot be attributed solely to environmental factors. In addition to the seemingly increased risk in relatives of those who experienced placental abruption, this is evidence that genetic factors may play a role and contribute to the risk of developing this disorder (Zdoukopoulos & Zintzaras, 2008). Hyperhomocysteinemia, thrombophilic mutations and angiogenic factors have been implicated as potential risk factors for placental abruption and will be discussed briefly.

i) Hyperhomocysteinemia

Homocysteine, the demethylated derivative of methionine, is either transsulphurated or remethylated to methioinine. The transsulphuration pathway requires the enzyme cystathionine synthase and vitamin B6 as a cofactor, while remethylation requires methionine synthase with vitamin B12 as a cofactor and 5, 10-methylenetetrahydrofolate reductase (MTHFR) with folate as a cofactor. The position of MTHFR in the methionine-homocysteine cycle is critical and for this reason homocysteine has become a sensitive marker for folate status (Eskes *et al.*, 2001). Various studies (Goddijn-Wessel *et al.*, 1996; Owen *et al.*, 1997) have found homocysteine level to be a risk factor, with specifically hyperhomocysteinemia being associated with abruptio placentae. Hyperhomocysteinemia occurs in two forms, based on its severity. Severe hyperhomocysteinemia, known as homocystinuria, is caused by deficiency in cystathionine β-synthase or MTHFR enzymes and has been linked to susceptibility to vascular disease. Mild hyperhomocysteinemia is associated with mutations in genes encoding methionine synthase, cystathionine β-synthase and MTHFR. The *MTHFR* C677T mutation is a major determinant of hyperhomocysteinemia. In homozygous form, it results in lowered enzyme activity of homocysteine and a subsequent elevation in plasma concentrations of the protein (van der Molen *et al.*, 2000).

Elevated homocysteine levels have been associated with a number of placental vasculopathies, including abruptio placentae, placental infarcts and fetal growth impairment, in the South African population (Owen *et al.*, 1997). This finding was confirmed in a Dutch population by Eskes in 2001. It is believed that damage to the vascular endothelium and resulting placental vasculopathy could be a result of elevated homocysteine levels (Gebhardt *et al.*, 2001). A study by Gebhardt *et al.* (2001) found combined heterozygosity for two *MTHFR* gene variants, A1298C and C677T, to be associated with abruptio placentae in a South African patient cohort.

ii) Thrombophilia

Genetic or acquired thrombophilia has been associated with many obstetric complications. The condition predisposes individuals to venous thromboembolism and the placentas of women with thrombophilia are characterized by an increase in vascular damage, infarcts and fibrinoid necrosis (Nath *et al.*, 2008). High frequencies of inherited thrombophilic factor V Leiden and prothrombin/factor II A20210 mutations were reported in patients with pregnancies complicated by abruptio placentae (Facchinetti *et al.*, 2003). This finding was significant in the Caucasian population, but not in the African population, in whom these mutations were absent (Hira *et al.*, 2002).

iii) Angiogenic factors

Vascular remodeling of the maternal uterine arteries is essential for normal placentation, and occurs under the influence of various angiogenic factors. These include vascular-endothelial growth factor (VEGF) and the pro-angiogenic placental growth factor (PlGF). Abnormalities in trophoblast invasion and subsequent vascular remodeling have been associated with the development of pre-eclampsia, intrauterine growth restriction and abruptio placentae. Circulating levels of human soluble fms-like tyrosine kinase (sFlt-1), a splice variant of VEGF receptor 1, and PlGF in the serum of women with abruptio placentae and pre-eclampsia were measured by ELISA (enzyme-linked immunosorbant assay). Levels of the PlGF were decreased, and sFlt-1/PlGF ratio increased mid-pregnancy in women who later went on to develop abruptio placentae and hypertension, but not in those who presented with abruption only (Signore *et al.*, 2006). In addition, variations in the Angiotensinogen gene, most recently the Thr235 mutation, have been associated with abnormal physiological changes related to abruptio placentae, such as abnormal artery remodeling and diminished placental perfusion (Zhang *et al.*, 2007).

1.5 BIOMARKERS IN PREGNANCY

Maternal serum screening during the first- and second-trimester of pregnancy became available in the 1980s to identify pregnancies at risk for trisomies 13, 18 and 21, as well as open neural tube defects and anencephaly. The use of maternal serum markers in screening programs is relatively inexpensive and non-invasive, unlike procedures such as amniocentesis and chorionic villus sampling (CVS) that are associated with a risk of miscarriage and physical damage to the fetus. Sensitivity, specificity and low false-positive rates are ensured by combining clinical markers with multiple serum analyte screening. For example, screening for Down Syndrome involves ultrasound to measure nuchal translucency in combination with a "quadruple screen" to ensure high sensitivity and detection. The analytes currently being used in this screening protocol include maternal serum alpha-fetoprotein (MSAFP), human chorionic gonadotrophin (hCG), unconjugated estriol (uE3) and inhibin-A (INH-A) (Driscoll, 2004).

Screening programs have become standard practice for prenatal diagnosis and risk assessment for chromosomal abnormalities and fetal defects, however not much attention has been focused on risk assessment in terms of placental vasculopathies. Conditions such as pre-eclampsia, abruptio placentae and preterm labour have serious consequences related to maternal and fetal well-being. The identification of pregnancies at risk of developing such conditions could be invaluable in clinical practice and result in decreased maternal and fetal morbidity and mortality. Recently, several attempts have been made to discover maternal serum markers for the identification of women at risk of developing pre-eclampsia and abruptio placentae. These studies have been met with varied results and a few will be discussed briefly in the sections which follow.

1.5.1 Maternal serum alpha-fetoprotein (MSAFP)

Maternal serum alpha-fetoprotein (MSAFP) is commonly measured in the second trimester to screen for fetal malformations. Elevated levels of this protein are associated with neural tube defects, whereas decreased levels are associated with Down's syndrome (Driscoll, 2004). In addition, elevated second trimester MSAFP

has been associated with a several pregnancy complications such as preterm birth, PIH, pre-eclampsia, placenta previa delivery of low birth weight infants and perinatal loss.

Recently, elevated MSAFP levels were observed in patients who developed abruptio placentae (Tikkanen *et al.*, 2007a). AFP is produced by the fetal liver, accumulates in the amniotic fluid and enters the maternal circulation. An explanation for these elevated levels is based on a key abnormality linked to abruptio placentae. Superficial implantation caused by acute atherosis of the spiral arteries may cause these arteries to rupture, culminating in the retroplacental haemorrhage and subsequent placental detachment. The arteries may leak fetal blood to the maternal side before the appearance of haematoma and placental detachment occurs. The presence of fetal blood, which is rich in AFP, may explain the increased levels of this protein in maternal serum of patients with abruptio placentae. However, the study found that although the MSAFP levels were higher in patients with placental abruption than in the control group, screening for the disorder with this protein was not sufficiently specific or sensitive (Tikkanen *et al.*, 2007a).

1.5.2 Activin A

High levels of the glycoprotein activin A are secreted by the placenta in pre-eclamptic patients and there is believed to be a link between this increased secretion and pathologies involving the trophoblast (Florio *et al.*, 2003). The excessive release has been implicated in the adaptive response of the placenta to adverse conditions such as tissue damage. Abnormally high maternal serum levels of activin A were found several weeks before the occurrence of abruptio placentae, with evidence of placental damage and dysfunction and disrupted placentation. However, this study was based on findings in a small patient cohort and therefore requires confirmation in an expanded cohort.

1.5.3 Angiogenic factors

A recent study (Tikkanen *et al.*, 2007b) aimed to identify a biomarker for placental abruption. The authors suggested that an imbalance in placental or maternal angiogenic factors may precede development of the condition. Measurement of maternal serum levels of proangiogenic placental growth factor (PIGF), soluble fms-like tyrosine kinase 1 (sFlt-1) and soluble endoglin (sEng) in the second trimester was found to be a predictive marker of pre-eclampsia, but not of subsequent abruption. In this regard, pre-eclampsia seems to differ from abruptio placentae and these factors are unlikely to be involved in the pathogenesis of this condition in early pregnancy.

1.5.4 Placental protein 13

In 2004, Burger *et al.* reported that PP13 levels in the first trimester were lower in pathological pregnancy conditions (pre-eclampsia, preterm labour and intrauterine growth restriction) and higher in second and third trimesters than in normal pregnancies. Significant differences were observed in the cases of early pre-eclampsia which developed before or at 34 weeks of gestation. The study showed that abnormal PP13 levels in the first and second trimesters are associated with placental insufficiency most likely due to abnormal placentation. The decreased levels of PP13 in the first trimester and increased second and third trimester levels in maternal serum may be attributed to impaired protein synthesis, protein structure, passage from the placenta to maternal serum, or a combination of the three (Burger *et al.*, 2004).

Recently, Than *et al.* (2008) proposed that increased third trimester PP13 levels in pre-eclamptic patients may be a consequence of increased syncytiotrophoblast microparticle (STBM) shedding in response to placental ischemia and under perfusion, which is characteristic of this disorder.

A polyclonal radioimmunoassay (RIA) and a two-monoclonal antibody sandwich ELISA have been used to measure PP13 levels in maternal serum, but the sandwich ELISA was found to be better suited for clinical use in the first trimester

and a kit was subsequently developed (Burger *et al.*, 2004). The sandwich ELISA comprises a pair of PP13 specific monoclonal antibodies (MAbs) and PP13-R (recombinant PP13, from expression of a cDNA clone) and can accurately detect PP13 in a pregnant woman's serum. The MAb sandwich ELISA was found to be better suited to this application than RIA because it allowed for large-scale detection. It was found to be 10 times more sensitive with a lower background, and is better for early detection while placental development is still proceeding (Burger *et al.*, 2004).
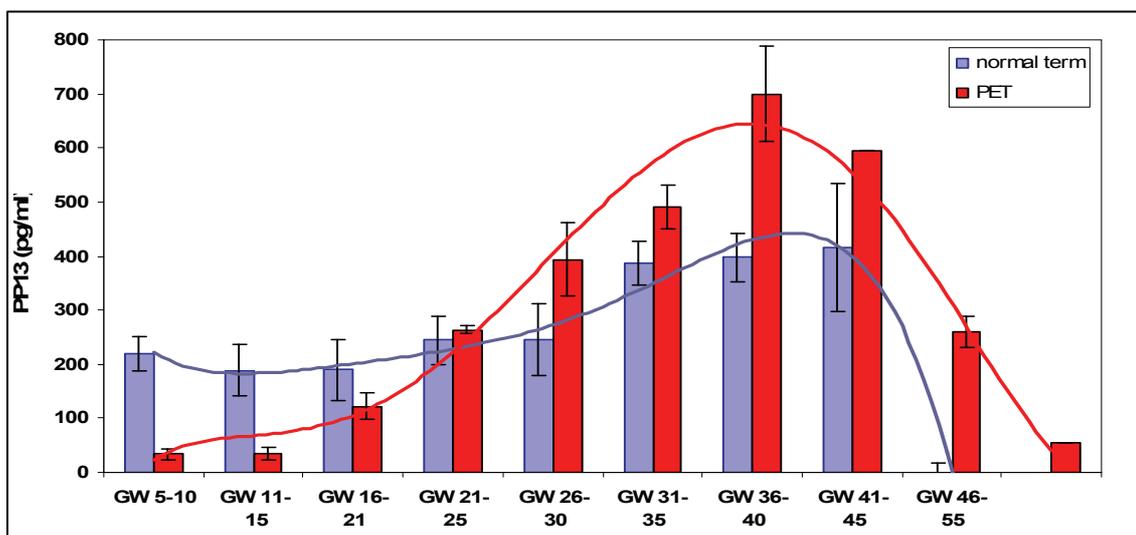
Figure 1.5 A comparison of PP13 levels in normal term versus pre-eclamptic (PET) pregnancies. GW = gestational week. (Image: Courtesy of Dr M Sammar, workshop presentation at the IFPA conference, 2008.).

These varying PP13 levels made it an ideal candidate for use as a biomarker for pre-eclampsia. Abnormal levels are observed in the first trimester, which allows for screening early in pregnancy so that women can begin prophylaxis relatively early and thereby decrease/reduce their risk of developing pre-eclampsia or its complications.

Recently, several studies have focused on the use of placental protein 13 (PP13) in combination with uterine artery Doppler pulsatility index in the first trimester as potential markers of early pre-eclampsia (Nicolaides *et al.*, 2006, Spencer *et al.*,

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2007a, 2007b, Chafetz *et al.*, 2007) and intrauterine growth restriction (Chafetz *et al.*, 2007).

In the study by Nicolaides *et al.* (2006), first-trimester maternal serum PP13 levels were measured together with the pulsatility index (PI) of blood flow in the uterine arteries, determined by Doppler ultrasound, in women who subsequently developed early pre-eclampsia (delivery before 34 weeks) as well as in unaffected control individuals. Doppler ultrasound is used to assess the impairment of blood flow in maternal uterine arteries and is useful for the identification of pregnancies with inadequate trophoblast invasion of the maternal arteries, the hallmark of pre-eclampsia. The median uterine artery PI was higher and median serum PP13 level lower in cases that developed early pre-eclampsia, when compared with unaffected pregnancies. Screening of maternal serum PP13 levels in combination with measurement of uterine artery PI had a 90% detection rate with 6% false-positives (Nicolaides *et al.*, 2006).

Currently, there is no biomarker for the prediction of abruptio placentae. Such a molecule would be invaluable in clinical practice among patients with a history of the condition, as well as other risk factors such as hypertension.

1.6 GALECTINS

The highly conserved galectin family originated more than 800 million years ago, and their trademark specificity for β-galactoside binding and various other molecular properties such as their characteristic amino acid sequences and protein architecture have been maintained throughout evolution (Visegrady *et al.*, 2001). Galectins have been identified in a wide range of vertebrates, including mammalian and non-mammalian vertebrates, as well as non-vertebrates such as nematodes and plants (Cooper, 2002). Vertebrate galectins have been identified in a number of tissues such as the skin, liver, kidney, intestine, brain and placenta (Kasai & Hirabayashi, 1996). Their biological functions seem to vary based on site and time of expression; however the basic molecular function of the galectin family as a whole is to decipher glycocodes.

On the basic of their structural features, mammalian galectins may be subdivided into three groups, namely (i) prototype, (ii) tandem-repeat and (iii) chimeric.

Three Architectural Types of Galectin

Proto	Chimera	Tandem-repeat
Two identical Galectin CRDs	Non-lectin Domain / Galectin CRD	Two distinct Galectin CRDs
Galectin-1,2,5,7,10	Galectin-3	Galectin-4,6,8,9
Cross-link homologous carbohydrate ligands	Cross-link carbohydrate and non-carbohydrate ligands	Cross-link two distinct carbohydrate ligands
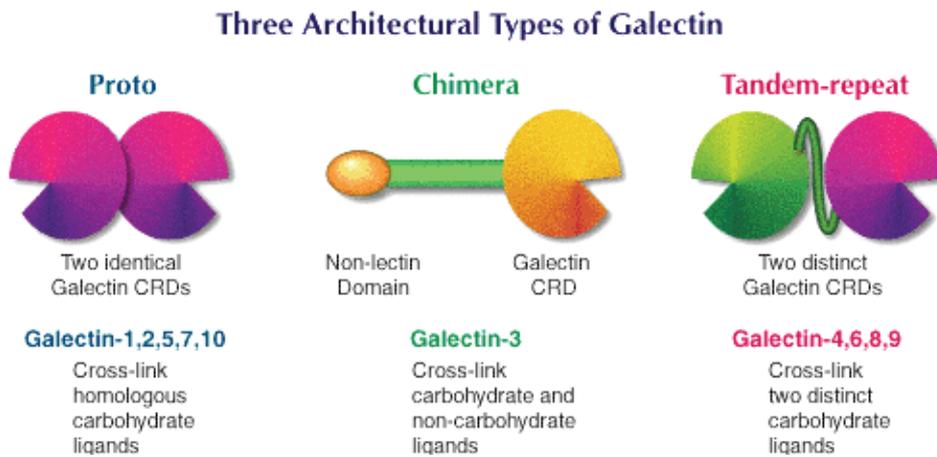
Figure 1.6 Classification of the galectin family into the prototype, tandem-repeat and chimera groups, based on their structural features. CRD = carbohydrate-recognition domain. (Image: Courtesy of Dr M Sammar, workshop presentation at the IFPA conference, 2008.)

(i) Prototype galectins

The galectins within this group are generally small proteins comprising a peptide chain with a single carbohydrate recognition domain (CRD) and are often found as monomers (gal-5,-7,-8,-10,-13,-14) or as monomers/dimers (gal-1,-2,-11) (Chiariotti *et al.*, 2004). Dimerization of the prototype galectins involves association of subunits at sides opposite to the CRD (Cooper *et al.*, 2002). Sequence alignment of some prototype galectins shows conservation surrounding exon 3, which encodes the CRD (Kasai & Hirabayashi, 1996). Most prototype galectins display distinct tissue specific and developmentally regulated expression (Cooper *et al.*, 2002).

(ii) Tandem-repeat galectins

These galectins are characterized by the presence of two non-identical carbohydrate recognition domains on the same peptide chain, connected by a

short linker peptide of variable length (gal-4,-6,-8,-9,-12) (Chiariotti *et al.*, 2004). Some of these galectins have their CRDs positioned in such a way to allow simultaneous binding on multivalent ligands and enhance binding avidity. The presence of two different galectin CRDs in the same protein means that the protein is able to specifically crosslink two distinct types of ligand, as opposed to homofunctional cross linking by dimeric prototype galectins (Cooper *et al.*, 2002). The structure of the linker peptide which joins the two CRDs is important. In mammals, tissue-specific splice variants of galectin-8,-9 and -12 differ only from the major form of galectin by their linker peptides. The apparent tissue-specificity and cross-species conservation implies that linker peptides play an important role beyond binding the CRDs (Cooper *et al.*, 2002).

(iii) Chimeric galectins

Galectin-3 is the only galectin in this group. It is comprised of a single CRD attached to distinct N- or C-terminal domains which possess different functions (Cooper *et al.*, 2002). The CRD at the C-terminal end of the protein is homologous to that of other galectins, whereas the domain at the N-terminal end is related to components of the heteronucleur ribonucleoprotein complex (hnRNP) (Kasai & Hirabayashi, 1996). In addition, a repetitive region which is rich in proline, glycine and tyrosine residues is found at the N-terminal domain. Deletion of the first 11 amino acids of the initial amino acid N-terminal peptide blocks galectin-3 secretion, which suggests that this sequence preceding the proline/glycine rich region is a functional domain (Cooper *et al.*, 2002).

1.6.1 Secretion of galectins

Galectins are soluble cytoplasmic proteins, characterized by an acetylated N-terminus and lack of glycosylation. Although some galectins are secreted they lack a typical secretion signal peptide and do not associate with the ER/Golgi pathways and are not localized in secretory compartments. Instead, they are secreted to the cell surface via a non-classical pathway, by mechanisms distinct from classical vesicle mediated exocytosis (Cooper *et al.*, 2004). It has been suggested that this mode of secretion is necessary to segregate galectins from their glycoconjugate

ligands, which are secreted by the classical pathway, to allow for interaction between the two only after externalization. Alternatively, multiple secretion pathways may exist to facilitate the selective secretion of galectins in response to specific cellular signals (Barondes *et al.*, 2004).

1.6.2 Functions of galectins

Galectins mediate processes such as the interactions between cells and extracellular matrix components, cell adhesion and cell signalling by cross-linking to β-galactoside-containing glycoconjugates. They exhibit developmentally-regulated expression in a variety of cell types and have the capacity for multiple interactions with carbohydrate ligands, which makes them important factors influencing cell-cell and cell-matrix interactions. Galectins are also thought to mediate cell migration, cell growth regulation, tissue differentiation and remodelling, apoptosis triggering or inhibition and may play role in neoplastic transformation, tumour progression, invasion and metastasis (Visegrady *et al.*, 2001).

Each member of the galectin family, in addition to the above mentioned properties, has additional specific functions. Galectin-1 and -3 are believed to be involved in a wide range of physiological and pathological processes such as carcinogenesis, tumour progression, metastatic potential, T-cell mediated immune disorders, acute inflammation, microbial infections and even pre-mRNA splicing (Chiariotti *et al.*, 2004). Galectin-7 plays a role in mediating the proliferation and differentiation of epithelial cells. Galectin-9, -10 and -14 have been implicated in a number of allergic processes. The gene encoding galectin-9 gives rise to two distinct isoforms of the protein, each with its own unique function, one involved in uric acid translocation (as a urate transporter/channel transmembrane protein), the other in immune/inflammatory processes (as an eosinophil chemoattractant). Galectin-10 is the Charcot-Leiden Crystal protein found in eosinophils and basophils.

It is clear that each galectin has a specific role intra- or extracellularly, which is largely dependant on the tissue type or on a specific developmental or differentiation stage and that galectins perform their necessary functions in a given

tissue, at a given time. This requires the precise regulation of expression and activity, coordinated by regulation at a transcriptional level as well as changes at a biochemical level, for example by changing location, or mediating glycosylation of specific ligands (Chiariotti *et al.*, 2004).

Most galectins are believed to play an important role in embryogenesis. Specific glycosidic structures are essential for a number of events during embryogenesis such as fertilisation and implantation, and galectins are likely to be involved in these processes, because at least five members of the galectin family are expressed in distinct and specific patterns during the processes of embryogenesis in the mouse. Galectin-1 is shown to be activated in trophectoderm cells of mouse embryo a few hours before implantation; however studies with knockout mice have shown that mice deficient in galectin-1 (homozygous galectin-1 null mutants) remain fertile and viable. Galectin-3 is another member of the galectin family that accumulates in the trophectoderm cells along with galectin-1 in the hours leading up to implantation, and it was therefore proposed that the two galectins may work together and have overlapping functions during this stage of embryogenesis. However, once again, the generation of galectin-3 null mutant mice, as well as galectin-1/galectin-3 double mutant mice, had no influence on implantation or early development (Colnot *et al.*, 1998).

1.7 PLACENTAL PROTEIN 13 / GALECTIN-13

Placental protein 13 (PP13) is a member of the pregnancy-related protein family, of which there are various maternal, fetal or fetoplacental proteins which are produced in increasing amounts during pregnancy. Although these proteins differ in structure and function, they all play a key role in the development of the fetus and placenta or in maintenance of the pregnancy (Than *et al.*, 1999). PP13 was first purified and characterized by Bohn *et al.* in 1983 and was found to be composed of two 16 kDa subunits linked by disulphide bonds. Based on sequence analysis and alignments, PP13 was found to possess high homology with the Charcot-Leyden Crystal (CLC) protein (galectin-10), as well as other members of the galectin family (Than *et al.*, 2004). Northern blot analysis detected the expression of PP13 mRNA in placental tissue alone; PP13 is not expressed in any

other human adult tissues (Than *et al.*, 1999) but has more recently been reported in human fetal liver and spleen tissues (Than *et al.*, 2004).
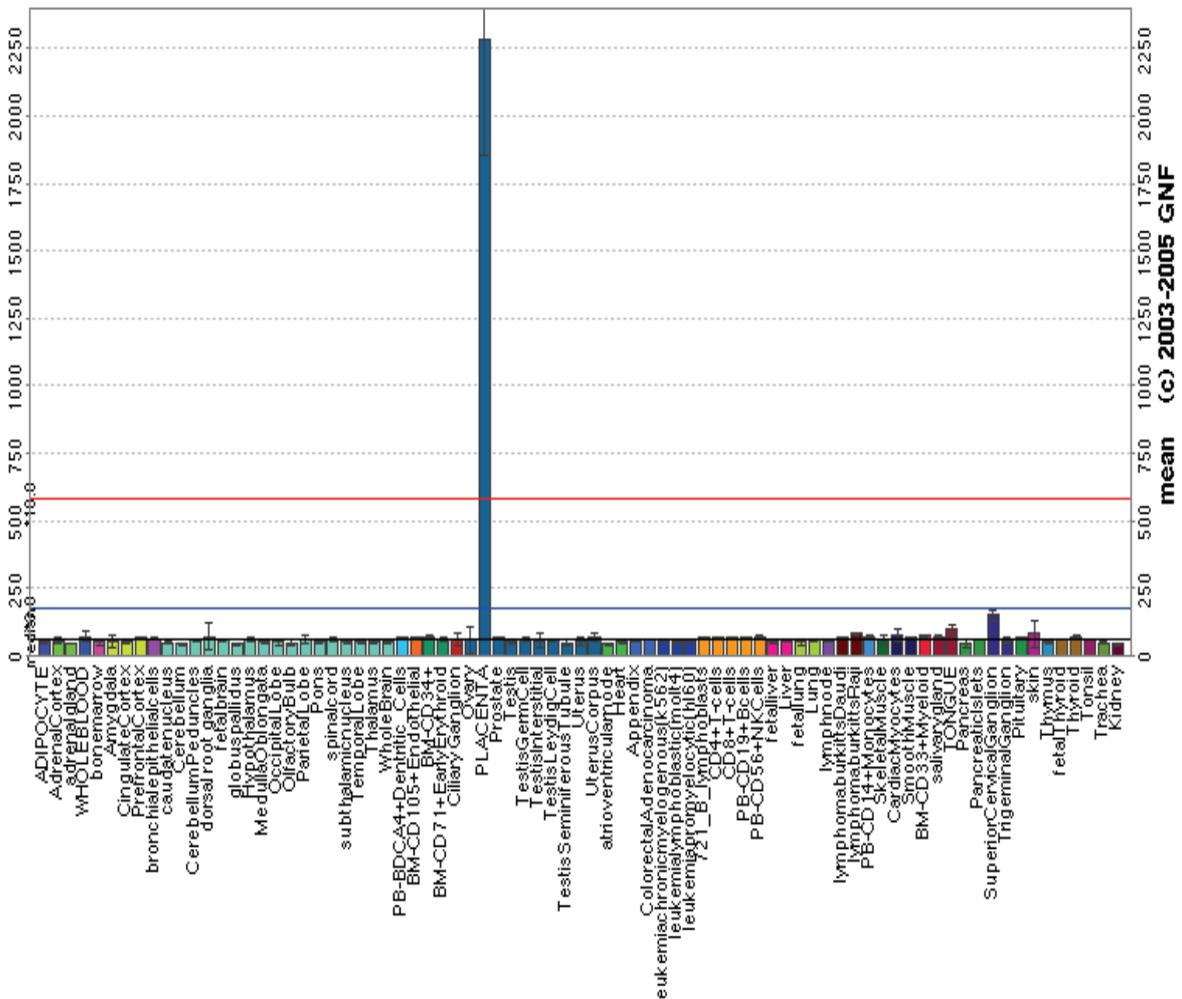
Figure 1.7 GNF SymAtlas expression profile of the *LGALS13* gene in various human tissues (http://wombat.gnf.org/SymAtlas/) - accessed Dec 2008

Placental protein 13 shares many features with its galectin family members. It too is a soluble cytoplasmic protein, shares an identical secondary structure with the CLC protein, possesses the highly conserved galectin carbohydrate recognition domain, as well as the overall "jellyroll" structural fold shared by the prototype galectins. Due to these shared structural and functional characteristics, PP13 was designated galectin-13.

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1.7.1 Biochemical features of PP13

1.7.1.1 Phosphorylation

Bioinformatic analysis of the protein revealed that phosphorylation by casein kinase II or tyrosine kinase may be involved in PP13 regulation. Putative serine and tyrosine kinase phosphorylation sites were found on the surface of PP13 close to its CRD at positions 44-52 (Ser48), 37-45 (Tyr41), 76-84 (Tyr80). Phosphorylation may have an influence on the functional properties of PP13 as it may modulate its carbohydrate affinity and act as an 'on/off' switch, as is the case with galectin-3 (Than *et al.*, 2004).

1.7.1.2 Lysophospholipase activity

PP13 was found to have weak lysophospholipase activity (Than *et al.*, 1999). Lysophospholipases are esterolytic enzymes, found in most cells, ranging in form and function. They catalyse the removal of a single fatty acid from the 1-position of lysophospholipids, which are generated by phospholipase A2 catalysing fatty acid hydrolysis from the 2-carbon position of phospholipids. It has been suggested that the weak lysophospholipase activity of PP13 may be involved in the regulation of vasoconstriction and vasodilation, as well as maternal artery remodelling (Burger *et al.*, 2004).

1.7.1.3 Sugar-binding activity

PP13 has sugar-binding activity (Visegrady *et al.*, 2001; Than *et al.*, 2004) and effectively binds several sugars with different binding affinities to the PP13 binding site. The sugar-binding domain of PP13 has been shown to bind with the highest affinity to N-acetyl lactosamine, which is found in glycoconjugates such as laminin and fibronectin, two major components of the uterine epithelia which are widely expressed in the placenta (Kasai & Hirabayashi, 1996).

Knowledge of the morphological localization of PP13 in various cells and tissues, together with its associated proteins, should be considered when trying to explain

and understand the biological functions of this protein and its exact interactions with glycoconjugates. This may elucidate the role of PP13 in placental development and its subsequent involvement in pregnancy complications.

1.7.2 Associated proteins

A study by Than *et al.* in 2004 reported that human annexin II and beta/gamma actin interact with PP13 intracellularly. The proposed role of this interaction is that actin filaments play a role in the translocation of lectins during the differentiation processes of trophoblasts and could be involved in concentration of cytosolic galectin at specific cytoskeletal regions. The exact mechanism of PP13 transport to the outer surface of the syncytiotrophoblast plasma membrane is unknown, but is assumed to involve secretion through a non-classical route similar to other galectins. Fibroblasts have been shown to secrete galectins by ectocytosis in microvesicles containing actin and annexin II. PP13 potentially uses the same pathway for externalization.

The most recent study in this regard (Than *et al.*, 2008) confirmed the presence of PP13 in villous endothelium and syncytiotrophoblast membrane blebs, which provides evidence of accumulation of the protein below the plasma membrane, followed by subsequent secretion via exovesicle shedding.

Annexin II is a member of the calcium- and phospholipid-binding protein family and is present on the apical extracellular surface of syncytiotrophoblasts. The annexin II protein is involved in placental differentiation and a variety of functions of mature microvilli. It acts as a co-receptor for tissue plasminogen activator and plasminogen on endothelial cells and functions by stimulating tissue plasminogen activator-dependent conversion of plasminogen to plasmin. In addition, the protein is also involved in regulation of ion channels and inactivation of PLA2 and prothrombin. These functions suggest that the interactions of this protein with PP13 at the feto-maternal interface may have implications in placental haemostatic processes (Than *et al.*, 2004).

1.7.3 Localization

Immunolocalization studies in human term placenta revealed a specific pattern on the brush border membrane of the syncytiotrophoblasts which line the common feto-maternal blood spaces of placenta (Than *et al.*, 2004). Figure 1.7 shows PP13 and annexin II co-localized at the brush border membrane.

(A)   (B)   (C)
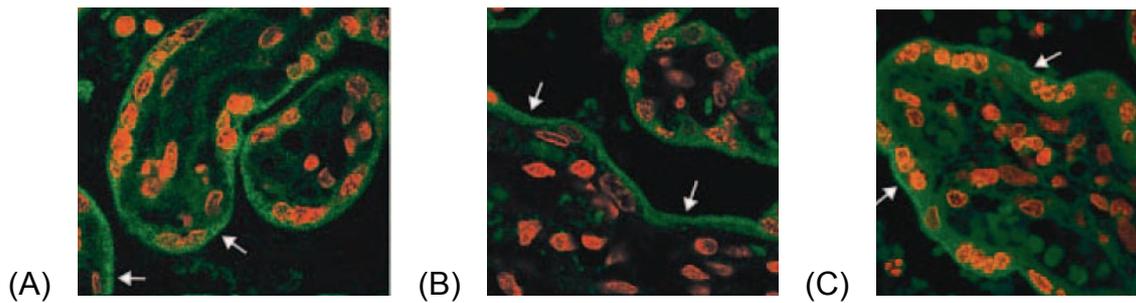
Figure 1.8 Localization of placental protein 13 and annexin II at the brush border membrane of syncytiotrophoblasts. (A) – (C) are confocal images (750x magnification) which have been stained with DRAQ5 nucleus dye (red). Arrows denote the brush border membrane. (A) and (B) show PP13 (green) localization, (C) shows annexin II (green) in the syncytiotrophoblasts, as well as on the brush border membrane (Than *et al.*, 2004).

The location of PP13 on brush border membranes of syncytiotrophoblasts corresponds with the structure and distribution of specific glycans in the human placenta. N-acetyl-lactosamine, mannose and N-acetyl-glucosamine have high affinity for the PP13 CRD and are commonly expressed on villous surfaces of the placenta (Than *et al.*, 2004).

1.7.4 *LGALS13*

PP13 is encoded by the *LGALS13* gene, which is located on the long arm of chromosome 19 (19q13.1), in close proximity to the genes of four known galectins (galectin-10, galectin-7, galectin-4 and placental protein 13-like protein) and three putative galectins. This cluster of genes have similar exon structures and their encoded proteins share 80% homology, which suggests the occurrence of a gene

multiplication event in the galectin subfamily (Than *et al.*, 2004). A number of placenta-specific transcription factor binding sites were identified in the promoter region, which corresponds with the differential placental-specific expression of this gene (Than *et al.*, 2004). There is not, however, much experimental data on the study of transcriptional regulation reported in these genes. Upstream regulatory regions of galectin-1,-2,-3,-4,-6,-10 from different species have been cloned, as well as human galectin-9 and -12 and rat galectin-11. Functional analysis of the promoter region has been performed for a few galectins (Chiariotti *et al.*, 2004); however, literature regarding the promoter region and upstream regulation of PP13 is very limited.

A cluster of galectin genes is present on chromosome 19, spanning the 19q13.1-13.2 region, with high conservation among these genes as well as their surrounding untranslated regions. The cluster includes several pseudogenes, galectin-13, PP13-like gene and *LGALS13* and is in close proximity to the genes encoding galectin-4 and galectin-7. The expression patterns and tissue specificity of these galectins was investigated by examination of the 5' untranslated regions (UTRs) of these genes and comparison with the well-characterized promoter region of galectin-10, with which *LGALS13* shares high homology. The galectin-10 gene, *LGALS10*, has an imperfect TATA box ~31 bp upstream of the transcription start site and a consensus cap site (-77 to -71). This cap site is conserved in all the galectin-10 relatives, PP13 included, but the TATA box is not. A CCAAT box is present ~35 bp upstream of the transcription start site. In galectin-10 promoter, three eosinophil transcription factor sites are present which are important for the regulation of eosinophil specific genes, none of which are conserved in PP13. Two potential GATA-1 binding sites (GATA-1a and b) were reported in the galectin-10 promoter, of which GATA-1b (-285 to -280) is conserved in PP13 (Cooper *et al.*, 2002).

Analysis of a placenta-specific promoter identified at least five different sequence elements required for placental specificity; however identification of these transcription factor binding sites does not mean that they are functionally active. A potential binding site for placental-enriched TEF-5 factor was identified in PP13, PP13-like and galectin-10 genes (-118 to -110). It is possible that the placenta-

specific relatives of galectin-10 evolved to play a major role in maintaining the immune balance at the feto-maternal interface (Cooper *et al.*, 2002).

Recent gene expression studies showed that *LGALS13* expression was lower in preterm pre-eclampsia than in controls matched for gestational age. The localization of PP13 to syncytiotrophoblasts, but not cytotrophoblasts, suggests that *LGALS13* gene regulation could be related to syncytialization (Than *et al.*, 2008).

1.7.5 Functions of PP13

Although the role of PP13 in pregnancy is not fully understood, it is clear that this protein is important in several biochemical and physiological processes in the trophoblast which may be implicated in implantation, blood pressure regulation and tissue oxygenation (Burger *et al.*, 2004). PP13 homologues, galectin-1 and galectin-3, bind several placental glycoconjugates (e.g. laminin and fibronectin), and they may therefore be involved in several important physiological events such as embryo implantation, trophoblast invasion and embryogenesis (Visegrady *et al.*, 2001). Galectin secretion is responsive to developmental events. During placentation, changes in distribution patterns of PP13 homologues, galectin-1 and galectin-3, were found to correlate with differentiation pathways of trophoblasts (Than *et al.*, 2004).

A number of processes are crucial for ensuring the normal development and organisation of the placental structure and environment. Most involve the binding of cells to the extracellular matrix proteins via surface receptors called adhesion molecules. These include integrins, cadherins, selectins and the immunoglobulin superfamily. These processes include cell migration, which is dependant on cell-matrix interactions for anchorage, and cell growth and differentiation. The galectins are involved in these processes of cell-cell and cell-matrix interaction as well as in cell-growth regulation and apoptosis. Galectins are developmentally regulated and associated with the presence of specific carbohydrate-rich structures in the placenta. As a member of the galectin family, PP13 is believed to play a critical

role in these biological processes as well as in placental development (Than *et al.*, 1999).

The lysophospholipase activity of PP13 has implications in implantation. Lysophospholipids play a role in implantation in rabbit embryos, by facilitating the cellular fusion of trophoblasts and the uterine epithelial cells and, as a result, the penetration of the embryo into the decidua. PP13 may therefore have a protective function during implantation and in maintenance of normal pregnancy (Than, 1999). In addition, the implication of several galectins in immune and inflammatory processes (Rabinovich *et al.*, 2002) suggests that the PP13 may also have specialised immune functions at the feto-maternal interface.

1.7.6 The hypothesis: PP13 and abruptio placentae

The functions of PP13 and its localization at the feto-maternal interface suggest that it plays a crucial role in normal developmental processes and specifically implantation and placentation.

DNA sequence variants in the encoding LGALS13 gene may ultimately lead to the expression of an aberrant form of the protein, which may affect functionality and subsequently disrupt normal implantation and placentation.

This may culminate in placental vasculopathies such as abruptio placentae, a disorder in which the underlying cause is believed to be related to faults in early placental development.

1.8 AIM AND OBJECTIVES

The aim of this study was to investigate whether DNA sequence variants in the *LGALS13* gene underlie and/or confer susceptibility to abruptio placentae.

This would be achieved by:

1) Screening and genotyping DNA sequence variants in the entire *LGALS13* gene in patients with abruptio placentae and matched control samples
2) Comparing allele and genotype frequencies
3) Using appropriate statistical tools to analyze the data
4) *In silico* analysis of the identified variants to predict the effects thereof
5) Investigating the possible functional effect of a 5'UTR DNA sequence variant using a reporter gene assay.

CHAPTER 2:

MATERIALS AND METHODS

MATERIALS AND METHODS

MATERIALS

2.1 STUDY COHORT

This project was approved by the Institutional Review and Ethics board (N05/07/122) and written, informed consent was provided by all participants. The study cohort comprised South African Coloured and Black women from the Western Cape, who were recruited at Tygerberg Hospital. The patient cohort comprised a total of 195 women, diagnosed with abruptio placentae, as well as their neonates. This total maternal group was represented by 138 Coloured and 57 Black women.

The control cohort comprised 237 Coloured and 99 Black women from Paarl Hospital. Controls were selected on the basis of their obstetric outcome and history - consequently, only healthy women with a good obstetric outcome and no history of placental vasculopathy were selected. The control group was matched to the patient group, regarding ethnicity.

Abruptio placentae was diagnosed clinically and confirmed upon postpartum inspection of the placenta, with a positive diagnosis based on the presence of blood clots covering more than 15% of the maternal surface of the placenta (Odendaal *et al.*, 2000).

METHODOLOGY

2.2 DNA EXTRACTION

Maternal whole blood was drawn from peripheral veins and fetal whole blood was obtained from the umbilical cord at delivery (by hospital staff). Blood samples were stored in EDTA tubes (Becton, Dickson and Company, New Jersey, USA) at -20°C. Genomic DNA was extracted from 5 - 10 ml of maternal and fetal whole

blood using an adaptation of the simple salting out extraction method described by Miller *et al.*, 1988 (Appendix 6.3). Collection of fetal blood from the umbilical cord sometimes yielded only small volumes of whole blood, and in these cases, when less than 1 ml of fetal blood was available, the Puregene ® DNA Purification System (Gentra Systems, MI, USA) was used for extraction of genomic DNA. Following extraction, DNA concentrations were determined by the NanoDrop® ND-100 Spectrophotometer (NanoDrop Technologies, USA) and samples were stored at 4°C.

2.3 POLYMERASE CHAIN REACTION

2.3.1 Oligonucleotide primers

LGALS13 reference sequences were obtained from the Ensembl Genome Browser (http://www.ensembl.org/) and oligonucleotide primers were designed using the IDT® Primer design software (Integrated DNA Technologies Inc., Coralville, USA) (http://www.idtdna.com/) and analysed for the formation of hairpins, as well as homo- and heterodimers using the OligoAnalyzer software from IDT®. Primers for mutation detection were designed to include ~170bp of the 5'UTR region of the *LGALS13* gene, as well as the four exons and ~60bp of their flanking intronic regions to ensure detection of variants which could affect splicing. Generally, large fragments do not resolve well on Multiphor SSCP-HD polyacrylamide gels and are not suited to this method of mutation detection. For this reason, exons 3 and 4 were each split into two smaller overlapping amplicons. Primer specificity was confirmed with the NCBI Basic Local Alignment Search Tool (BLAST) (http://www.ncbi.nlm.nih.gov/BLAST/). All primers were synthesized by the Synthetic DNA Laboratory (UCT, Cape Town, South Africa).
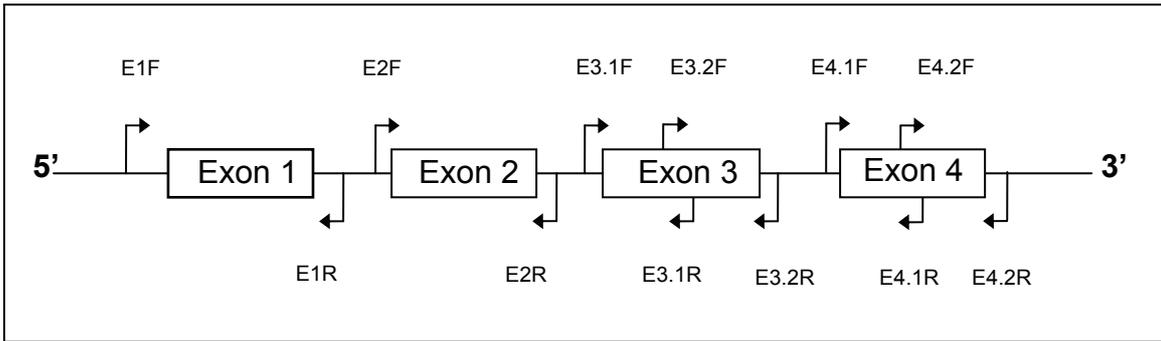
Figure 2.1 Schematic representation of *LGALS13* oligonucleotide primer placement.

2.3.2 DNA amplification

The coding and flanking non-coding regions of the *LGALS13* gene were amplified using the oligonucleotide primers described in Table 2.1. PCR reactions for amplification of exons 2 - 4 included: 1 X GoTaq buffer (Promega, Madison, USA), 1.5 mM $MgCl_2$ (Promega), 0.5 U GoTaq® Flexi DNA Polymerase (Promega), 0.25 mM of each dNTP (dATP, dCTP, dGTP, dTTP) (Fermentas Inc., Hanover, USA), 10 – 20 pmol each primer and ~20 ng gDNA. The amplification of exon 1 required the use of 1 X Flexi buffer (Promega) and 2 mM $MgCl_2$ (Promega), with the concentrations of other PCR components remaining the same as described above. All reactions were made up to a final volume of 50 µl with nuclease-free water.

PCR amplifications were performed using the GeneAmp® PCR System 2700 (Applied Biosystems, California, USA). The PCR cycling conditions were: initial denaturation for 3 min at 95°C, followed by 35 cycles of 20 s denaturation at 95°C, 30 s annealing at a temperature specifically optimized for each primer set, an elongation step of 20 s at 72°C, followed by final extension step of 5 min at 72°C and cooling at 10°C.

Table 2.1 Oligonucleotide primers for PCR amplification of the coding and surrounding non-coding regions of the *LGALS13* gene

Position	Primer	Primer sequence (5'→3')	Ta (°C)	Size (bp)
Exon 1	E1F	GTTTCTTCCTAACAACTAAACCTG	52	324
	E1R	TATTCCACACCTCAATAGCTCTA		
Exon 2	E2F	CAACCTCCTGCACCATGAG	52	246
	E2R	CATCACCCACATGTAAGGTC		
Exon 3	E3.1F	GGCCATCAGTATTATCTGGGAG	54	213
	E3.1R	GATTGCCAAAGTGCACTCGG		
	E3.2F	CAGGTGGATTTCTACACTGAC	53	288
	E3.2R	CCCTGACGGACTACTGAG		
Exon 4	E4.1F	TGTACCAGGACAGAGTGGAG	53	196
	E4.1R	CTATTGCAGACACACACTGA		
	E4.2F	GAGAGATATCTCCCTGACCTC	54	260
	E4.2R	CCTGATGCCTCCCATAGAATG		

2.3.3 Agarose gel electrophoresis

All PCR amplicons were analyzed by agarose gel electrophoresis as follows: Briefly, 5 µl of 6 X Orange Loading Dye Solution (Fermentas) was added to 10 µl of each PCR product and resolved by electrophoresis on 1% (w/v) agarose gels, containing 0.5 µg/ml Ethidium Bromide, in 1 X TBE buffer (Appendix 6.2). Amplicons were resolved for 30 minutes at 120 V, with a 100 bp ladder (O'GeneRuler^{TM}, Fermentas). DNA fragments were visualized with an ultraviolet (UV) light at A_{260}nm, and photographed using the MultiGenius Bio Imaging Capture System (Syngene, Cambridge, UK).

2.4 MUTATION DETECTION

2.4.1 SSCP-HD analysis

Single Stranded Conformational Polymorphism and Heteroduplex (SSCP-HD) analysis was the chosen method of mutation detection for this study. The Pharmacia LKP 2117 Multiphor^{TM} II Electrophoresis System (Amersham Pharmacia Biotech, Amersham, UK) allows for the detection of ~97% of mutations under optimal conditions (Liechti-Gallati *et al*. 1999). Briefly, 3 µl of each PCR product was heat denatured at 95ºC for 5 minutes in an equal volume of formamide- based loading dye (Appendix 6.2) and subsequently quenched on ice. Products were resolved by electrophoresis at 355V on 12% polyacrylamide gels (Appendix 6.2) at a constant temperature for the identification of conformational variants, using an adaptation of the protocol described by Liechti-Gallati *et al.*, 1999. The time and temperatures at which the PAGE gels were resolved were optimized for each amplicon in order to ensure maximum sensitivity, and are indicated in Table 2.2. The single-stranded conformational polymorphism and heteroduplex components of the polyacrylamide gel were visualized by 0.1% silver staining (Appendix 6.2).

Table 2.2 Experimental conditions for *LGALS13* amplicons using the Multiphor system for mutation detection.

Amplicon	Temperature (°C)	Time (hr, min)
E1	4	3h00
E2	9	2h30
E3.1	11	2h45
E3.2	4	1h30
E4.1	11	2h30
E4.2	11	2h30

2.4.2 Semi-automated DNA sequencing

Samples demonstrating conformational variation by SSCP-HD analysis were subjected to semi-automated DNA sequencing. PCR products were purified prior to sequencing to ensure removal of excess primers, primer dimers and dNTPs using the SureClean kit (Bioline, London, UK) according to the manufacturers' protocol. The concentration of these purified products was determined by spectrophotometric measurement at 260nm with the NanoDrop ® ND-100 (Nanodrop Technologies Inc., USA). Sequencing reactions were performed using the BigDye ® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, California, USA) and products were analyzed by electrophoresis on an ABI 3130XL Genetic Analyzer (Applied Biosystems, California, USA). Sequencing reactions and electrophoresis were performed at the Central Analytical Facility (Stellenbosch University, Stellenbosch, South Africa). Sequences and electropherograms were subsequently analyzed using BioEdit Sequence Alignment Editor v7.0.5 software (Hall, 1999). Visual inspection of the electropherograms, as well as alignment of the sequences with published reference sequences obtained from Ensembl was performed to identify sequence variation.

2.4.3 Restriction enzyme analysis

Sequence variants which were identified by semi-automated DNA sequencing analysis were characterized using the restriction map feature of the BioEdit Sequence Alignment Editor v7.0.5 software (Hall, 1999), together with RestrictionMapper version 3 (http://www.restrictionmapper.org/), in order to identify any restriction endonuclease sites that were created or abolished by these identified variants. The respective restriction enzymes were subsequently used for confirmation of the conformational variants identified by SSCP-HD analysis. Restriction enzyme digestion reactions were set up according to manufacturer recommendations. Briefly, 10 - 15 µl of PCR product was digested with 1 - 2 units of restriction enzyme in a total reaction of 20 µl at the recommended temperature for 16 hours. Table 2.3 shows the various restriction enzymes (and their conditions) used in this study. Restricted PCR products were resolved on 2% (w/v)

agarose gels at 80 V for ~90 minutes with a 100bp ladder (O'GeneRuler™, Fermentas). Visualization of the DNA fragments was by UV light and images were captured as described in section 3.2.3.

Table 2.3 Restriction enzyme digestion conditions for genotyping of the conformational variants identified in the *LGALS13* gene.

Variant	dbSNP rs	Enzyme	Recognition site	Conditions	Manufacturer
-98A/C	3764843	*Ava*I	5' C↓YCGRG '3 5' GRGCY↑C '3	37°C 16 hours	Fermentas*
-36G/A	NOVEL	*Nla*IV	5' GGN↓NCC '3 5' CCN↑NGG '3	37°C 16 hours	NEB**
-22A/G	2233706	*Acl*I	5' AA↓CGTT '3 5' TTGC↑AA '3	37°C 16 hours	NEB
-15G/A	NOVEL	*Nla*III	5' CATG↓ '3 5' GTAC↑ '3	37°C 16 hours	NEB
Hotspot	NOVEL	*Apo*I	5' R↓AATTY '3 5' YTTAA↑R '3	55°C 16 hours	NEB
+72T/A	2233708	*Stu*I	5'AGG ↓CCT '3 5' TCC↑GGA '3	37°C 16 hours	NEB

* Fermentas, Hanover, USA   ** NEB, New England Biolabs Inc., Beverly, USA

2.5 STATISTICAL ANALYSIS

The allele and genotype frequencies of the patient and control groups' samples were determined and the Hardy-Weinberg equilibrium (HWE) Chi squared test was performed. HWE was considered when the observed and expected genotype frequencies did not differ significantly from each other ($P>0.05$). Both the patient and control groups were stratified according to ethnicity and the data was analyzed using contingency tables in StatCalc (EpiInfo, CDC, Atlanta, GA) and Fishers Exact Test, with a P-value of less than 0.05 indicating statistical significance.

2.6 PREPARATION OF LUCIFERASE REPORTER GENE CONSTRUCTS

The *LGALS13* gene and its upstream regulatory region had not previously been characterized and very little information was available regarding the structure of the 5' untranslated region and transcriptional regulation, in general. The following section describes the construction of luciferase reporter gene constructs in order to investigate the functionality of a variant identified in the 5'UTR of the gene, - 98A/C (dbSNP rs:3764843).

2.6.1 Oligonucleotide primers

Reference sequences for the *LGALS13* 5' untranslated region were obtained from Ensembl and oligonucleotide primers were designed as described in Section 2.3.1. Primers were designed to amplify a 700bp region, immediately upstream of the start codon, ATG in exon 1. The selection of the 700bp region specifically was due the existence of a highly repetitive region immediately upstream of this amplicon. In order to ensure the specific amplification of *LGALS13*, and not one of its highly homologous family members, the forward primer was placed immediately 3' of this repeat-rich block in a position which ensures maximum binding specificity.

To facilitate the ligation of this amplicon into the pGL4.23 minimal promoter vector (Promega; Appendix 6.5), primers were designed to include restriction endonuclease recognition sites *Xho*I (5'C↓TCGAG3') and *Bgl*II (5'A↓GATCT3') at their 5' and 3' ends, respectively. *Xho*I and *Bgl*II were selected because (i) they do not have recognition sites in the 700bp insert, (ii) they yield non-compatible sticky ends, and (iii) digestion with these particular enzymes ensures orientation-specific ligation of the insert into the vector.

Table 2.4 Oligonucleotide primers for PCR amplification of the 5' untranslated region of the *LGALS13* gene. Restriction enzyme recognition sites are shown in red.

Primer	Primer sequence (5'→3')	Cut site
F	AGT**CTCGAG**AATCTGCAGGAGAAACAGTGTCTCT	*Xho*I
R	TGA**AGATCT**TGTTCTCTCCTTCTTGGCGACCTTC	*Bgl*II

Primer specificity was confirmed with the NCBI Basic Local Alignment Search Tool (BLAST) (http://www.ncbi.nlm.nih.gov/BLAST/) and primers were synthesized by IDT ® Integrated DNA Technologies (Whitehead Scientific, Cape Town, South Africa).

2.6.2 DNA amplification

DNA samples harboring a homozygous status for the -98 wild type (A) and variant (C) alleles, respectively, were used for amplification of the 700bp region of the 5' untranslated region. Sequencing confirmed the homozygosity status of these samples and excluded the presence of additional DNA sequence variants in this amplicon.

The 700bp region was amplified using the KAPA Long Range Kit (KAPA Biosystems, Cape Town, South Africa) with the following 25 µl reaction setup: 1 X KAPA Long Range Buffer, 2.5 mM $MgCl_2$, 0.30 mM of each dNTP, 0.50 µM of each primer (F1 and R1), 0.625 U Long Range Enzyme, ~200 ng gDNA and brought to volume with dH_2O. This fragment was amplified using the cycling conditions described in Table 2.5.

Table 2.5 PCR Cycling conditions for amplification of the 5' *LGALS13* 700bp fragment.

Cycle	Temperature (°C)	Time	# of cycles
Initial denaturation	95	5 min	1
Denaturation	95	20 s	
Annealing	55	20 s	35
Elongation	68	40 s	
Final extension	72	4 min	1
Cooling (Hold)	10	Indefinite	1

min = minutes; s = seconds

All PCR amplifications were performed on the GeneAmp® PCR System 2700 (Applied Biosystems, California, USA) and PCR products were analyzed by agarose gel electrophoresis as described in Section 2.3.3.

2.6.3 Digestion and purification

The pGL4.23 minimal promoter plasmid and the two *LGALS13* inserts (-98A and -98C) were each restricted in "double digest" reactions comprising the following: 2 X Buffer Tango (Fermentas), 1 U *Xho*I (Fermentas), 1 U *Bgl*II (Fermentas) and nuclease – free water to a final volume of 20 µl. Approximately 500 ng PCR product of the inserts and 1000 ng plasmid DNA was double digested at 37°C for 16 hours. Digested products were subsequently resolved on a 0.8% (w/v) agarose gel at 80 V for 1 hour, excised from the gel with a sterile scalpel and purified using the Wizard ® SV Gel & PCR Clean-up System (Promega) according to the manufacturers' instructions (Appendix 6.3). Concentrations of the purified digestion products were determined by the NanoDrop® ND-100 Spectrophotometer (NanoDrop Technologies, USA).

2.6.4 Ligation into the pGL4 vector

The digested and purified *LGALS13* inserts (-98 A and C alleles) were ligated into the pGL4.23 minimal promoter luciferase reporter vector, using a 3:1 insert:vector molar ratio. Briefly, an overnight ligation was performed at 4°C comprising the following: 50 ng pGL4.23 vector DNA, 25 ng insert DNA, 1 X T4 Ligase Buffer (Promega) and 1 U T4 Ligase (Promega) in a final volume of 20 µl.

2.6.5 Transformation of competent cells

Following ligation, E.cloni® Chemically Competent Cells (Lucigen Corporation, Middleton, USA) were transformed with the pGL4:*LGALS13* (-98A and C) ligation products following the manufacturers' protocol. The ligation reaction was inactivated at 70°C for 15 min prior to transformation and all tubes were chilled on ice. Briefly, 5 µl of ligation reaction was added to 40 µl of competent cells, followed by incubation on ice for 30 min and subsequent heat shock at exactly 42°C for 45 seconds. Following a 2 min incubation step on ice, 960 µl Recovery Medium (Lucigen) was added to the cells and they were incubated at 37°C with shaking (250 rpm) for 1 hour. Transformed cells were then plated out onto LB agar plates (Appendix 6.2) supplemented with 100µg/ml Ampicillin (Sigma-Aldrich, Aston Manor, South Africa) and incubated overnight at 37°C.

2.6.6 Colony PCR

Colony PCR was performed to select positive colonies, using a vector-specific forward primer (RV3 - Promega) and an insert-specific reverse primer (*LGALS13* R). The primer sequences are shown in Table 2.6. The 25 µl PCR reaction setup was as follows: 1 X Flexi buffer (Promega) and 2 mM $MgCl_2$ (Promega), 1 U GoTaq® Flexi DNA Polymerase (Promega), 0.25 mM of each dNTP (dATP, dCTP, dGTP, dTTP) (Fermentas) and 10 pmol of each primer. The PCR cycling conditions were: initial denaturation for 5 min at 95°C, followed by 35 cycles of 30 s denaturation at 95°C, 30 s annealing at 55°C, an elongation step of 30 s at 72°C, followed by final extension step of 7 min at 72°C and cooling at 10°C.

Electrophoresis and visualization of the amplification products are described in Section 2.3.3.

Table 2.6 Oligonucleotide primers for colony screening

Primer	Primer sequence (5'→3')
RV3 F	CTAGCAAAATAGGCTGTCCC
LGALS13 R	TGAAGATCTTGTTCTCTCCTTCTTGGCGACCTTC

2.6.7 Plasmid extractions

Positive colonies were picked from the master LB plate, inoculated into 5 ml LB medium (Appendix 6.2) supplemented with 100 µg/ml Ampicillin and incubated overnight at 37°C, shaking at ~200 rpm. Small-scale plasmid extractions were performed using the GenElute™ Plasmid MiniPrep Kit (Sigma-Aldrich) according the manufacturers' protocol (Appendix 6.3). Glycerol stocks were made with the remaining bacterial cultures by adding 500 µl of the culture to 500 µl 80% sterilized glycerol, thorough mixing by vortex and immediate storage at -80°C. Plasmid DNA was quantified using the NanoDrop® ND-100 Spectrophotometer (NanoDrop Technologies, USA) and subjected to semi-automated DNA sequencing analysis in order to confirm the sequences and integrity of the constructs. Upon verification, 100 ml LB medium supplemented with 100 µg/ml Ampicillin was inoculated with a scraping of the glycerol stock of the positive colony and incubated overnight at 37°C, shaking at ~200 rpm. The QIAGEN EndoFree Plasmid Purification Maxi Kit (QIAGEN GmbH, Hilden, Germany) was used according to the manufacturers' protocol (Appendix 6.3) for the preparation of highly concentrated, pure, endotoxin-free plasmid DNA for use in subsequent transfections.

2.7 CELL CULTURE

The human choriocarcinoma JAr cell line (ATCC, Rockville MD, USA) was used in this study to establish the effect of the *LGALS13* -98 alleles on transcriptional activity of the gene.

JAr cells were cultured in 75 cm^2 sterile culture flasks (Cellstar ®, Greiner Bio-One, Frickenhausen, Germany) in 20 ml RPMI-1640 (Lonza, Walkersville, USA) with 2 mM L-glutamine, 2000 mg/ml sodium bicarbonate, 2000 mg/ml glucose, fully supplemented with 10 mM HEPES (Lonza), 1 mM sodium pyruvate (Lonza), 10% FBS (Sigma-Aldrich), 50 U/ml Penicillin and 50 µg/ml Streptomycin (Lonza). Supplementation with HEPES increases the buffer strength of the culture medium and allows for the maintenance of physiological pH despite changes in CO_2 levels. Sodium pyruvate served as an additional source of energy, and is believed to protect cells against oxidative stress. Fetal bovine serum (FBS) provides hormones, growth factors and proteins which aid with the attachment of cells to the culture vessel. The cells were grown at 37°C with an atmosphere of 5% CO_2 (Heraeus Cell 150, Kendro Laboratory Products, USA). Growth medium was replenished as required and cells were subcultured at approximately 80% confluency.

Briefly, the subculturing procedure was as follows: growth medium was decanted from the flask and cells were rinsed with fresh RPMI 1640 medium free of serum and additional supplements. Cells were trypsinized by the addition of 3 ml Trypsin/Versene (Lonza) and subsequent incubation at 37°C for 4 minutes. Following trypsinization, flasks were tapped lightly to facilitate detachment of the cells, 5 ml fresh, fully supplemented RPMI 1640 was added, cells were resuspended by gently pipetting, transferred to a sterile 15 ml tube (Corning, NY, USA) and spun for 5 min at 800 x g. After removal of the supernatant, the cells were resuspended in an appropriate volume of fresh, fully supplemented RPMI 1640 medium (dependant on confluence of the cells), seeded to fresh flasks and brought to a final volume of 20 ml with fully supplemented RPMI 1640 medium.

2.8 TRANSFECTIONS

JAr cells were transiently transfected by lipofection with the *Trans*IT-LT1 transfection reagent (Mirus, Madison, USA). All transfections were performed in triplicate. Cells were seeded into 6-well plates (Greiner Bio-One) with 2.5 ml complete growth medium per well, 24 hours prior to transfection. The *Trans*IT-LT1 reagent - DNA complex was formed, immediately before transfection, as follows (per well): 7.5 µl transfection reagent was added directly to 250 µl serum-free RPMI 1640 in a sterile tube and gently mixed. A total of 2.5 µg DNA - 2 µg *LGALS13* construct and 500 ng pGL4.73 [hRluc/SV40] vector - was added to the tube and incubated at room temperature for 30 minutes. The DNA-reagent complex was then added drop wise to each well, gently rocked to ensure even distribution and incubated for 24 hours at 5% CO_2 and 37°C. The pGL4.73 vector was used as a control for transfection efficiency and was selected specifically for use in the Dual-Luciferase ® reporter assay described in section 2.9.

2.9 LUCIFERASE ASSAYS

The luciferase assays were performed with the Dual-Luciferase ® reporter assay system (Promega). The two vectors used in this study – pGL4.23 [luc2/minP] and pGL4.73 [hRluc2/SV40] - acted as dual reporters in the assay i.e. two reporters simultaneously expressed and measured within a single assay. The pGL4.23 [luc2/minP] vector (Appendix 6.5) contains the *Luc* reporter gene which encodes the firefly (*Photinus pyralis)* luciferase protein. This served as the experimental reporter vector into which the *LGALS13* amplicon was cloned. The co-transfected control vector, pGL4.73 [hRluc/SV40] (Appendix 6.5) contains the gene encoding Renilla (*Renilla reniformis*) luciferase, which is under the control of the SV40 promoter.

Cell lysates were prepared for use in the luciferase reporter assay approximately 24 hours after transfection. Briefly, growth medium was removed from the 6-well plates and the cells were washed with cold DPBS (Lonza). Following the removal of DPBS, 500 µl 1 X Passive Lysis Buffer (PLB) was added to each well, plates were placed on a rocking platform at room temperature for 15 min and then placed

at -80°C for 1-2 hours to facilitate cell lysis. Cells were then allowed to thaw at room temperature, the lysates were collected in sterile 2 ml microcentrifuge tubes, centrifuged at 14 000 x g for 30 s and 20 µl of the cleared lysate was transferred to a white 96-well plate (Promega).

The GloMax™ 96 Luminometer (Promega) was used for the measurement of luciferase activity. The Luciferase Assay Reagent II (LARII) and Stop & Glo ® reagents were used to prime injectors one and two of the luminometer, and the system was programmed to perform measurements for a period of 10 s with a 2 s pre-measurement delay. The addition of 50 µl LARII initiated a luminescent signal from the firefly luciferase. Following measurement of this signal, 50 µl Stop & Glo ® reagent was added, which quenched the firefly signal and initiated the renilla reaction.

The values representing the firefly luciferase activity were normalized for each sample by division with the corresponding renilla luciferase activity. An average of the normalized values was calculated per construct and these values were used to determine the relative expression per construct.

2.10 STATISTICAL ANALYSIS OF LUCIFERASE ASSAY DATA

As mentioned in the previous section, a normalized value was calculated from the raw data by dividing the firefly by the renilla luciferase activity per sample. Values that were not within 20% of each other were excluded from further analysis. The normalized values that were within the appropriate range were used to calculate the average, standard deviation and standard error. Statistically significant differences were determined using the t-test, with two samples assuming equal variances. A p-value of less than 0.05 was regarded as significant.

CHAPTER 3:

RESULTS AND DISCUSSION

RESULTS AND DISCUSSION

3.1 CLINICAL PROFILING OF ABRUPTIO PLACENTAE

This study cohort is one of the largest abruptio placentae cohorts in South Africa to date, for which we have extensive clinical records. This allowed for various comparisons to be made in an attempt to identify environmental/behavioural risk factors and correlations in this South African cohort. Patient demographic information available for analysis included:

- Race
- Diastolic blood pressure
- Maternal age (years)
- Gravidity (the number of times a woman has been pregnant)
- Parity (the number of times a woman has given birth)
- Medications administered during pregnancy
- Smoking and alcohol consumption during pregnancy
- History of complications in previous pregnancies
- The gestational age (weeks) at which the complication is diagnosed, as well as the gestation at delivery
- The severity of the abruption, which was recorded as the percentage of the maternal placental surface covered with infarcts/clots
- Pregnancy complications in addition to abruptio placentae, namely pre-eclampsia
- Birth weight (grams) at delivery
- Gender
- Neonatal outcome

Table 3.1 General demographic characteristics of the patient cohort (n=195). The range is shown in *parentheses*.

Demographic characteristics	Mean
Maternal age (years)	27 (14-44)
Gravidity	2 (1-7)
Parity	1 (0-5)
Severity (%)*	65 (15-100)
Gestational age at delivery (weeks)	32 (22-40)
Birth weight (grams)	1757 (166-5000)

* % clot coverage on placental surface.

The general demographic characteristics of the abruptio placentae cohort are shown in Table 3.1. The corresponding information was not available for the control cohort and therefore further demographic analyses and comparisons were performed within the patient cohort exclusively. The following evaluations were performed in order to gain insight into the clinical profile of South African women whose pregnancies were complicated by abruptio placentae.

3.1.1 Clinical features

The study cohort comprised a total of 195 women, of whom 138 (70.8%) were South African Coloured and 57 (29.2%) were Black African Xhosa individuals. These patients were stratified according to their ethnicity for the following comparisons, in order to elucidate aspects of the clinical data which may be specific to different racial groups. The following clinical features were investigated: the 'status/type' of abruption (with or without pre-eclampsia), neonatal outcome and the severity of placental abruption.

3.1.1.1 Abruptio placentae with and without pre-eclampsia

Abruptio placentae can occur as an isolated event or in combination with pre-eclampsia. The specific etiologies of abruptio placentae and pre-eclampsia are largely unknown, although differences have been identified in the underlying pathology associated with these disorders. A large percentage of the patient cohort (59%) presented with abruptio placentae together with pre-eclampsia, whereas the isolated cases of abruption represented only 41% of the cohort. The cohort was therefore grouped accordingly into the AP-only (isolated abruptio placentae) and AP+PE (abruptio placentae with pre-eclampsia) subgroups. Stratification within these groups in terms of other clinical features such as neonatal outcome, severity, etc. may reveal specific aspects which may be involved in the pathogenesis of one or both of these subgroups.

3.1.1.2 Neonatal outcome

Abruptio placentae can impact on neonatal outcome in a number of ways. Partial separation of the placenta from the uterine wall increases the risk of fetal brain damage (Eskes, 2001), whereas complete separation results in intrauterine death in most cases (Ananth *et al.*, 1999). It is evident that the neonatal outcomes resulting from pregnancies complicated by abruptio placentae are, more often than not, poor. The cohort was therefore grouped and analyzed according to neonatal outcome of the Coloured and Black patients. A normal, healthy baby was considered as a good outcome. Poor outcomes included intrauterine death, miscarriage and neonatal- and child deaths. Table 3.2 shows the representation of these outcomes in the cohort.

Table 3.2 Neonatal outcomes of the abruptio placentae cohort

Neonatal Outcomes	n=195	%
Good	48	24.6
Poor	147	75.4
Intrauterine death	143	97.3
Miscarriage	1	0.7
Neonatal death*	2	1.4
Child death**	1	0.7

* 7-28 days after delivery, ** after 28 days of delivery

As expected, the majority of the study cohort (75%) comprised poor outcomes. The percentages of poor outcomes in the Black and Coloured populations were 82% and 72%, respectively. The increased frequency of poor outcomes in the Black population is notable and it is tempting to speculate that Black patients are more likely to have poor neonatal outcomes; however the overall frequency of poor outcomes in the general population could follow the same trend and this data is needed for comparison in order to clarify this matter. Table 3.2 shows that the majority of poor outcomes (97%) were due to intrauterine death (IUD), which could be associated with the severity of AP in this group. Analysis of the frequency of IUD when this data was stratified for ethnicity showed that this specific complication was the cause of 100% of Black, and 96% of the Coloured poor neonatal outcomes.

3.1.1.3 Severity of placental abruption

Placental abruption is diagnosed clinically and confirmed at delivery by visual inspection of the placenta. A positive diagnosis is made based on the presence of retroplacental clots which cover more than 15% of the placental surface. The percentage of the maternal surface of the placenta which is covered by infarcts or clots is documented by the clinician in theatre immediately after delivery of the baby. Based on this percentage, the severity of the abruption can be assessed.

Studies have shown that the more severe cases are associated with poor outcomes such as intrauterine death and brain damage (Ananth *et al.*, 1999; Eskes, 2001). In an attempt to correlate severity with neonatal outcome in our population, groupings were made based on the percentage of clot coverage on the placenta (% abruption), as shown in Table 3.3.

Table 3.3 Classification of the severity of abruption, based on clot coverage of the placenta

Severity	% Abruption
Mild	15-50
Moderate	60-75
Severe	80-100

The comparison of the severity, in terms of the degree of abruption (%), in good and poor outcome groups is shown in Figure 3.1.
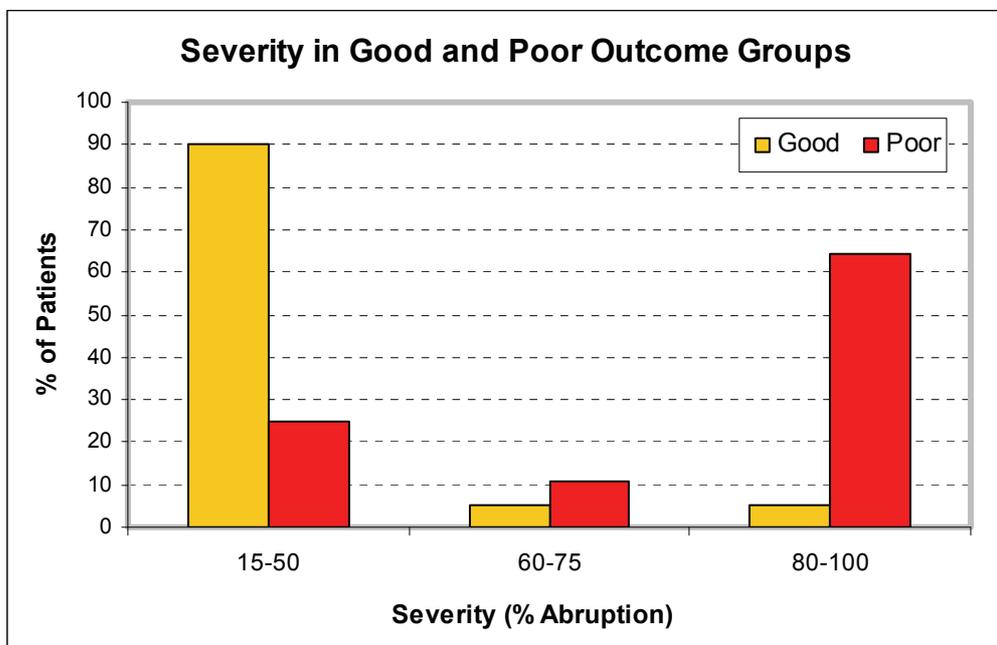
Figure 3.1 Assessment of neonatal outcome in relation to the severity of abruption

The results shown in Figure 3.1 indicate that the milder abruptions resulted in mostly good outcomes (90%), whereas the moderate and more severe abruptions resulted largely in a poor neonatal outcome. While the percentage of clot coverage of the placenta is a good indicator of severity, there are a few factors which need to be considered regarding this analysis. Severity is represented by more than just the percentage of abruption - there are other factors involved in determining whether abruption is mild, moderate or severe. Even a small degree of placental separation can jeopardize the safety and well-being of the fetus.

3.1.2 Risk Factors

A number of risk factors have been associated with abruptio placentae, including cigarette smoking and alcohol consumption during pregnancy, hypertensive disorders and/or abruption in a prior pregnancy and male fetal gender. Although the validity of these factors as potential risk factors in our population cannot be commented upon due to the absence of corresponding demographic information for the control cohort, the following comparisons were made in order to investigate whether any of these factors are more frequent with regards to neonatal outcome and severity of the abruption.

3.1.2.1 Smoking and alcohol consumption

Smoking and alcohol consumption during pregnancy have been associated with a number of pregnancy complications and have serious implications for the health and well-being of the fetus, because the outcome of such pregnancies is usually poor. Figure 3.2 shows the percentage of women who smoked cigarettes and/or used alcohol during pregnancy and the comparison between these frequencies in good and poor outcome pregnancies.
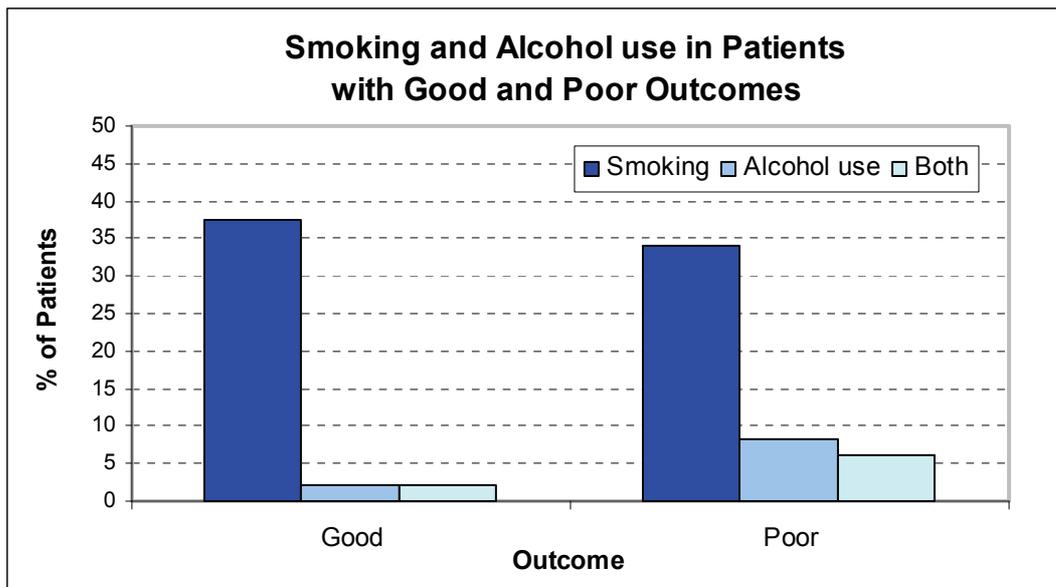
Figure 3.2 Comparison of smoking and alcohol consumption in the good and poor outcome groups.

Approximately 35% of the total cohort smoked cigarettes during pregnancy, the percentage of which is relatively the same in both good and poor outcome groups. The percentage of women who used alcohol during pregnancy, however, is higher in the poor outcome (~8%) than good outcome (~2%) group. Similarly, alcohol consumption together with smoking is also higher in the poor outcome (~6%) than the good outcome group (~2%). It is important to note that because this information is self-reported by patients, these numbers may actually be higher – because of the social stigma associated with alcohol consumption and smoking during pregnancy factors are often under reported. These frequencies are low, but they are in keeping with other studies that demonstrated the relationship between alcohol consumption during pregnancy, abruptio placentae (Marbury *et al.*, 1983) and other adverse outcomes (Aliyu *et al.*, 2008).

3.1.2.2 Previous pregnancy complications

A history of abruptio placentae, hypertension, pre-eclampsia and related disorders are known risk factors for the development of abruptio placentae in the current pregnancy. Figure 3.3 shows the percentage of multigravida women in this cohort

whose previous pregnancies were complicated by miscarriage, hypertensive disorders (including pregnancy-induced hypertension, pre-eclampsia and eclampsia), abruptio placentae and other conditions, such as preterm labour and intrauterine growth restriction.
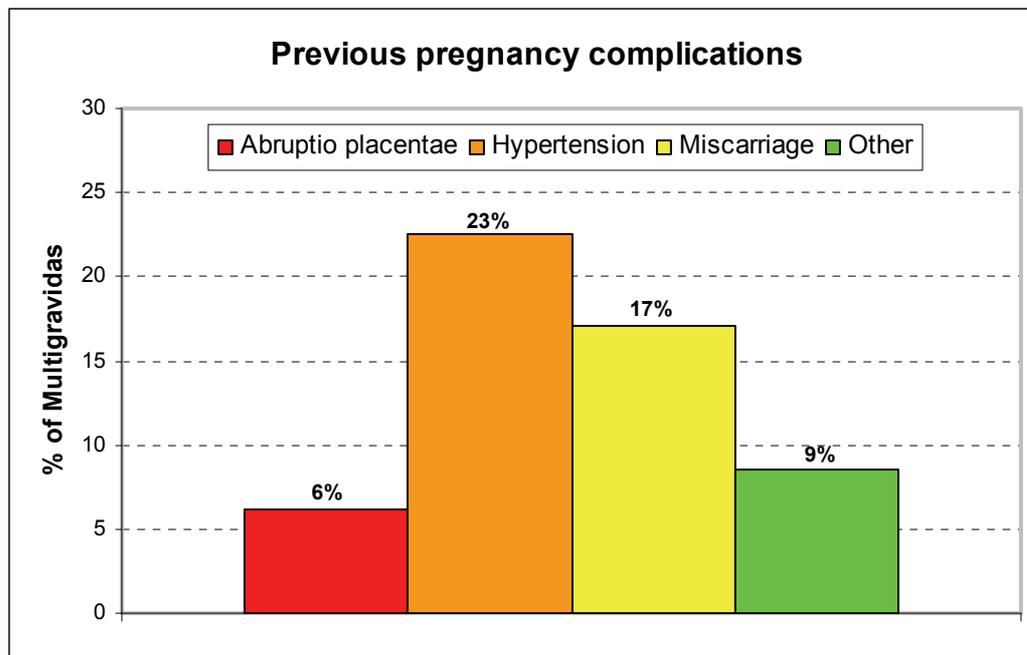
Figure 3.3 Previous pregnancy complications in multigravidas.

The percentage of multigravidae women in this cohort that did not experience complications in a prior pregnancy was 46%. The remaining 54% of women mostly experienced hypertensive disorders and miscarriage. A history of abruptio placentae only featured in 6% of the cohort, which is surprising based on the recurrence rate of this complication. It seems that a history of hypertensive disorders in pregnancy is more of a risk factor for abruptio placentae in our population.

3.1.2.3 Male fetal gender and sex ratio of the offspring

Several studies have reported associations between various pregnancy disorders and a skewed sex ratio, with abruptio placentae associated with an excess of male deliveries (Ananth *et al.*, 1996, Kramer *et al.*, 1997, Odendaal *et al.*, 2000). Male

infants are thought to be more likely to be preterm, have lower birth weights and generally be less likely to thrive than their female counterparts. To investigate the validity of this in our cohort, comparisons of male:female ratios examined according to ethnicity and neonatal outcome.
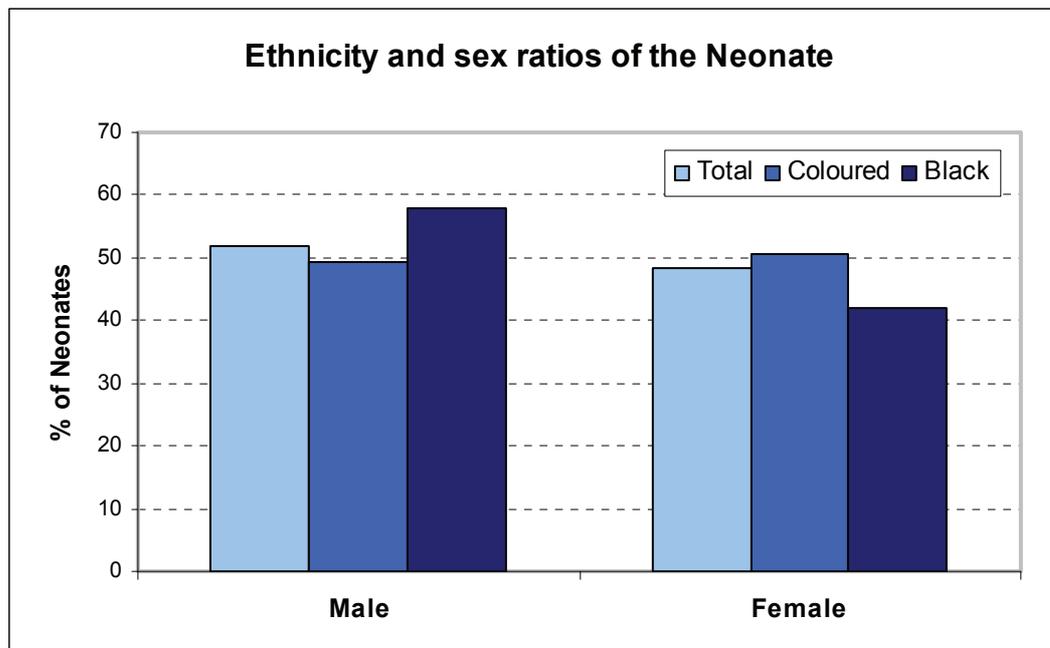
Figure 3.4 Neonatal sex ratios in the total, Coloured and Black groups

The sex ratios for the total and Coloured groups are more or less 50%, as is expected, however the ratio seems to be skewed in the Black group, with more males (58%) than females (42%). Took sentence out here. Stratification of this group of Black individuals according to neonatal outcome revealed that the sex ratio in the poor outcome group is 64% males to 36% females. This suggests that perhaps the Black male infants are more likely to have poor neonatal outcomes as a result of placental abruption. Investigation of the male:female ratio in the good and poor outcome groups, regardless of ethnicity, again showed a higher percentage of male infants (54%) overall in the poor outcome group with 56% of the good neonatal outcomes represented by females. Took sentence out here.

In summary, poor neonatal outcomes were prevalent, observed in 75% of the cohort, with intrauterine death representing 97% of these poor outcomes. These findings correspond with other studies which report the high fetal mortality and morbidity rates associated with abruptio placentae. With regard to risk factors, a history of hypertension in pregnancy is more likely to be associated with abruptio placentae in the current pregnancy than a previous abruption. The frequency of alcohol consumption is slightly higher in the poor outcome group and, although we cannot comment on the significance thereof, it seems to be in keeping with trends observed in other studies. The frequency of male neonates in the poor outcome group appears to be higher than that of the good outcomes in the Black population, but once again the statistical significance thereof cannot be commented upon due to the unavailability of corresponding control data.

The major strength of this study is that the study cohort is possibly the largest abruptio placentae cohort in South Africa and extensive clinical information is available on the patients. A weakness is the lack of demographic information for the control cohort. This information is essential for thorough demographic analysis and the characterization of risk factors in the South African population. Future demographic analyses should aim to include a control cohort with the necessary information. In addition to this, pathology reports on the placentas of the abruptio placentae cases may introduce new aspects to the research. For example, information regarding infection, which is believed to contribute to the pathogenesis of abruption, would contribute to our understanding of the mechanisms involved in progression of this devastating complication.

3.2 SCREENING OF THE *LGALS13* GENE

The coding and non-coding regions of the *LGALS13* gene were amplified by PCR as described in Section 2.3 in maternal and corresponding neonatal samples, as well as ethnically matched controls. The amplicons were resolved by electrophoresis together with a 100bp molecular weight marker to ensure that they were the correct size, no amplification was present in the negative control and products were suitable for downstream applications. The six amplicons representing the 5' UTR, intronic and exonic regions of the *LGALS13* gene were screened by Multiphor SSCP-HD analysis in order to identify conformational variants, which were confirmed by restriction enzyme analysis and DNA sequencing, where appropriate. Mutation detection by Multiphor SSCP-HD analysis identified variation in the exon 1, 3.1 and 3.2 amplicons, but none in the exon 2, 4.1 and 4.2 amplicons. All variants identified in this study were previously identified by our research group in another study cohort (Bruiners N; MSc Thesis, 2006).

3.2.1 Exon 1

Mutation detection by Multiphor SSCP-HD analysis resulted in the identification of three conformational variants, exclusively in the SSCP component of the polyacrylamide gel. Samples representing each of the conformations (shown in Figure 3.6) were purified and sequenced. A single nucleotide polymorphism was identified in the 5' untranslated region as -98A/C (dbSNP rs: 3764843).
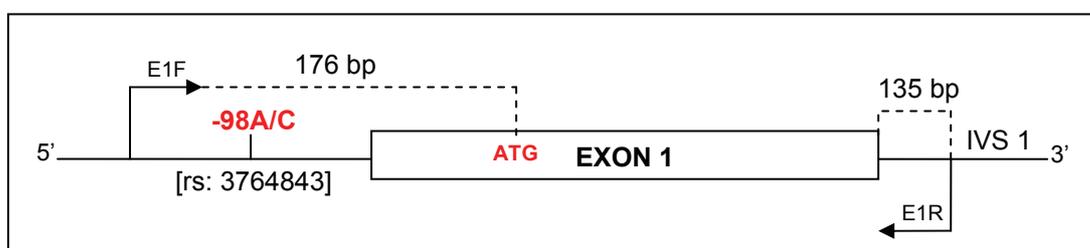
Figure 3.5 Schematic diagram of the exon 1 amplicon and positions of the variants identified by SSCP analysis, sequencing and restriction enzyme digestion. ATG = start site/codon; IVS = intervening sequence
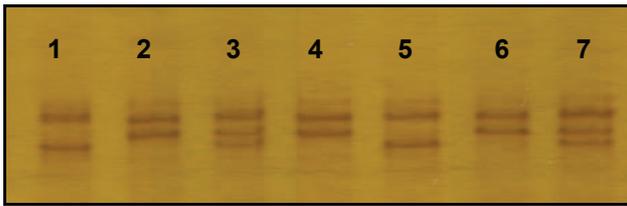
Figure 3.6 Conformational variants identified in the exon 1 amplicon. Conformations are shown in the SSCP component of the polyacrylamide gel. Lanes 1 & 5: homozygous for the variant allele (-98C/C); lane 2, 4 & 6: homozygous for the wild type allele (-98A/A); lanes 3 & 7: heterozygous individuals (-98A/C).

Restriction mapping of the amplicon showed that the enzyme *Ava*I could be used for specific genotyping of this variant. *Ava*I cuts once in the presence of the variant allele (C), therefore digestion with this enzyme yields two fragments for individuals homozygous for the variant allele; 247bp and 77bp (which is not visible on the gel). Homozygous wild type genotypes are reflected by a single undigested 324bp fragment.
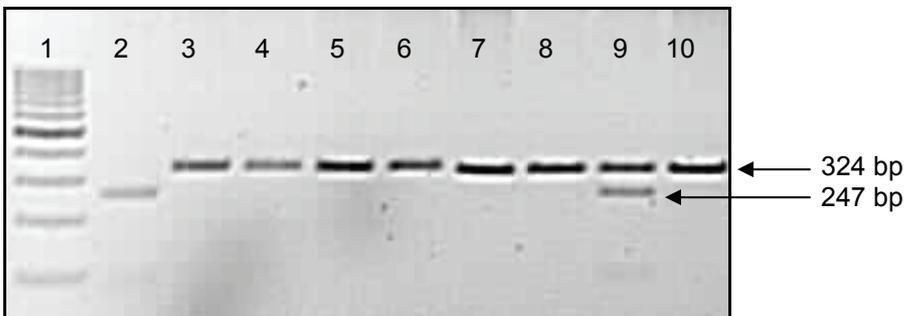
Figure 3.7 Restriction enzyme digestion of the exon 1 amplicon with *Ava*I for genotyping of the -98A/C variant. The digestion products were resolved on a 2% agarose gel for 120 minutes at 100V. Lane 1: 100bp molecular marker; lane 2: CC; lanes 3 – 8, 10: AA; lane 9: AC.

The patient and control groups were genotyped for the -98 A/C variant as described above and the genotype and allele frequencies of this variant in both groups were determined. These frequencies are shown in Appendix 6.1, Table 1.

3.2.2 Exon 3

Exon 3 of the *LGALS13* gene was represented by 2 amplicons designated 3.1 and 3.2. The positions of the variants present in these amplicons, together with the positions of the primers relative to exon 3, are shown in Figure 3.8.
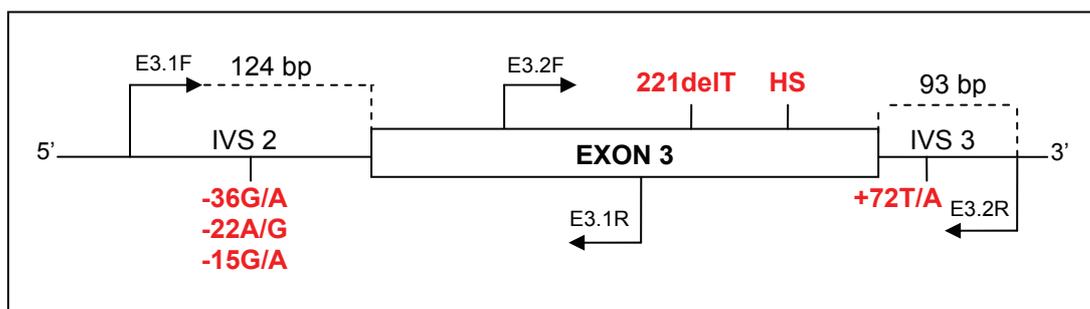
Figure 3.8 Schematic diagram showing the positions of all variants identified in the exon 3 amplicons

Exon 3.1

A total of six SSCP conformational variants were identified by SSCP-HD analysis (Figure 3.9) in the SSCP component of the polyacrylamide gels. Samples representative of each of these conformations were purified and sequenced, and were identified as the following SNPs: IVS2 -36G/A (novel), IVS2 -22A/G (dbSNP rs: 2233706) and IVS2 -15G/A (novel). Figure 3.8 shows the position of these variants in the exon 3.1 amplicon. Conformations were designated 1 - 5 for genotyping purposes and correspond to the genotypes shown in Table 3.4. Restriction enzymes identified for confirmation of the conformational variants are shown in Table 2.3. The exon 3.1 amplicons were digested for confirmation of the genotypes only when conformations on the polyacrylamide gels were not distinguishable from one another. For example, under certain electrophoretic conditions, conformations 3 and 5 closely resemble each other. The genotypes of these conformations differ at the -36 G/A locus as shown in Table 3.4, therefore digestion with the enzyme *Nla*IV was performed in order to distinguish them.
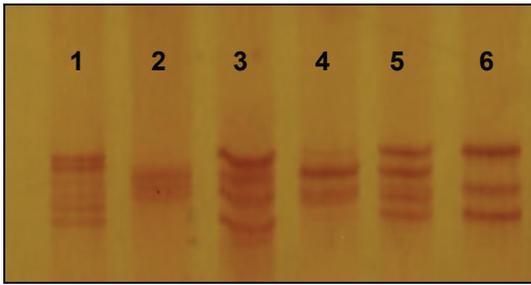
Figure 3.9 Conformational variants identified in the exon 3.1 amplicon. These conformations are representative of the various genotypes of the IVS2 -36G/A, IVS2 -22A/G and IVS2 -15G/A single nucleotide polymorphisms present in this amplicon.

Table 3.4 Summary of exon 3.1 conformations and their corresponding genotypes, confirmed by automated sequencing

Conformation	IVS2 -36G/A	IVS2 -22A/G	IVS2 -15G/A
1	GG	AA	GA
2	GA	AA	GG
3	GA	AG	GG
4	GG	GG	GG
5	GG	AG	GG
6	GG	AA	GG

The patient and control groups were genotyped for the following intronic variants: IVS2 -36G/A, IVS2 -22A/G and IVS2 -15G/A. Genotype and allele frequencies of these variants are shown in Appendix 6.1, Tables 3, 5 and 7 respectively.

Exon 3.2

Multiphor SSCP-HD analysis and subsequent sequencing of the amplicons representative of the various conformations resulted in the identification of the following variants in the exon 3.2 amplicon: IVS3 +72T/A (dbSNP rs: 2233708), 221delT (L74W) and a series of 6 single nucleotide polymorphisms within an 11bp region (beginning at nucleotide position 230), which will be referred to as the hotspot (HS). Figure 3.10 shows the conformations of the exonic variants, 221delT and the hotspot, in the SSCP and HD components of the Multiphor polyacrylamide gel.
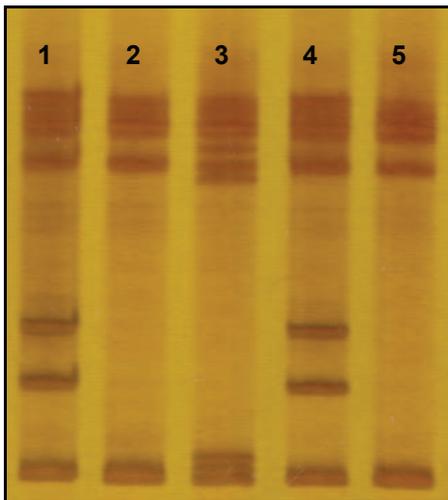
Figure 3.10 Conformational variants identified in the exon 3.2 amplicon by Multiphor SSCP-HD. Lanes 1 and 4 show the conformations of the hotspot variant and lane 3 shows the 221delT conformation in both SSCP and HD components of the polyacrylamide gel.

Despite extensive optimization of the Multiphor SSCP-HD electrophoresis conditions, the IVS3 +72T/A single nucleotide polymorphism could not be reliably genotyped on the polyacrylamide gels. Restriction mapping of the exon 3.2 amplicon identified the restriction enzyme *Stu*I as a suitable enzyme for use in genotyping this SNP. The enzyme lacks a recognition site in the presence of the

wild type allele (T). Digestion of an individual homozygous for the variant allele (A) yields two fragments – a 269bp, and a 19bp fragment which is not visible on the agarose gels due to its size. Figure 3.11 shows the results of a restriction digest with *Stu*I.
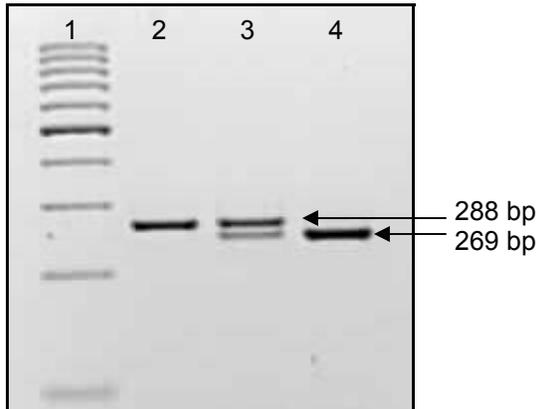
Figure 3.11 Restriction enzyme digestion of the exon 3.2 amplicon with *Stu*I for genotyping of the IVS3 +72T/A variant. Digested products were resolved on a 3% agarose gel for 3 hours at 100V. Lane 1: 100bp molecular marker; lane 2: TT; lane 3: TA; lane 4: AA.

The patient and control groups were genotyped for the variants identified in the exon 3.2 amplicon. Genotype and allele frequencies of these variants are shown in Appendix 6.1, Tables 9, 11 and 13.

3.2.3 Exon 2 and 4

No conformational variants were identified in the SSCP or HD components of the polyacrylamide gels for the exon 2 and 4 amplicons, despite the use of various electrophoresis conditions (such as varied temperature) for mutation detection. The results shown in Figure 3.12 are the SSCP components of the resolved exon 2, 4.1 and 4.2 amplicons.
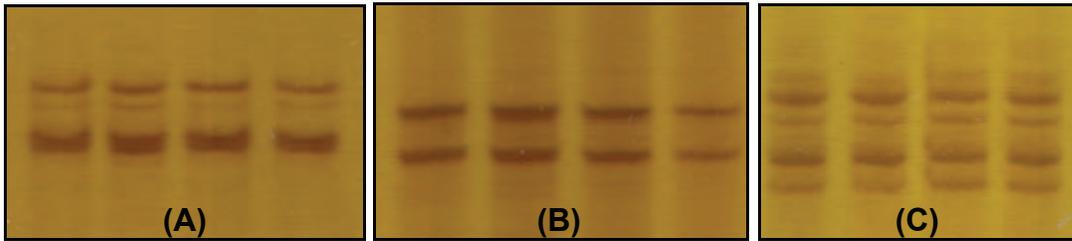
Figure 3.12 Conformations of the (A) exon 2 and (B) exon 4.1 and (C) 4.2 amplicons.

In summary, a total of seven variants were identified in the *LGALS13* gene in this study – one variant in the 5' untranslated region, 4 intronic variants and two exonic variants. These variants have been previously identified by our research group in a different patient group. *LGALS13* screening in the abruptio placentae cohort is therefore a novel study, which was one of the strengths of this study. Although the sensitivity of the Multiphor SSCP-HD system of mutation detection was optimized extensively for each amplicons, the clear resolution of some amplicons remained a challenge, resulting in conformations which were hard to distinguish and genotype. This problem was combated by the use of restriction enzyme analysis for confirmation of these genotypes, which greatly minimized genotyping errors. It may be of value to consider the use of additional mutation detection techniques such as dHPLC (denaturing high performance liquid chromatography) or high resolution melt (HRM) analysis. Both of these techniques have demonstrated high sensitivity and are relatively inexpensive and suitable for high throughput mutation detection (D'Apice *et al.*, 2004; Krypuy *et al.*, 2006).

3.3 STATISTICAL ANALYSIS

Genotype and allele frequencies were determined for all variants in both patient and control groups and these frequencies were in Hardy-Weinberg Equilibrium (p > 0.05) at all examined loci. Statistically significant differences (p ≤ 0.05) between the patient and control groups were identified using Fishers Exact Test and contingency tables in StatCalc (EpiInfo, CDC, Atlanta, GA). Maternal and fetal frequencies for all subgroups were compared to the same ethnically matched maternal control frequencies in all cases. Patients were stratified according to ethnicity (to prevent the effects of population admixture), neonatal outcome and the 'type' of abruptio placentae (AP), i.e. with or without pre-eclampsia (PE). Stratification into these subgroups may shed light on susceptibility in the specific groups – for example, associations in a certain ethnic group may be indicative of ethnicity-specific susceptibility factors.

All tables showing the genotype and allele frequencies, together with the p-values, in the total and poor outcome groups for each variant can be found in Appendix 6.1.

3.3.1 Variants in the *LGALS13* 5' UTR

The genotype and allele frequencies of the -98A/C variant in the patient and control groups are shown in Table 1 and 2, along with the relevant p-values from Hardy-Weinberg analysis and StatCalc.

A statistical significance was observed in the poor outcome group, specifically the Coloured fetal subgroup (p=0.036). Comparison of the genotype and allele frequencies showed a higher frequency of heterozygotes in this specific subgroup of patients (56%) when compared to the Coloured controls (40%), with a slightly higher frequency of the C allele in patients (33%) than controls (28%). Although the frequency of the C allele is not remarkably higher than that of the

control group, it could potentially act as a susceptibility factor for poor neonatal outcome in abruptio placentae in the Coloured population.

3.3.2 Intronic variants

3.3.2.1 IVS2 -36G/A

Table 3 and 4 show the genotype and allele frequencies of the IVS2 -36G/A variant, as well as the p-values from Hardy-Weinberg analysis and StatCalc in both patient (total and poor outcome) and control groups.

Statistical significance was identified in the total group of patients, specifically the Coloured maternal AP+PE group (p=0.043). Stratification of this total group into the poor outcome group, by elimination of the genotypes of good neonatal outcomes from statistical analyses, revealed an association in the Coloured maternal group once again. The significance was observed in the poor outcome maternal group as a whole (p=0.043), as well as in the poor outcome AP+PE group (p=0.009). The strength of this association appears to increase as the group is further stratified. In all three groupings, the carrier frequency is higher in patients (13%) than controls (6%) and comparison of the allele distribution revealed a higher frequency of the A allele amongst patients. Interestingly, individuals homozygous for the variant allele were not identified in either patient or control groups, which could have implications in risk assessment and susceptibility to abruptio placentae with pre-eclampsia.

3.3.2.2 IVS2 -22A/G

Genotype and allele frequencies for the IVS2 -22A/G variant, as well as the p-values from Hardy-Weinberg analysis and StatCalc are shown in Tables 5 and 6 for the patient and control groups.

Statistically significant differences were observed between the patient and control groups in both the total (p=0.039) and poor outcome (p=0.037) groupings for the Coloured fetal AP only subgroup. There was a higher carrier frequency (~43%) in both patient subgroups when compared to controls (25%), with the variant G allele being more prevalent in the patient groups (21% vs. 14% in controls).

3.3.2.3 IVS2 -15G/A

Genotype and allele frequencies for the -15G/A variant, as well as the p-values from Hardy-Weinberg analysis and StatCalc are shown in Tables 7 and 8 for the patient and control groups. No statistical significance was identified for this variant – it seems to be a low frequency polymorphism, occurring only in heterozygous state and only in 1 – 2% of patient and control individuals.

3.3.2.4 IVS3 +72T/A

Table 13 and 14 show the genotype and allele frequencies in patients and controls for the IVS3 +72T/A variant, as well as the p-values from Hardy-Weinberg analysis and StatCalc results.

The total and poor outcome groups' frequencies were all in Hardy-Weinberg Equilibrium and no statistically significant differences were observed between these groups and the control group. Further stratification of these groups according to AP status (with or without PE) resulted in deviation from HWE in a few of the Black subgroups. This is probably due to the small sample size of these stratified groups (n ranged from 23 to 29).

Statistical significance was observed in the Black AP+PE group in both total and poor outcome analyses. Unfortunately, this subgroup of the total patient cohort was not in HWE and the statistical significance (p=0.004) in inconclusive. The group did not deviate from HWE when analyzed in the poor outcome cohort and

a p-value of 0.016 was observed. The genotype and allele frequencies of the patients in the Maternal Black AP+PE subgroup were very different from the Black control group, but again these frequencies are questionable due to the small sample size of this extensively stratified group.

3.3.3 Exonic variants

3.3.3.1 221delT

Genotype and allele frequencies for the 221delT variant, as well as the p-values from Hardy-Weinberg analysis and StatCalc results are shown for the patient and control groups in Table 9 and 10. No statistically significant associations were identified for this variant.

3.3.3.2 The hotspot

Table 11 and 12 show the genotype and allele frequencies for the hotspot variant, as well as the p-values from Hardy-Weinberg analysis and StatCalc results for the patient and control groups.

Statistically significant differences were observed between the Coloured control group and the following patient groupings: The total group (good and poor neonatal outcomes) of Coloured maternal patients (p=0.038), the total group of Coloured maternal patients who presented with pre-eclampsia in addition to abruptio placentae (AP+PE) (p=0.011) and finally, the poor outcome group (excludes the genotypes of patients with good neonatal outcome) of Coloured patients with AP+PE (p=0.005).

The carrier frequency of this variant in the above mentioned groups was considerably greater than that of the control group – the frequency ranged from 12 to 18% in patients vs. 6% in controls. In all patient groups, the prevalence of

the hotspot allele was higher (ranged from 6 - 9%) than in the corresponding control group (3%). Individuals homozygous for the hotspot were not identified in either patient or control groups.

In summary, statistical significance was observed in the Coloured population at the following loci: -98A/C, IVS2 -36G/A, IVS2 -22A/G and the hotspot. These variants could represent a susceptibility profile in the Coloured population or they may have implications in the pathogenesis of abruptio placentae. In order to gain insight into the role these variants play in disease susceptibility, their functional effects need to be established. The major As most of the associations were demonstrated in the subgroup of abruptio placentae patients who also presented with pre-eclampsia (AP+PE group), it is not clear whether these variants are involved in the etiology of placental abruption or pre-eclampsia specifically. The frequencies of these variants should therefore be established in a cohort of patients with isolated pre-eclampsia (i.e. not complicated with placental abruption) in order to investigate whether associations with these variants exist in the PE only cohort, or whether they exist only in combination with abruptio placentae. weakness of this aspect of the analysis was that the stratification of patients into a number of subgroups drastically reduced the sample size of each subgroup, which was not ideal for statistical analysis and resulted in deviation from HWE in some cases. The strength was that the availability of the clinical information allowed for patient stratification. Although it yielded some undesirable effects, stratification has the potential to identify aspects of importance in the subgroups which would go unnoticed in analysis of the total group. An example of this is the association observed specifically in the subgroup of abruptio placentae with pre-eclampsia for the IVS2 -36G/A and hotspot variants.

3.4 HAPLOTYPE ANALYSIS

Haplotypes have been associated with a number of diseases and pregnancy disorders. One of the more recent examples of such an association was between a haplotype in the *ANXA5* (annexin A5) promoter region and recurrent pregnancy loss in a German population (Bogdanova *et al.*, 2007). The identification of a haplotype in the *LGALS13* gene which could potentially be associated with abruptio placentae could have potential for the identification of those at risk, which would in turn impact clinical management of these women significantly.

Haplotype analysis was performed using the Haploview software (www.broad.mit.edu/haploview/haploview) in order to identify regions of linkage disequilibrium (LD) and possible haplotypes which may be associated with either patient or control phenotypes (Barrett *et al.*, 2005). The algorithms available for construction of the haplotype blocks include Gabriel's method, the four gamete rule and solid spine of LD. For most of the analyses, the four gamete rule (Wang *et al.*, 2002) was employed because it provided the most appropriate and accurate grouping of markers. The patient and control groups were stratified according to ethnicity for the analyses in order to identify potential race-specific haplotypes.

The LD plots representing analysis of the Coloured maternal and fetal groups are shown in Figures 3.13 (A) and (B), respectively. The four gamete rule was implemented for the analysis of both groups.
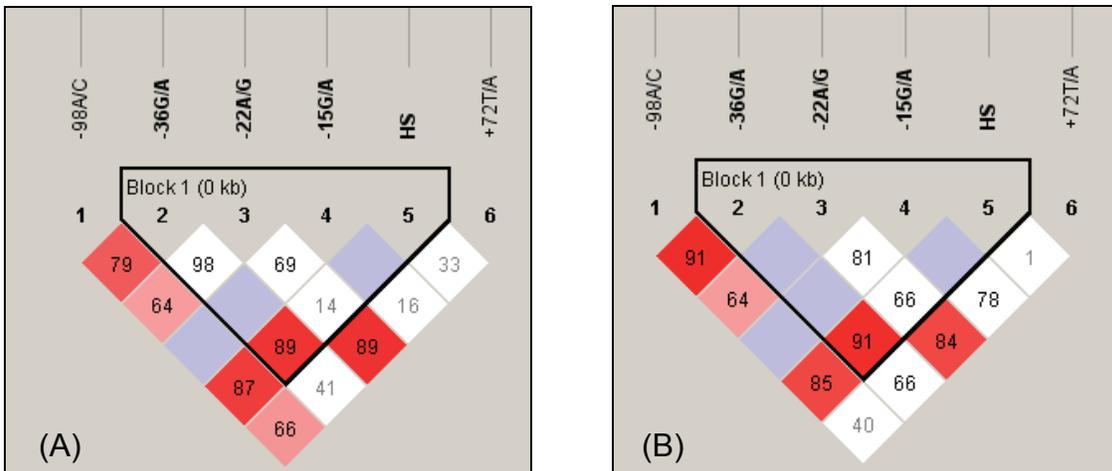
Figure 3.13 LD plot between the SNPs identified in the Coloured (A) maternal and (B) fetal groups.

The LD plots representing analysis of the Black maternal and fetal groups are shown in Figures 3.14 (A) and (B), respectively. The four gamete rule was implemented for analysis of the Black fetal group, but the LD plot generated with this algorithm was not suitable in the Black maternal group. For this reason, the blocks were defined for this group using the custom setting in Haploview to include the SNPs with the highest D', LOD and r^2 values.
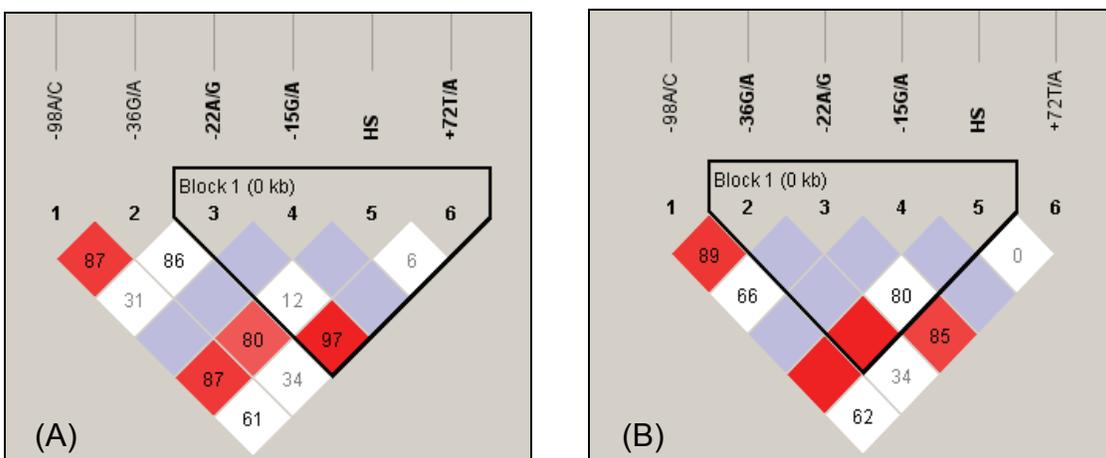
Figure 3.14 LD plot between the SNPs identified in the Black (A) maternal and (B) fetal groups.

The D', LOD and r^2 values determined in Haploview for the above analyses are shown in Table 3.5. These values are all indicative of the degree or strength of LD existing between markers. D' is the standardized measure of LD; the closer this value is to one, the stronger the LD between the markers. The r^2 value or correlation coefficient is an additional measure of LD and these values should range from 0.7 - 0.8 for positive LD. The LOD (logarithm of the odds) score is a statistical indication of linkage i.e. the likelihood of two markers being inherited together. A LOD score of greater than 3 indicates linkage between two markers.

Table 3.5 LD values of the proposed haplotype groupings

	Coloured		Black	
	Maternal	**Fetal**	**Maternal**	**Fetal**
Markers	-36G/A → HS*	-36G/A → HS	-22A/G → +72T/A**	-36G/A → HS
D'	0.891	0.913	0.976	1.0
LOD	30.96	27.35	34.51	18.49
r^2	0.741	0.737	0.790	0.876

Genomic distance between the markers: * 172bp, **303bp

The D', r^2 and LOD scores all indicate linkage between the markers. The frequencies of the specific combinations of markers in the proposed haplotype did not differ significantly between patient and control groups and therefore no statistically significant associations ($p>0.05$) were observed for a single marker or a specific haplotype combination in the any of the patient subgroups. The haplotypes identified are therefore not associated with abruptio placentae, nor do they confer a protective effect.

Linkage disequilibrium exists between the IVS2 +36G/A variant and the hotspot in all groups. The scores for the Black maternal group indicated LD between these variants (D' 0.8, LOD 12.65, r^2 0.64), however the values were higher for the IVS2 -22G/A → IVS 3 +72T/A and this custom grouping of markers was

therefore chosen for further analysis within this group. The degree of LD observed may be related to selection and recombination frequencies in this region of the gene, the genetic history of the South African populations being studied and possibly the conservation of this specific region of exon 3. The existence of strong LD is believed to be associated with conservation of important coding regions which are critical for functionality of the protein product (Kato *et al.*, 2006). The strong LD observed between the variants identified in the exon 3 amplicons suggests that the conservation of this region is important and is being selected for. This hypothesis is strengthened by the fact that exon 3 is the site of the protein's carbohydrate-recognition domain – a feature which is absolutely essential for the biological functions performed by PP13.

3.5 BIOINFORMATIC ANALYSIS

3.5.1 Variants in the *LGALS13* 5' UTR

Variants which are located in the 5' untranslated region of a gene have the potential to affect gene regulation by disruption of important regulatory elements, the core promoter sequence or transcription factor binding sites. Little information is available regarding the 5' UTR of the *LGALS13* gene – the positions of important regulatory regions are unknown. In order to investigate whether the -98A/C variant may impact on gene regulation by disruption of transcription factor binding sites, *in silico* analysis was performed using the following internet-based tools: PATCH[TM] Pattern Search for Transcription Factor Binding Sites (http://www.gene-regulation.com/cgi-bin/pub/programs/patch/bin/patch.cgi), MATCH[TM] Matrix Search for Transcription Factor Binding Sites (http://www.gene-regulation.com/cgi-bin/pub/programs/match/bin/match.cgi), P-MATCH[TM] Combined Pattern-Matrix Search for Transcription Factor Binding Sites (http://www.gene-regulation.com/cgi-bin/pub/programs/pmatch/bin/p-match.cgi), TESS: Transcription Element Search System (http://www.cbil.upenn.edu.cgi-bin/tess/tess?RQ=WELCOME) and AliBaba2.1 (http://www.gene-regulation.com/cgi-bin/pub/programs/alibaba2/index.html).

The results of these predictions are shown in Table 3.6. PATCH predicted the disruption of putative NF1, GR and GATA1 sites, together with the creation of novel Sp1,2,3,4 and NF-E4 sites. MATCH and AliBaba supported the predicted disruption of the NF1 site, while TESS supported the predicted disruption of the GR site. In addition to this, AliBaba predicted the creation of an NF1 site further upstream of its original site, together with a Sp1 site. P-MATCH produced conflicting results by predicting that the -98 variant would not alter any transcription factor binding sites that are present in the wild type sequence.

Table 3.6 Putative transcription factor binding sites predicted to be abolished or created by the presence of the -98 variant C allele.

Program	Abolishes	Creates
PATCH	**NF1** **GR** GATA1	**Sp1**,2,3,4 NF-E4
MATCH	**NF1**	-
P-MATCH	-	-
TESS	**GR**	-
AliBaba	**NF1**	NF1 **Sp1**

Disruption of the NF1 and GR transcription factor binding sites and creation of the Sp1 site were validated by more than one prediction program and the discussion will therefore focus on these factors exclusively.

NF1 or Nuclear factor 1 regulates transcription in a number of ways and can either activate or repress transcription by associations with a number of different regulators. The interaction of its transcriptional activation domain with basal transcription factors facilitates assembly of the transcription initiation complex. In addition, this transcription factor may contribute to the processes of chromatin-regulated control (Alevizopoulos et al., 1995). The importance of NF1 was demonstrated in a study of the core promoter of the $P2X_1$ gene, in which site-directed mutagenesis of the NF1 site resulted in a significant reduction of transcriptional activity (Zhao & Ennion, 2006).

The glucocorticoid receptor (GR) is a ligand-activated transcription factor which regulates the transcription of steroid responsive genes (Kumar & Thompson, 2005; Barnes 2006). The transcriptional activity of a gene can be increased or decreased in response to steroids via the binding of GR to glucocorticoid response elements. Interestingly, mutation of the NF1 site has resulted in the inability of the promoter to respond to glucocorticoids, implicating additional regulatory elements in the process of hormone induction together with GR (Strahle et al, 1988).

The predicted disruption of these putative NF1 and GR sites may affect transcriptional activity of the *LGALS13* gene by interference with assembly of the basal transcription machinery or by reducing potential hormone induction of the gene.

The Sp1 protein (Specificity protein 1) is a general transcription factor which is involved in the expression of a variety of different genes (Suske 1999) and can regulate transcriptional activity of a gene by functioning as an activator or as a basal promoter element (Zhao & Ennion, 2006). Recently, Sp1 was implicated in the protection of CpG islands from hypermethylation in various cancer cell lines (Boumber et al., 2008). In this study, variation in the promoter region of a tumor suppressor gene was found to create a Sp1 binding site, the presence of which resulted in reduced methylation in cancer cell lines. The creation of a Sp1 site in the upstream regulatory region of *LGALS13* could therefore have implications in epigenetic control and the methylation status of this gene.

These *in silico* results need to be confirmed experimentally in order to elucidate the mechanisms of transcriptional regulation of the *LGALS13* gene.

3.5.2 Intronic variants

The process of intron splicing is essential for gene regulation. Motifs in proximity to intron-exon boundaries are essential for the success of splicing and variants in these regions, and in some cases further into the intron, can impact negatively on the splicing process. Variants which affect splicing could do so by resulting in exon skipping or activation of cryptic splice sites, which may result in the production of aberrant mRNA transcripts and ultimately a translated protein product which is not functional (Krawczak *et al.*, 1992; Pagani & Baralle, 2004).

The intronic variants identified in this study, namely IVS2 -36G/A, IVS2 -22A/G, IVS2 -15G/A and IVS3 +72T/A, were analyzed using two internet-based programs: NetGene2 (http://www.cbs.dtu.dk/services/NetGene2/) and ASSP or Alternative Splice Site Predictor (http://www.es.embnet.org/~mwang/assp.html). The predictions made by these programs were conflicting. NetGene2 predicted the creation of an acceptor site in the presence of the IVS -22 variant G allele, whereas the ASSP results indicated that none of the intronic variants were predicted to affect splicing. Nonetheless, by virtue of their positions in intron 2 (close to the intron-exon boundary), it is possible that these variants may have an impact on splicing.

Predicting whether or not a given variant will affect splicing by analysis of the nucleotide sequence alone is not always feasible and results are often inconsistent. The reason for this is that the regions involved in splicing or regions which are critical for recruitment of the splicing machinery are comprised of very few conserved nucleotides. It is therefore necessary to investigate the functionality of these variants experimentally by, for example, use of a minigene assay (Baralle *et al.*, 2003).

3.5.3 Exonic variants

Exonic variants can affect the structure and function of a protein in a number of ways. These variants could, for example (a) result in amino acid substitutions which may change the nature of the protein, (b) create premature stop codons, which results in protein truncation, or (c) have an effect on exonic splicing. The exonic variants identified in this study were analyzed *in silico* in order to predict the effects they may have on the structure and function of placental protein 13.

3.5.3.1 The Hotspot

The hotspot (HS), a series of six single nucleotide polymorphisms within an 11bp region, results in three amino acid changes: T77N, T78L, D79H (shown in Figure 3.15).
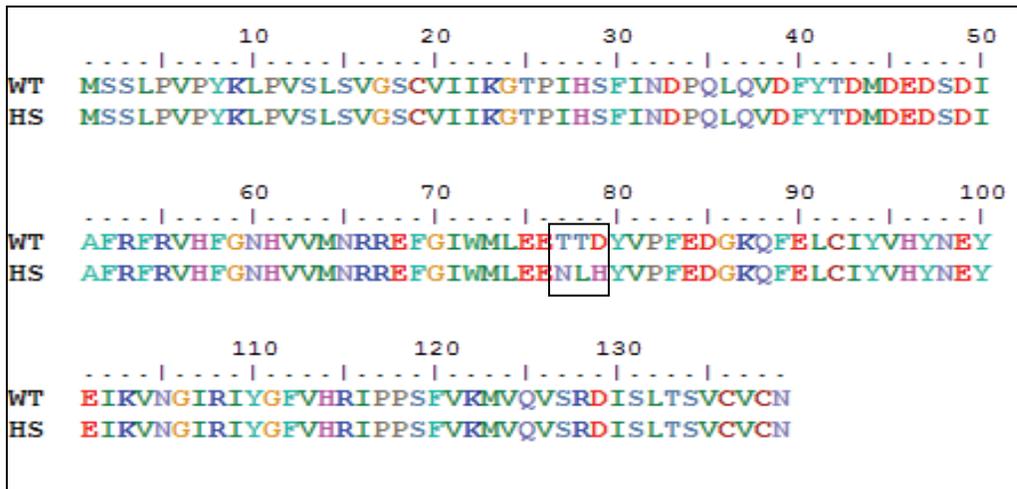
Figure 3.15 Alignment of the wild type and hotspot amino acid sequences. The boxed region shows the amino acid changes caused by the hotspot. WT = wild type, HS = hotspot.

Analysis with the amino acid substitution prediction program SIFT (Sorting Intolerant From Tolerant) (http://blocks.fhcrc.org/sift/SIFT.html) was performed in order to predict whether these amino acid changes would be tolerated or alter the functionality of PP13. The predictions showed that the amino acid substitutions

resulting from the hotspot variant do not to alter protein function. In addition to protein functionality and tolerance of the amino acid changes, another aspect to consider is exonic splicing. Studies have shown that approximately 15% of point mutations result in splicing defects, which ultimately cause human genetic diseases (Krawczak *et al.*, 1992). Most research has focused on intronic mutations as a cause for splicing defects, however exonic mutations are also a common cause of these defects (Wu *et al.*, 2005).

Disruption of ESEs or exonic splicing enhancers by exonic mutations could affect splicing significantly, and such mutations that inactivate functional ESEs have been implicated in diseases, such as neurofibromatosis type 1 (Wu *et al.*, 2005). Exonic splicing enhancers mostly serve as binding sites for SR proteins, a family of proteins which have specialized motifs and domains that facilitate recruitment of the splicing machinery to the specified regions (Wang *et al.*, 2005).

ESE Finder 3.0 is a program designed to identify motifs in a protein sequence which may act as exonic splicing enhancers, in response to specific SR proteins, namely SF2/ASF, SC35, SRp40 and SRp55.
(http://rulai.cshl.edu/cgi-bin/tools/ESE3/esefinder.cgi?process=home)

The hotspot is predicted to disrupt two SRp40 and two SF2/ASF motifs and one SRp55 motif, and create a novel SRp40 motif. The major problem with this prediction method is that the short and degenerate sequence motifs characteristic of ESEs occur relatively frequently in the average human exon and are not necessarily functional (Wu *et al.*, 2005). Nevertheless, disruption of these motifs may affect splicing by exon skipping or creation of cryptic splice sites, resulting in aberrant mature mRNA transcripts.

3.5.3.2 221delT (L74W)

The deletion of a single thymine at nucleotide position 221 results in a few notable changes to the amino acid sequence, as shown in Figure 3.16.

```
              10         20         30         40         50
     ....|....|....|....|....|....|....|....|....|....|....|
WT   MSSLPVPYKLPVSLSVGSCVIIKGTPIHSFINDPQLQVDFYTDMDEDSDI
delT MSSLPVPYKLPVSLSVGSCVIIKGTPIHSFINDPQLQVDFYTDMDEDSDI

              60         70         80         90        100
     ....|....|....|....|....|....|....|....|....|....|....|
WT   AFRFRVHFGNHVVMNRREFGIWMLEETTDYVPFEDGKQFELCIYVHYNEY
delT AFRFRVHFGNHVVMNRREFGIWMWRRQQTTCPLRMANNLSCASTYITMSM

             110        120        130
     ....|....|....|....|....|....|....|....|....
WT   EIKVNGIRIYGFVHRIPPSFVKMVQVSRDISLTSVCVCN
delT R
```
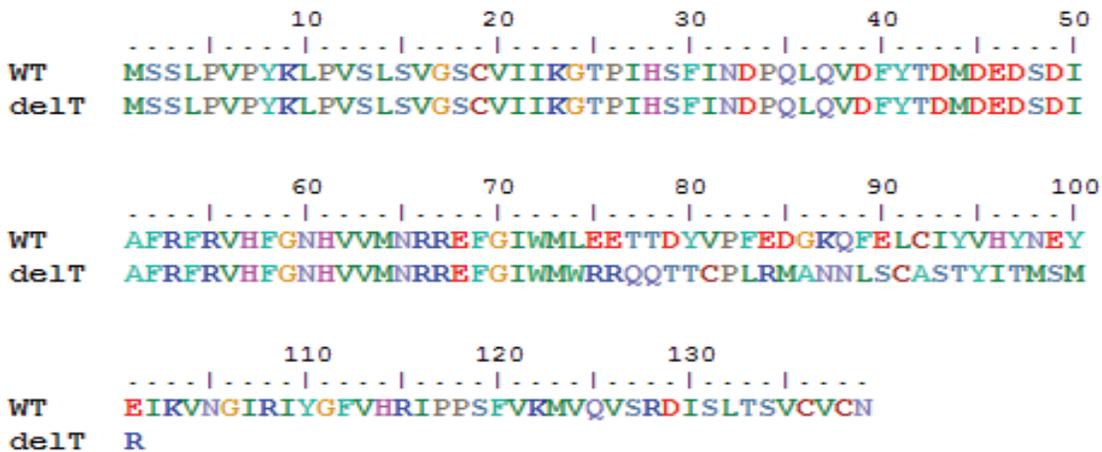
Figure 3.16 Alignment of the wild type and 221delT amino acid sequences.

The deletion results in truncation of the protein due to the introduction of a premature stop codon, which shortens the protein by 38 amino acids. In addition, the last 28 amino acids which form the C-terminal of the protein are altered as a result of the deletion. The structural changes resulting from the delT are likely to have a major effect on functionality and have may have implications in the progression of abruptio placentae. Protein truncation due to introduction of a premature stop codon is common in human disease – an example of this is neurofibromatosis type 1, which is in part caused by the expression of truncated forms of the neurofibromin protein as a result of a mutation which introduces a premature stop codon into the coding sequence (Park & Pivnick, 1998).

The effect that the 221delT variant has on PP13 structure and, presumably, function, together with the lack of delT homozygosity observed in both patients and controls suggests that (a) delT homozygosity may be incompatible with life and (b) the deletion may affect PP13 levels and functionality to such an extent

that it could impact on early implantation and placentation processes which are crucial for embryonic survival and development. To gain insight into this aspect of the research, delT genotyping should be extended to include conception products from spontaneous miscarriages.

Bioinformatic analysis of PP13 performed by Than *et al.* in 2004 revealed the presence of putative serine and tyrosine kinase phosphorylation sites (Ser48, Tyr41, Tyr80) and cysteine residues (Cys 19, 92, 136, 138), which may be involved in dimerization of the protein. The predicted positions of these sites are shown in Figure 3.17.


```
              10         20         30         40         50
     ----|----|----|----|----|----|----|----|----|----|
WT   MSSLPVPYKLPVSLSVGSCVIIKGTPIHSFINDPQLQVDFYTDMDEDSDI
HS   MSSLPVPYKLPVSLSVGSCVIIKGTPIHSFINDPQLQVDFYTDMDEDSDI
delT MSSLPVPYKLPVSLSVGSCVIIKGTPIHSFINDPQLQVDFYTDMDEDSDI
                                        ↑          ↑

              60         70         80         90        100
     ----|----|----|----|----|----|----|----|----|----|
WT   AFRFRVHFGNHVVMNRREFGIWMLEETTDYVPFEDGKQFELCIYVHYNEY
HS   AFRFRVHFGNHVVMNRREFGIWMLEENLHYVPFEDGKQFELCIYVHYNEY
delT AFRFRVHFGNHVVMNRREFGIWMWRRQQTTCPLRMANNLSCASTYITMSM
                                  ↑

              110        120        130
     ----|----|----|----|----|----|----|
WT   EIKVNGIRIYGFVHRIPPSFVKMVQVSRDISLTSVCVCN
HS   EIKVNGIRIYGFVHRIPPSFVKMVQVSRDISLTSVCVCN
delT R
```

Figure 3.17 Alignment of the wild type, hotspot and delT amino acid sequences indicating putative phosphorylation sites and Cys residues involved in dimerization. The arrows indicate the phosphorylation sites and the boxed regions represent the cysteine residues.

All putative phosphorylation sites and cysteine residues remain unaffected by the amino acid changes caused by the hotspot. The presence of the delT, however,

80

alters the Tyr80 and Cys92 residues and abolishes Cys136 and 138. The absence/alteration of the majority of cysteine residues in the delT sequence is likely to impact on dimerization and, subsequently, the structure and function of the protein. The phosphorylation of galectin-3 has been shown to modulate its affinity for carbohydrates, and thereby control the functioning of the protein (*Than et al.*, 2004). Similarly, the phosphorylation status of galectin-13 (PP13) may affect its carbohydrate/sugar-binding activity and consequently its biological functions. Variants in these regions, such as the 221delT, may therefore impact on the functionality of PP13.

In summary, *in silico* analysis revealed the following:

- The -98A/C variant disrupts or abolishes putative NF1 and GR transcription factor binding sites, and creates a Sp1 site;
- The hotspot disrupts a number of ESEs sites;
- The 221delT truncates PP13 which abolishes a number of Cys residues, together with putative phosphorylation sites.

These findings may impact significantly on our knowledge of *LGALS13* gene regulation, together with insight into the molecular and physiological functions of PP13. These variants have the potential to affect gene regulation, and protein structure and function considerably. Disruption of putative NF1 and GR sites is likely to affect transcriptional activity of the gene, whereas the creation of a Sp1 site could therefore have implications in epigenetic control by methylation. The abolishment of ESE motifs by the hotspot could ultimately result in the production of abnormal mRNA transcripts. Evidence exists that strongly suggests the negative impact of the deletion on the structure and functionality of PP13. It is important to note that the prediction programs employed for *in silico* analysis in this study, while providing a good starting point, are not always compatible, accurate and reliable. Therefore it is necessary to perform a number of independent analyses in order to gain a consensus result and more importantly, findings of potential importance should be confirmed experimentally.

3.6 FUNCTIONAL ANALYSIS OF THE -98A/C VARIANT

Transcription is a complex process which requires the precise assembly of the preinitation complex, comprising RNA polymerase II and the basal transcription factors, in order to ensure that transcription initiation occurs correctly. Additional transcription factors can regulate transcription initiation by binding to their transcription factor binding sites in the core and proximal promoter regions, as well as the distal enhancer elements, in response to environmental or physiological signals (Nikolov & Burley, 1997). Transcriptional activity can also be regulated by activators, which can recruit and stimulate transcription machinery, and repressors which prevent or interfere with binding of the activators, thereby inhibiting transcription (Lee & Young, 2000).

Variation in the 5' UTR could affect these closely regulated processes in many ways, impacting on transcriptional activity of the gene. In order to investigate the functionality of the -98 A/C variant identified in the 5' untranslated region of the *LGALS13* gene, the wild type and variant alleles were each cloned into the pGL4 luciferase reporter vector and transiently transfected into JAr cells. The expression of luciferase is dependant on the activity of the promoter region or regulatory element being studied, and therefore the relative luciferase activity of each construct provides information regarding the transcriptional activity of the *LGALS13* sequences cloned into the reporter vectors.

Figure 3.18 shows the relative luciferase activity of the pGL4 constructs. The graph represents the normalized mean values (±SEM) from three independent experiments. A value of 1 was assigned to the pGL4 empty vector (minP) control and the relative luciferase activity of the other constructs was determined relative to this value. The activity of the empty pGL4 vector represents the baseline expression of the luciferase gene, under the control of the minimal promoter.
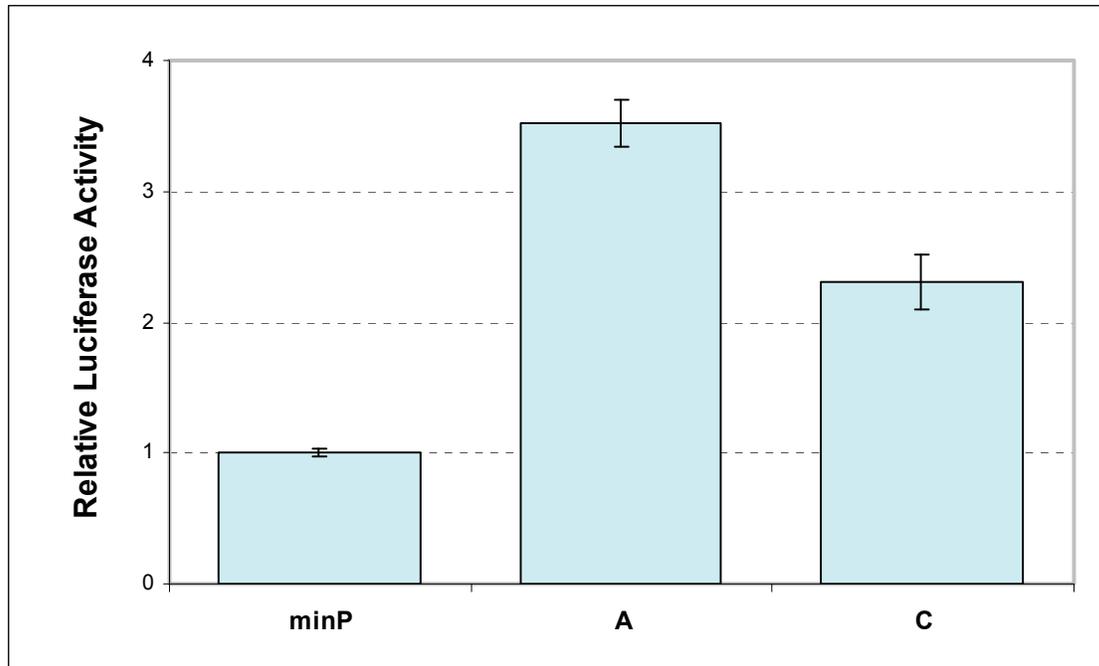
Figure 3.18 Luciferase activity of the constructs relative to that of the pGL4 minimal promoter vector. minP = the empty pGL4 vectors, A = the wild type (-98A) construct, C = the variant (-98C) construct.

The results indicate an increased expression of the wild type and variant constructs, when compared to that of the empty vector. This is evidence that the 700bp fragment, which was cloned into the reporter vector, probably contains regulatory elements which enhance transcriptional activity.

Comparison of the expression of the wild type and variant constructs is shown in Figure 3.19. Once again, the graph represents the normalized mean values (±SEM) from three independent experiments. In this analysis, a value of 1 was assigned to the wild type construct (-98A) and the relative luciferase activity of the variant (-98C) construct was determined relative to this value.
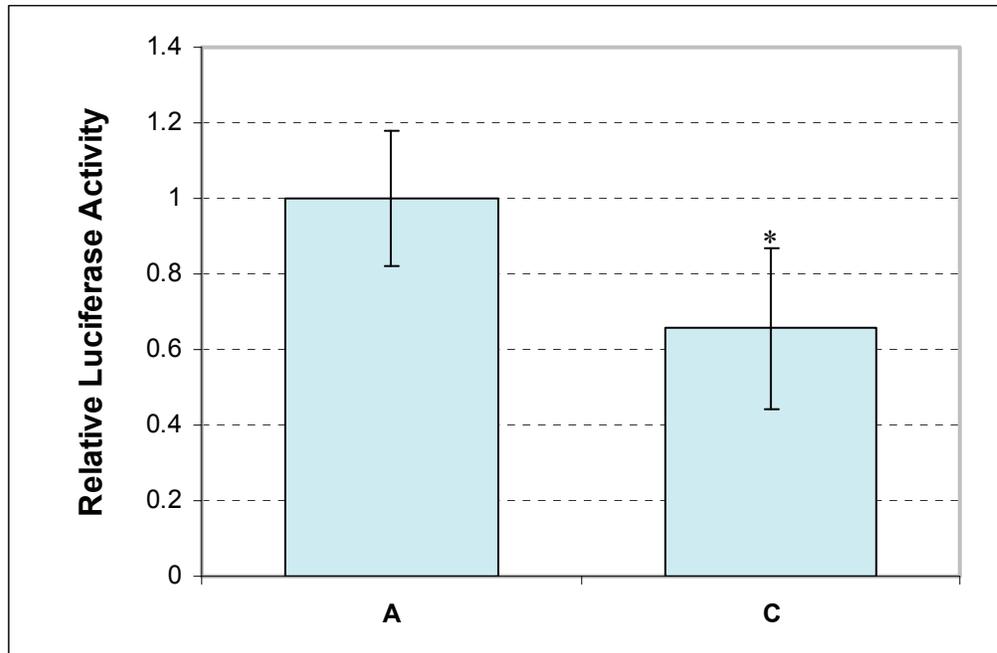
Figure 3.19 Luciferase activity of the -98 C (variant) construct relative to that of the (-98A) wild type construct. * indicates statistical significance (p<0.05) relative to the reference, which is the wild type construct in this case.

Results indicate that the expression of the construct containing the -98 variant allele is significantly reduced (p=0.004). This reduction could be due to disruption of transcription factor binding sites or recognition sites of transcriptional activators by the variant allele. Alternatively, the basal transcription machinery could also be affected, causing a decrease in the transcriptional activity of the gene. Based on the *in silico* predictions presented in Section 3.5.1, it is possible that the reduced expression is due to the disruption of an NF1 or GR transcription factor binding site.

Currently, there is no information regarding the regions of functional importance in the 5' UTR of *LGALS13.* Screening of this region in patient and control cohorts, together with the creation of deletion constructs and reporter gene assays would provide great insight into regulation of this gene.

In summary, the -98C allele was shown to decrease the expression of the luciferase reporter gene. This reduction may be due to the disruption of transcription factor binding sites by the variant C allele and considering the bioinformatics predictions from the previous section, it seems likely that the binding sites may be those of NF1 and GR. Regardless, this decrease in expression may result in decreased PP13 levels in maternal circulation, which may ultimately impact on the physiological processes underlying the pathogenesis of abruption.

CHAPTER 4:

CONCLUSIONS AND FUTURE PROSPECTS

CONCLUSIONS AND FUTURE PROSPECTS

The aim of this study was to investigate whether DNA sequence variants in the *LGALS13* gene underlie and/or confer susceptibility to abruptio placentae.

The novel approach of screening and genotyping of the variants identified in this gene in an abruptio placentae study cohort and subsequent statistical, *in silico* and functional analyses resulted in the:

- Identification of a number of potential susceptibility factors (environmental/behavioural and genetic) in the Coloured population
- Predicted disruption of normal protein structure and functionality by the deletion of a single thymine base at nucleotide position 221
- Establishment of the functionality of the -98A/C variant and insight into possible mechanisms of *LGALS13* gene regulation

The following hypothesis was proposed upon commencement of this research:

"DNA sequence variants in the encoding LGALS13 gene may ultimately lead to the expression of an aberrant form of the protein, which may affect functionality and subsequently disrupt normal implantation and placentation."

In light of the results presented in this study, this hypothesis seems plausible. This study, the first of its kind on PP13/*LGALS13*, provided evidence for a functional effect of the -98C allele. The presence of the C allele was shown to reduce expression of the luciferase reporter gene and *in silico* results suggest that this effect could be caused by the disruption of NF1 or GR transcription factor binding sites by the SNP. This finding is a positive step towards understanding *LGALS13*/PP13 regulation. The association observed at a number of loci in the Coloured group of this study cohort could represent mild and/or cumulative susceptibility factors specific for this ethnic group. This needs to be investigated further, using appropriate experimental methods. For example, the

intronic variants could be examined by the minigene system (Baralle *et al.*, 2003) which proves whether alternative splicing, caused by these polymorphisms, has arisen. As previously mentioned, the majority of statistically significant associations were observed in the subgroup of patients whose pregnancies were complicated by pre-eclampsia as well as abruptio placentae. In order to clarify the true nature of these associations, future work should aim to establish the validity of these findings in a cohort of pre-eclampsia patients whose pregnancies were not additionally complicated with abruptio placentae.

The 221delT, identified in exon 3 of the *LGALS13* gene, results in the truncation of PP13, disruption of cysteine residues believed to be important in dimerization of the protein, and disruption of putative phosphorylation sites.

The 'hotspot' polymorphism represents 6 single nucleotide polymorphisms within an 11bp region, which always occur together as a haplotype. Bioinformatic analysis suggested that the mechanism underlying this polymorphism could be gene conversion - a process whereby genetic information is transferred from a donor sequence/gene to a homologous acceptor sequence/gene (Chen *et al.*, 2007). A "predicted" gene [LOC148003] is located immediately downstream of *LGALS13* and, based on sequence homology comparison, is likely to be the donor sequence/gene of the hotspot. This is not unexpected since many gene conversion events involve pseudogenes or related genes and, being a form of homologous recombination, this event requires homology between interacting sequences. A recent study has shown that most disease-associated gene conversion events result from the participation of non-functional pseudogenes in the transfer of DNA sequences to closely related functional genes. This usually results in a loss-of-function by the acceptor gene (Chen *et al.*, 2007). There is no literature/information available on the predicted gene LOC148003, except its documentation on the NCBI database. The origin, nature and impact of the hotspot polymorphism therefore warrants further investigation by extended bioinformatic analysis (eg, phylogenetic analysis, expression profiling, etc) and

wet-bench experimentation (eg, PP13-immunohistochemistry on placental sections, mutation screening, quantitative real-time PCR to investigate its effect on mRNA levels, etc).

Individually, and possibly collectively, these effects are likely to impact on the structure and function of the protein, and possibly PP13 levels in maternal circulation. Assuming PP13 is as involved in early pregnancy and development as the literature suggests, it is probable that decreased levels of the protein or disruption of normal protein function could impact negatively on implantation and/or placentation. This could have consequences not only for the pathogenesis and development of abruptio placentae, but a wide range of additional pregnancy complications and disorders, and could impact on fetal survival and development.

From a clinical point of view, it is imperative that circulating levels of PP13 are measured in the South African population in order to correlate these protein levels with pregnancy outcome, including complications such as abruptio placentae. Previous studies have investigated PP13 levels in pre-eclampsia, intrauterine growth restriction and preterm delivery (Burger *et al.*, 2004) but none have focused on the measurement of this protein in abruptio placentae. The recruitment of patients for such a study could prove challenging because abruptio placentae is an obstetric emergency and patients are usually recruited shortly before delivery. A possible solution would be to recruit a group of high risk individuals, for example women who experienced abruption or severe hypertension in a previous pregnancy. In well-defined cases of abruptio placentae, the following is necessary:

- Measurement of maternal serum PP13 levels at regular intervals throughout pregnancy to determine whether it has value as a biomarker for abruption
- The composition of full clinical data sets on these patients
- Thorough examination of the placenta and the full pathology reports , with the focus on infection and staining for PP13 in these affected placentas

- The collection of maternal and cord blood at delivery, for maternal and fetal *LGALS13* genotyping, respectively
- The collection of placental tissue for further investigations, for example, mRNA level determination by quantitative real-time PCR.

With regards to PP13 measurements, several aspects should be considered. Protein measurement will be performed with an ELISA kit, developed by DTL (Diagnostic Technologies Limited, Israel). This kit is currently used in Europe as a diagnostic assay for the first-trimester prediction of pre-eclampsia. The impact of the exonic variants on this diagnostic assay requires further investigation, because there is evidence that they could affect protein levels, structure and function which could, in turn, influence the specificity of the assay. In addition, the frequency of the *LGALS13* DNA polymorphisms should be established in extended European populations, as well as other global population groups, since the diagnostic kit is destined for world marketing.

The major findings of this study have revealed several avenues of future research which should be considered, investigated and expanded upon.

The mechanisms involved in regulation of the *LGALS13* gene are unknown, and there is limited information available regarding the regions of functional importance in the 5'UTR of this gene. Future research in this regard should therefore include the following:

- Screening of a larger genomic region upstream of the *LGALS13* gene in the patient and control cohorts in order to identify additional DNA sequence variants in the 5'UTR which could potentially impact on gene regulation;
- Creation of a series of deletion constructs of this extended region and subsequent analysis with luciferase reporter gene assays to identify regions of functional importance;

- Investigation into the effects of various stimuli on gene regulation – for example, inductions with glucocorticoids to investigate the validity of GR disruption by the -98 C allele. In order to gain insight into drug response, the effects of treatment with conventional anti-hypertensive drugs should also be investigated, within this setting;
- Experimental confirmation of the predicted disruption of putative NF1 and GR sites by the -98 variant allele by electrophoretic mobility shift assays (EMSA), to detect the interaction of these transcription factors with their DNA binding sites.

Elucidation of *LGALS13* gene function is critical for future studies. The focus of this research should therefore shift to include techniques such as RNA interference (RNAi), a post-transcriptional gene silencing method (Wheeler *et al.*, 2005), and the use of knock-out- or animal- models. The implementation of these experimental procedures is essential to investigate the effects of gene silencing and subsequent loss-of-function and the impact thereof on embryonic development and the maintenance of pregnancy.

Future research into pregnancy complications such as abruptio placentae should strive towards a functional genomics approach. Microarray technology, for example, could result in some major scientific breakthroughs in this regard.
Recently, this technology has been used to investigate and identify differentially expressed genes in the myometrium, which are related to labour (Esplin *et al.*, 2005). In addition, the use of microarrays for investigation into endometrial function resulted in novel findings that may impact on fertility and contraception, as well as providing insight into the process of implantation (Giudice *et al.*, 2003). The identification of differentially expressed genes in abruptio placentae, pre-eclampsia and preterm labour by the employment of microarray technologies could lead to significant advances regarding our understanding of the molecular etiology and pathogenesis of these pregnancy disorders.

Screening for specific DNA sequence variants in genes that are either involved in susceptibility to abruptio placentae or the pathogenesis thereof in the first trimester of pregnancy could have implications in a clinical setting, after the appropriate assessment thereof in a suitably sized, matched population. The high mortality rate associated with this complication is, in part, due to its sudden and unexpected occurrence. The identification of women who are susceptible to placental abruption could potentially impact on the associated mortality rates by 'flagging' high risk pregnancies and allowing for closer observation thereof.

All future studies should therefore strive towards insight into the molecular etiology of abruptio placentae, using fully integrated and multi-disciplinary approaches, towards the establishment of a predictive screening program. The existence of such a program could result in a decrease of morbidity and mortality associated with this complication in South African mothers and their babies, which is the ultimate goal of our research.

CHAPTER 5:

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REFERENCES

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CHAPTER 6:

APPENDICES

APPENDIX 6.1

Genotype and allele frequencies of the variants identified in this study, as well as p-values from statistical analysis

The following appendix contains the tables showing genotype and allele frequencies for all variants identified in this study, together with their Chi-squared values and p-values from Chi-squared testing for departure from HWE (this value is represented in all tables as p-value**), as well as p-values from StatCalc analyses (represented as p-value*). In all cases the statistical analysis is shown for the total cohort (representing patients with good and poor neonatal outcomes), as well as for the poor outcome cohort (which excludes the good neonatal outcomes from analysis). Shaded blocks indicate statistical significance ($p < 0.05$).

Table 1 Genotype- and allele frequencies of the *LGALS*13 -98 A/C variant in the total patient and control cohorts

	Patients (Total)						Controls		
	Maternal			Fetal			Maternal		
	Total	Coloured	Black	Total	Coloured	Black	Total	Coloured	Black
	n=182	n=132	n=50	n=159	n=114	n=45	n=336	n=237	n=99
AA	90 (0.49)	62 (0.47)	28 (0.56)	73 (0.46)	50 (0.44)	23 (0.51)	174 (0.52)	125 (0.53)	49 (0.50)
AC	80 (0.44)	62 (0.47)	18 (0.36)	76 (0.48)	57 (0.50)	19 (0.42)	139 (0.41)	93 (0.40)	46 (0.47)
CC	12 (0.07)	8 (0.06)	4 (0.08)	10 (0.06)	7 (0.06)	3 (0.07)	23 (0.07)	19 (0.08)	4 (0.04)
A	0.71	0.70	0.74	0.7	0.69	0.72	0.72	0.72	0.72
C	0.29	0.30	0.26	0.3	0.31	0.28	0.28	0.28	0.28
p-value*	0.85	0.33	0.35	0.4	0.16	0.75	-	-	-
p-value**	0.5836	0.338	0.9014	0.2399	0.2084	0.9403	0.7968	0.9584	0.234
Chi^2	1.0769	2.1696	0.2077	2.8554	3.1366	0.1231	0.4543	0.085	2.9049

		Coloured		Black			Coloured		Black	
		AP only	AP+PE	AP only	AP+PE		AP only	AP+PE	AP only	AP+PE
		n=59	n=73	n=18	n=32		n=51	n=63	n=17	n=28
AA		28 (0.47)	34 (0.46)	11 (0.61)	17 (0.53)		24 (0.47)	26 (0.41)	11 (0.65)	12 (0.43)
AC		29 (0.49)	33 (0.45)	5 (0.28)	13 (0.41)		26 (0.51)	31 (0.49)	5 (0.29)	14 (0.50)
CC		2 (0.03)	6 (0.08)	2 (0.11)	2 (0.06)		1 (0.02)	6 (0.10)	1 (0.06)	2 (0.07)
A		0.72	0.69	0.75	0.73		0.72	0.66	0.80	0.68
C		0.28	0.31	0.25	0.27		0.28	0.34	0.20	0.32
p-value*		0.25	0.64	0.21	0.78		0.14	0.26	0.42	0.7
p-value**		0.2399	0.8766	0.5461	0.9731		0.1356	0.7551	0.9177	0.7414
Chi^2		2.8547	0.2634	1.2099	0.0546		3.9962	0.5617	0.1718	0.5985

Table 2 Genotype- and allele frequencies of the *LGALS*13 -98 A/C variant in the poor outcome patient and control cohorts

	Patients (Poor Outcome)						Controls		
	Maternal			Fetal			Maternal		
	Total	Coloured	Black	Total	Coloured	Black	Total	Coloured	Black
	n=131	n=91	n=40	n=119	n=81	n=38	n=336	n=237	n=99
AA	64 (0.49)	43 (0.47)	21 (0.53)	51 (0.43)	32 (0.40)	19 (0.50)	174 (0.52)	125 (0.53)	49 (0.50)
AC	55 (0.42)	40 (0.44)	15 (0.38)	62 (0.52)	45 (0.56)	17 (0.45)	139 (0.41)	93 (0.40)	46 (0.47)
CC	12 (0.09)	8 (0.09)	4 (0.10)	6 (0.05)	4 (0.05)	2 (0.05)	23 (0.07)	19 (0.08)	4 (0.04)
A	0.70	0.69	0.72	0.69	0.68	0.72	0.72	0.72	0.72
C	0.30	0.31	0.29	0.31	0.33	0.28	0.28	0.28	0.28
p-value*	0.69	0.67	0.31	0.13	0.036	0.95	-	-	-
p-value**	0.9891	0.9552	0.8664	0.0625	0.0622	0.7654	0.7968	0.9584	0.234
Chi^2	0.0219	0.0917	0.2867	5.5462	5.5559	0.5346	0.4543	0.085	2.9049

		Coloured		Black			Coloured		Black	
		AP only	AP+PE	AP only	AP+PE		AP only	AP+PE	AP only	AP+PE
		n=39	n=52	n=15	n=25		n=36	n=45	n=14	n=24
AA		17 (0.44)	26 (0.50)	8 (0.53)	13 (0.52)		14 (0.39)	18 (0.40)	8 (0.57)	11 (0.46)
AC		20 (0.51)	20 (0.38)	5 (0.33)	10 (0.40)		21 (0.58)	24 (0.53)	5 (0.36)	12 (0.50)
CC		2 (0.05)	6 (0.12)	2 (0.13)	2 (0.08)		1 (0.02)	3 (0.07)	1 (0.07)	1 (0.04)
A		0.70	0.69	0.70	0.72		0.68	0.67	0.75	0.71
C		0.30	0.31	0.30	0.28		0.31	0.33	0.25	0.29
p-value*		0.35	0.71	0.26	0.65		0.08	0.21	0.7	0.95
p-value**		0.4452	0.7821	0.7266	0.9992		0.1224	0.4066	0.9843	0.5888
Chi^2		1.6183	0.4915	0.6387	0.0016		4.2012	1.8	0.0317	1.0592

Table 3 Genotype- and allele frequencies of the *LGALS*13 -36G/A variant in the total patient and control cohorts

	Patients (Total)									Controls		
	Maternal						Fetal			Maternal		
	Total	Coloured		Black		Total	Coloured		Black	Total	Coloured	Black
	n=183	n=133		n=50		n=163	n=118		n=45	n=336	n=237	n=99
GG	167 (0.91)	119 (0.89)		48 (0.96)		150 (0.92)	109 (0.92)		41 (0.91)	309 (0.92)	222 (0.94)	87 (0.88)
GA	16 (0.09)	14 (0.11)		2 (0.04)		13 (0.08)	9 (0.08)		4 (0.09)	27 (0.08)	15 (0.06)	12 (0.12)
AA	-	-		-		-	-		-	-	-	-
G	0.96	0.95		0.98		0.96	0.96		0.96	0.96	0.97	0.94
A	0.04	0.05		0.02		0.04	0.04		0.04	0.04	0.03	0.06
p-value*	0.78	0.15		0.11		0.98	0.65		0.57	-	-	-
p-value**	0.826	0.8144		0.9896		0.9925	0.9114		0.9525	0.745	0.8811	0.8138
Chi^2	0.3824	0.4105		0.0208		0.015	0.1855		0.0973	0.5888	0.2531	0.4121
		Coloured		Black			Coloured		Black			
		AP only	AP+PE	AP only	AP+PE		AP only	AP+PE	AP only	AP+PE		
		n=60	n=73	n=18	n=32		n=52	n=66	n=17	n=28		
GG		56 (0.93)	63 (0.86)	18 (1.00)	30 (0.94)		49 (0.94)	60 (0.91)	16 (0.94)	25 (0.89)		
GA		4 (0.07)	10 (0.13)	-	2 (0.06)		3 (0.06)	6 (0.09)	1 (0.06)	3 (0.11)		
AA		-	-	-	-		-	-	-	-		
G		0.97	0.93	1	0.97		0.97	0.96	0.96	0.94		
A		0.03	0.07	-	0.03		0.03	0.04	0.03	0.06		
p-value*		0.92	0.043	0.12	0.35		0.88	0.44	0.45	0.84		
p-value**		0.965	0.8209		0.9835		0.9773	0.9279	0.9922	0.9561		
Chi^2		0.0713	0.3947		0.0333		0.0459	0.1497	0.0156	0.0897		

Table 4 Genotype- and allele frequencies of the *LGALS*13 -36G/A variant in the poor outcome patient and control cohorts

	Patients (Poor Outcome)									Controls		
	Maternal						Fetal			Maternal		
	Total	Coloured		Black	Total	Coloured		Black		Total	Coloured	Black
	n=131	n=91		n=40	n=123	n=85		n=38		n=336	n=237	n=99
GG	117 (0.89)	79 (0.87)		38 (0.95)	113 (0.92)	78 (0.92)		35 (0.92)		309 (0.92)	222 (0.94)	87 (0.88)
GA	14 (0.11)	12 (0.13)		2 (0.05)	10 (0.08)	7 (0.08)		3 (0.08)		27 (0.08)	15 (0.06)	12 (0.12)
AA	-	-		-	-	-		-		-	-	-
G	0.94	0.93		0.98	0.96	0.96		0.96		0.96	0.97	0.94
A	0.06	0.07		0.02	0.04	0.04		0.04		0.04	0.03	0.06
p-value*	0.36	0.043		0.21	0.97	0.55		0.48		-	-	-
p-value**	0.8116	0.7971		0.9869	0.8955	0.9246		0.9684		0.745	0.8811	0.8138
Chi^2	0.4175	0.4534		0.0263	0.2208	0.1568		0.0642		0.5888	0.2531	0.4121
		Coloured		Black		Coloured		Black				
		AP only	AP+PE	AP only	AP+PE	AP only	AP+PE	AP only	AP+PE			
		n=39	n=52	n=15	n=25	n=37	n=48	n=14	n=24			
GG		36 (0.92)	43 (0.83)	15 (1.00)	23 (0.92)	35 (0.95)	43 (0.90)	13 (0.93)	22 (0.92)			
GA		3 (0.08)	9 (0.17)	-	2 (0.08)	2 (0.05)	5 (0.10)	1 (0.07)	2 (0.08)			
AA		-	-	-	-	-	-	-	-			
G		0.96	0.91	1	0.96	0.98	0.95	0.97	0.96			
A		0.04	0.09	-	0.04	0.02	0.05	0.03	0.04			
p-value*		0.75	0.009	0.15	0.56	0.83	0.31	0.58	0.6			
p-value**		0.9693	0.7919		0.9785	0.9858	0.9301	0.9904	0.9776			
Chi^2		0.0624	0.4667		0.0434	0.0285	0.1449	0.0192	0.0454			

Table 5 Genotype- and allele frequencies of the *LGALS*13 -22A/G variant in the total patient and control cohorts

	Patients (Total)						Controls		
	Maternal			Fetal			Maternal		
	Total	Coloured	Black	Total	Coloured	Black	Total	Coloured	Black
	n=183	n=133	n=50	n=163	n=118	n=45	n=336	n=237	n=99
AA	130 (0.71)	98 (0.74)	32 (0.64)	107 (0.66)	79 (0.67)	28 (0.62)	232 (0.69)	174 (0.73)	58 (0.59)
AG	49 (0.27)	32 (0.24)	17 (0.34)	53 (0.33)	38 (0.32)	15 (0.33)	96 (0.29)	60 (0.25)	36 (0.36)
GG	4 (0.02)	3 (0.02)	1 (0.02)	3 (0.01)	1 (0.01)	2 (0.04)	8 (0.02)	3 (0.01)	5 (0.05)
A	0.85	0.86	0.81	0.83	0.83	0.79	0.83	0.86	0.77
G	0.15	0.14	0.19	0.17	0.17	0.21	0.17	0.14	0.23
p-value*	0.89	0.75	0.61	0.64	0.38	0.92	-	-	-
p-value**	0.9701	0.9797	0.7606	0.4659	0.2948	1	0.8718	0.6882	0.9814
Chi^2	0.608	0.0409	0.572	1.5276	2.4427	2.47E-0.5	0.2743	0.7472	0.0375

	Coloured		Black			Coloured		Black	
	AP only	AP+PE	AP only	AP+PE		AP only	AP+PE	AP only	AP+PE
	n=60	n=73	n=18	n=32		n=52	n=66	n=17	n=28
AA	45 (0.75)	53 (0.73)	7 (0.39)	25 (0.78)		30 (0.58)	49 (0.74)	9 (0.53)	19 (0.68)
AG	13 (0.22)	19 (0.26)	11 (0.61)	6 (0.19)		22 (0.42)	16 (0.24)	7 (0.41)	8 (0.29)
GG	2 (0.03)	1 (0.01)	-	1 (0.03)		-	1 (0.02)	1 (0.06)	1 (0.04)
A	0.86	0.86	0.7	0.88		0.79	0.86	0.73	0.81
G	0.14	0.14	0.3	0.12		0.21	0.14	0.27	0.19
p-value*	0.47	0.99	0.12	0.14		0.039	0.97	0.91	0.67
p-value**	0.6998	0.8891	0.1751	0.7214		0.1539	0.9722	0.972	0.9905
Chi^2	0.7139	0.2352	3.4848	0.6531		3.743	0.0564	0.0568	0.019

Table 6 Genotype- and allele frequencies of the *LGALS*13 -22A/G variant in the poor outcome patient and control cohorts

	Patients (Poor outcome)						Controls		
	Maternal			Fetal			Maternal		
	Total	Coloured	Black	Total	Coloured	Black	Total	Coloured	Black
	n=131	n=91	n=40	n=123	n=85	n=38	n=336	n=237	n=99
AA	95 (0.73)	69 (0.76)	26 (0.65)	80 (0.65)	57 (0.67)	23 (0.61)	232 (0.69)	174 (0.73)	58 (0.57)
AG	33 (0.25)	20 (0.22)	13 (0.33)	40 (0.33)	27 (0.32)	13 (0.34)	96 (0.29)	60 (0.25)	36 (0.36)
GG	3 (0.02)	2 (0.02)	1 (0.03)	3 (0.02)	1 (0.01)	2 (0.05)	8 (0.02)	3 (0.01)	5 (0.05)
A	0.86	0.87	0.80	0.82	0.83	0.78	0.83	0.86	0.77
G	0.14	0.13	0.20	0.18	0.17	0.22	0.17	0.14	0.23
p-value*	0.76	0.7	0.69	0.71	0.52	0.97	-	-	-
p-value**	0.9978	0.9295	0.9149	0.7426	0.5283	0.9958	0.8718	0.6882	0.9814
Chi^2	0.0045	0.1462	0.1778	0.5952	1.276	0.0085	0.2743	0.7472	0.0375

		Coloured		Black			Coloured		Black	
		AP only	AP+PE	AP only	AP+PE		AP only	AP+PE	AP only	AP+PE
		n=39	n=52	n=15	n=25		n=37	n=48	n=14	n=24
AA		30 (0.77)	39 (0.75)	7 (0.47)	19 (0.76)		21 (0.57)	36 (0.75)	8 (0.57)	15 (0.63)
AG		8 (0.21)	12 (0.23)	8 (0.53)	5 (0.20)		16 (0.43)	11 (0.23)	5 (0.36)	8 (0.33)
GG		1 (0.03)	1 (0.02)	-	1 (0.04)		-	1 (0.02)	1 (0.07)	1 (0.04)
A		0.88	0.87	0.73	0.86		0.79	0.87	0.75	0.80
G		0.14	0.13	0.27	0.14		0.21	0.13	0.25	0.20
p-value*		0.68	0.89	0.36	0.27		0.037	0.86	0.95	0.94
p-value**		0.8761	0.9976	0.3709	0.6985		0.2447	0.9829	0.9843	0.9987
Chi^2		0.2645	0.0047	1.9835	0.7177		2.8157	0.0218	0.0317	0.0266

Table 7 Genotype- and allele frequencies of the *LGALS*13 -15G/A variant in the total patient and control cohorts

	Patients (Total)						Controls		
	Maternal			Fetal			Maternal		
	Total	Coloured	Black	Total	Coloured	Black	Total	Coloured	Black
	n=183	n=133	n=50	n=163	n=118	n=45	n=336	n=237	n=99
GG	181 (0.99)	132 (0.99)	49 (0.98)	161 (0.99)	117 (0.99)	44 (0.98)	330 (0.98)	233 (0.98)	97 (0.98)
AG	2 (0.01)	1 (0.01)	1 (0.02)	2 (0.01)	1 (0.01)	1 (0.02)	6 (0.02)	4 (0.02)	2 (0.02)
AA	-	-	-	-	-	-	-	-	-
G	0.995	0.99	0.99	0.99	0.99	0.99	0.99	0.99	0.99
A	0.005	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
p-value*	0.54	0.45	0.99	0.64	0.53	0.94	-	-	-
p-value**	0.9972	0.9991	0.9975	0.9969	0.9989	0.9972	0.9865	0.9915	0.9949
Chi^2	0.0055	0.0019	0.0051	0.0062	0.0021	0.0057	0.0273	0.0172	0.0103

		Coloured		Black			Coloured		Black	
		AP only	AP+PE	AP only	AP+PE		AP only	AP+PE	AP only	AP+PE
		n=60	n=73	n=18	n=32		n=52	n=66	n=17	n=28
GG		59 (0.98)	73 (1.00)	17 (0.94)	32 (1.00)		52 (1.00)	65 (0.98)	16 (0.94)	28 (1.00)
AG		1 (0.02)	-	1 (0.06)	-		-	1 (0.02)	1 (0.06)	-
AA		-	-	-	-		-	-	-	-
G		0.99	1	0.97	1		1	0.99	0.97	1
A		0.01	-	0.03	-		-	0.01	0.03	-
p-value*		0.99	0.26	0.38	0.42		0.36	0.92	0.35	0.45
p-value**		0.9979	-	0.9927	-		-	0.9981	0.9922	-
Chi^2		0.0042	-	0.0147	-		-	0.0038	0.0156	-

Table 8 Genotype- and allele frequencies of the *LGALS*13 -15G/A variant in the poor outcome patient and control cohorts

	Patients (Poor Outcome)						Controls		
	Maternal			Fetal			Maternal		
	Total	Coloured	Black	Total	Coloured	Black	Total	Coloured	Black
	n=131	n=91	n=40	n=123	n=85	n=38	n=336	n=237	n=99
GG	129 (0.98)	90 (0.99)	39 (0.98)	121 (0.98)	84 (0.99)	37 (0.97)	330 (0.98)	233 (0.98)	97 (0.98)
AG	2 (0.02)	1 (0.01)	1 (0.02)	2 (0.02)	1 (0.01)	1 (0.03)	6 (0.02)	4 (0.02)	2 (0.02)
AA	-	-	-	-	-	-	-	-	-
G	0.99	0.99	0.99	0.99	0.99	0.97	0.97	0.99	0.99
A	0.01	0.01	0.01	0.01	0.01	0.03	0.03	0.01	0.01
p-value*	0.85	0.7	0.86	0.91	0.74	0.83	-	-	-
p-value**	0.9961	0.9986	0.9968	0.9959	0.9985	0.9966	0.9865	0.9915	0.9949
Chi^2	0.0078	0.0028	0.0064	0.0083	0.003	0.0068	0.0273	0.0172	0.0103

		Coloured		Black			Coloured		Black	
		AP only	AP+PE	AP only	AP+PE		AP only	AP+PE	AP only	AP+PE
		n=39	n=52	n=15	n=25		n=37	n=48	n=14	n=24
GG		38 (0.98)	52 (1.00)	14 (0.93)	25 (1.00)		37 (1.00)	47 (0.98)	13 (0.93)	24 (1.00)
AG		1 (0.04)	-	1 (0.07)	-		-	1 (0.02)	1 (0.07)	-
AA		-	-		-		-	-	-	-
G		0.98	1	0.965	1		1	1	0.97	1
A		0.02	-	0.035	-		-	-	0.03	-
p-value*		0.7	0.36	0.29	0.47		0.43	0.85	0.26	0.48
p-value**		0.9967	-	0.9911	-		-	0.9973	0.9904	-
Chi^2		0.0066	-	0.0178	-		-	0.0053	0.0192	-

Table 9 Genotype- and allele frequencies of the *LGALS*13 221delT variant in the total patient and control cohorts

	Patients (Total)						Controls		
	Maternal			Fetal			Maternal		
	Total	Coloured	Black	Total	Coloured	Black	Total	Coloured	Black
	n=183	n=133	n=50	n=163	n=118	n=45	n=336	n=237	n=99
TT	179 (0.98)	130 (0.98)	49 (0.98)	159 (0.98)	115 (0.97)	44 (0.98)	328 (0.98)	233 (0.98)	95 (0.96)
T-	4 (0.02)	3 (0.02)	1 (0.02)	4 (0.02)	3 (0.03)	1 (0.02)	8 (0.02)	4 (0.02)	4 (0.04)
--	-	-	-	-	-	-	-	-	-
T	0.99	0.99	0.99	0.99	0.985	0.99	0.99	0.99	0.98
-	0.01	0.01	0.01	0.01	0.015	0.01	0.01	0.01	0.02
p-value*	0.89	0.7	0.51	0.96	0.59	0.58	-	-	-
p-value**	0.9889	0.9914	0.9975	0.9875	0.9903	0.9972	0.9759	0.9915	0.9792
Chi^2	0.0223	0.0173	0.0051	0.0251	0.0196	0.0057	0.0488	0.0172	0.0421

		Coloured		Black			Coloured		Black	
		AP only	AP+PE	AP only	AP+PE		AP only	AP+PE	AP only	AP+PE
		n=60	n=73	n=18	n=32		n=52	n=66	n=17	n=28
TT		59 (0.98)	71 (0.97)	17 (0.94)	32 (1.00)		51 (0.98)	64 (0.97)	17 (1.00)	27 (0.96)
T-		1 (0.02)	2 (0.03)	1 (0.06)	-		1 (0.02)	2 (0.03)	-	1 (0.04)
--		-	-	-	-		-	-	-	-
T		0.99	0.99	0.97	1		0.99	0.99	1	0.98
-		0.01	0.01	0.03	-		0.01	0.01	-	0.02
p-value*		0.99	0.57	0.77	0.25		0.91	0.49	0.4	0.91
p-value**		0.9979	0.993	0.9927			0.9976	0.9922		0.9954
Chi^2		0.0042	0.0141	0.0147			0.0049	0.0156		0.0093

Table 10 Genotype- and allele frequencies of the *LGALS*13 221delT variant in the poor outcome patient and control cohorts

	Patients (Poor Outcome)						Controls		
	Maternal			**Fetal**			**Maternal**		
	Total	**Coloured**	**Black**	**Total**	**Coloured**	**Black**	**Total**	**Coloured**	**Black**
	n=131	**n=91**	**n=40**	**n=123**	**n=85**	**n=38**	**n=336**	**n=237**	**n=99**
TT	128 (0.98)	89 (0.98)	39 (0.98)	121 (0.98)	83 (0.98)	38 (1.00)	328 (0.98)	233 (0.98)	95 (0.96)
T-	3 (0.02)	2 (0.02)	1 (0.02)	2 (0.02)	2 (0.02)	-	8 (0.02)	4 (0.02)	4 (0.04)
--	-	-	-	-	-	-	-	-	-
T	0.99	0.99	0.99	0.99	0.99	1	0.99	0.99	0.98
-	0.01	0.01	0.01	0.01	0.01	-	0.01	0.01	0.02
p-value*	0.95	0.76	0.66	0.62	0.7	0.21	-	-	-
p-value**	0.9913	0.9944	0.9968	0.9959	0.994	-	0.9759	0.9915	0.9792
Chi^2	0.0176	0.0112	0.0064	0.0083	0.012	-	0.0488	0.0172	0.0421

		Coloured		**Black**			**Coloured**		**Black**	
		AP only	**AP+PE**	**AP only**	**AP+PE**		**AP only**	**AP+PE**	**AP only**	**AP+PE**
		n=40	**n=52**	**n=15**	**n=25**		**n=37**	**n=48**	**n=14**	**n=24**
TT		38 (0.95)	51 (0.98)	14 (0.93)	25 (1.00)		36 (0.97)	47 (0.98)	14 (1.00)	24 (1.00)
T-		1 (0.05)	1 (0.02)	1 (0.07)	-		1 (0.03)	1 (0.02)	-	-
--		-	-	-	-		-	-	-	-
T		0.98	0.99	0.99	1		0.99	0.99	1	1
-		0.02	0.01	0.01	-		0.01	0.01	-	-
p-value*		0.7	0.91	0.64	0.31		0.67	0.85	0.44	0.32
p-value**		0.9967	0.9976	0.9911	-		0.9965	0.9973	-	-
Chi^2		0.0066	0.0049	0.0178	-		0.0069	0.0053	-	-

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Table 11 Genotype- and allele frequencies of the *LGALS*13 hotspot variant in the total patient and control cohorts

	Patients (Total)								Controls		
	Maternal				Fetal				Maternal		
	Total	Coloured		Black	Total	Coloured		Black	Total	Coloured	Black
	n=181	n=132		n=49	n=162	n=117		n=45	n=354	n=249	n=105
+/+	161 (0.89)	116 (0.88)		45 (0.92)	146 (0.90)	105 (0.90)		41 (0.91)	328 (0.93)	234 (0.94)	94 (0.90)
+/-	20 (0.11)	16 (0.12)		4 (0.08)	16 (0.10)	12 (0.10)		4 (0.09)	26 (0.07)	15 (0.06)	11 (0.10)
-/-	-	-		-	-	-		-	-	-	-
+	0.95	0.94		0.96	0.95	0.95		0.96	0.96	0.97	0.95
-	0.05	0.06		0.04	0.05	0.05		0.04	0.04	0.03	0.05
p-value*	0.15	0.038		0.65	0.33	0.15		0.77	-	-	-
p-value**	0.7338	0.7598		0.9566	0.8037	0.8429		0.9525	0.7732	0.8869	0.8518
Chi^2	0.619	0.5494		0.0887	0.4372	0.3419		0.0973	0.5145	0.2402	0.3208
		Coloured		Black		Coloured		Black			
		AP only	AP+PE	AP only	AP+PE	AP only	AP+PE	AP only	AP+PE		
		n=60	n=72	n=18	n=31	n=52	n=65	n=17	n=28		
+/+		55 (0.92)	61 (0.85)	18 (1.00)	27 (0.87)	46 (0.88)	59 (0.91)	16 (0.94)	25 (0.89)		
+/-		5 (0.08)	11 (0.15)	-	4 (0.13)	6 (0.12)	6 (0.09)	1 (0.06)	3 (0.11)		
-/-		-	-	-	-	-	-	-	-		
+		0.96	0.92	1	0.93	0.94	0.96	0.97	0.94		
-		0.04	0.08	-	0.07	0.06	0.04	0.03	0.06		
p-value*		0.51	0.011	0.15	0.71	0.16	0.36	0.56	0.97		
p-value**		0.9449	0.7817	-	0.9289	0.9071	0.9267	0.9922	0.9561		
Chi^2		0.1134	0.4925	-	0.1474	0.1949	0.1522	0.0156	0.0897		

Table 12 Genotype- and allele frequencies of the *LGALS*13 hotspot variant in the poor outcome patient and control cohorts

	Patients (Poor Outcome)							Controls		
	Maternal				Fetal			Maternal		
	Total	Coloured	Black	Total	Coloured	Black		Total	Coloured	Black
	n=129	n=90	n=39	n=112	n=84	n=28		n=354	n=249	n=105
+/+	115 (0.89)	79 (0.88)	36 (0.92)	101 (0.90)	76 (0.90)	25 (0.92)		328 (0.93)	234 (0.94)	94 (0.90)
+/-	14 (0.11)	11 (0.12)	3 (0.08)	11 (0.10)	8 (0.10)	3 (0.08)		26 (0.07)	15 (0.06)	11 (0.10)
-/-	-	-	-	-	-	-		-	-	-
+	0.95	0.94	0.96	0.95	0.95	0.96		0.96	0.97	0.95
-	0.05	0.06	0.04	0.05	0.05	0.04		0.04	0.03	0.05
p-value*	0.22	0.06	0.62	0.4	0.27	0.65		-	-	-
p-value**	0.8087	0.8264	0.9693	0.8613	0.9003	0.9561		0.7732	0.8869	0.8518
Chi^2	0.4247	0.3813	0.0624	0.2987	0.21	0.0897		0.5145	0.2402	0.3208

		Coloured		Black			Coloured		Black	
		AP only	AP+PE	AP only	AP+PE		AP only	AP+PE	AP only	AP+PE
		n=39	n=51	n=15	n=24		n=37	n=47	n=14	n=24
+/+		37 (0.95)	42 (0.82)	15 (1.00)	21 (0.87)		33 (0.89)	43 (0.91)	13 (0.93)	22 (0.92)
+/-		2 (0.05)	9 (0.18)	-	3 (0.13)		4 (0.11)	4 (0.09)	1 (0.07)	2 (0.08)
-/-		-	-	-	-		-	-	-	-
+		0.98	0.91	1	0.93		0.94	0.96	0.97	0.96
-		0.02	0.09	-	0.07		0.06	0.04	0.03	0.04
p-value*		0.83	0.005	0.19	0.77		0.28	0.52	0.69	0.75
p-value**		0.9866	0.7876	-	0.9481		0.9414	0.9546	0.9904	0.9776
Chi^2		0.027	0.4776	-	0.1067		0.1208	0.0928	0.0192	0.0454

Table 13 Genotype- and allele frequencies of the *LGALS*13 +72T/A variant in the total patient and control cohorts

	Patients (Total)						Controls		
	Maternal			Fetal			Maternal		
	Total	Coloured	Black	Total	Coloured	Black	Total	Coloured	Black
	n=178	n=131	n=47	n=150	n=108	n=42	n=336	n=237	n=99
TT	132 (0.74)	97 (0.74)	35 (0.74)	108 (0.72)	78 (0.72)	30 (0.71)	233 (0.69)	172 (0.72)	61 (0.62)
TA	41 (0.23)	31 (0.24)	10 (0.36)	36 (0.24)	27 (0.25)	9 (0.21)	97 (0.29)	63 (0.27)	34 (0.34)
AA	5 (0.03)	3 (0.02)	2 (0.04)	6 (0.04)	3 (0.03)	3 (0.07)	6 (0.02)	2 (0.01)	4 (0.04)
T	0.86	0.86	0.92	0.84	0.84	0.81	0.84	0.86	0.79
A	0.14	0.14	0.08	0.16	0.16	0.19	0.16	0.14	0.21
p-value*	0.3	0.45	0.27	0.22	0.37	0.27	-	-	-
p-value**	0.713	0.9618	0.545	0.4228	0.9385	0.2174	0.5221	0.3425	0.9633
Chi^2	0.6766	0.0779	1.214	1.7219	0.1269	3.0519	1.2999	2.143	0.0747

		Coloured		Black			Coloured		Black	
		AP only	AP+PE	AP only	AP+PE		AP only	AP+PE	AP only	AP+PE
		n=60	n=71	n=18	n=29		n=48	n=60	n=17	n=25
TT		45 (0.75)	52 (0.73)	9 (0.50)	26 (0.90)		31 (0.65)	47 (0.78)	11 (0.65)	19 (0.76)
TA		14 (0.23)	17 (0.24)	9 (0.50)	1 (0.03)		17 (0.35)	10 (0.17)	4 (0.24)	5 (0.20)
AA		1 (0.02)	2 (0.03)	-	2 (0.07)		-	3 (0.05)	2 (0.12)	1 (0.04)
T		0.87	0.85	0.75	0.91		0.82	0.87	0.77	0.86
A		0.13	0.15	0.25	0.09		0.18	0.13	0.24	0.14
p-value*		0.76	0.41	0.35	0.004		0.39	0.03	0.37	0.38
p-value**		0.9972	0.9151	0.3679	0.0001		0.3291	0.097	0.3611	0.6985
Chi^2		0.0055	0.1774	2	17.695		2.2227	4.6653	2.037	0.7177

Table 14 Genotype- and allele frequencies of the *LGALS*13 +72T/A variant in the poor outcome patient and control cohorts

	Patients (Poor Outcome)						Controls		
	Maternal			Fetal			Maternal		
	Total	Coloured	Black	Total	Coloured	Black	Total	Coloured	Black
	n=127	n=89	n=38	n=112	n=77	n=35	n=336	n=237	n=99
TT	97 (0.76)	67 (0.75)	30 (0.79)	78 (0.70)	53 (0.69)	25 (0.71)	233 (0.69)	172 (0.72)	61 (0.62)
TA	26 (0.20)	19 (0.21)	7 (0.18)	30 (0.27)	23 (0.30)	7 (0.20)	97 (0.29)	63 (0.27)	34 (0.34)
AA	4 (0.03)	3 (0.03)	1 (0.03)	4 (0.04)	1 (0.01)	3 (0.09)	6 (0.02)	2 (0.01)	4 (0.04)
T	0.86	0.86	0.88	0.84	0.84	0.81	0.84	0.86	0.79
A	0.14	0.14	0.12	0.16	0.16	0.19	0.16	0.14	0.21
p-value*	0.14	0.18	0.16	0.51	0.79	0.21	-	-	-
p-value**	0.4186	0.5505	0.7684	0.8731	0.6894	0.5958	0.5221	0.3425	0.9633
Chi^2	1.7418	1.1938	0.5268	0.2715	0.744	1.6356	1.2999	2.143	0.0747

		Coloured		Black			Coloured		Black	
		AP only	AP+PE	AP only	AP+PE		AP only	AP+PE	AP only	AP+PE
		n=39	n=50	n=15	n=23		n=34	n=43	n=14	n=21
TT		29 (0.74)	38 (0.76)	9 (0.60)	21 (0.91)		21 (0.62)	32 (0.74)	9 (0.64)	16 (0.76)
TA		9 (0.23)	10 (0.20)	6 (0.40)	1 (0.04)		13 (0.38)	10 (0.23)	3 (0.21)	4 (0.19)
AA		1 (0.03)	2 (0.04)	-	1 (0.04)		-	1 (0.02)	2 (0.14)	1 (0.05)
T		0.86	0.86	0.8	0.93		0.81	0.86	0.75	0.86
A		0.14	0.14	0.2	0.06		0.19	0.14	0.25	0.14
p-value*		0.58	0.16	0.7	0.016		0.33	0.22	0.22	0.39
p-value**		0.957	0.4879	0.6258	0.086		0.3868	0.9789	0.2765	0.5954
Chi^2		0.088	1.4354	0.9375	9.5215		1.8995	0.0428	2.5714	1.037

APPENDIX 6.2
Solutions and Buffers

Cell Lysis Buffer:

8.3g NH_4Cl (0.155M)

1.1g $KHCO_3$ (0.01M)

0.03g EDTA (0.0001M)

Bring to volume (1L) with dH_2O, adjust pH to 7.4 with HCl and store at 4°C

Phosphate Buffered Saline (PBS):

2g KCl (0.027M)

8g NaCl (0.137M)

1.14g Na_2HPO4 (0.008M)

0.2g KH_2PO4 (0.0015M)

Bring to volume (1L) with dH_2O and store at 4°C

Nucleur Lysis Buffer:

1.211g TRIS-Cl (0.01M)

23.4g NaCl (0.4M)

0.6g EDTA (0.002M)

Bring to volume (1L) with dH_2O, adjust pH to 8.2 with 1M NaOH and store at 4°C

10% SDS:

10g SDS

Bring to volume (100ml) with dH_2O

Store at room temperature

Proteinase K:

10mg/ml, dissolve in dH2O

Store at –20°C

6M NaCl (saturated solution):

175.32g NaCl

Bring to volume (500ml) with dH_2O

40% Acrylamide PDA Solution:

396g Acrylamide

4g Piperazine diacrylamide

Bring to volume (1L) with dH_2O

Wrap in foil and store @ 4°C

0.75M TRIS-Formate Buffer:

90.8g TRIS

600ml dH_2O

Adjust pH to 9.0 with formic acid

Bring to volume (1L) with dH2O

41% Glycerol:

41ml Glycerol

59ml dH_2O

TRIS-Borate Buffer:

125.9g TRIS

17.3g Boric acid

700ml dH_2O

Adjust pH to 9.0

50µl Bromophenol blue (4% solution)

Bring to volume (1L) with dH2O

SSCP Loading Dye:

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% Ammonium persulphate (APS):

0.15g APS

Bring to volume (1.5ml) with dH_2O

10% Glacial acetic acid:

10µl Glacial acetic acid

Bring to volume (100µl) with dH_2O

Gel mix (10X):

53ml 40% Acrylamide PDA solution

85ml TRIS-Formate buffer

30ml 41% Glycerol

Wrap in foil and store at 4°C

Silver staining solution I:

0.3g Silver nitrate

Bring to volume (300ml) with dH_2O

Silver staining solution II:

4.5 g NaOH

300ml dH_2O

3ml 15% Formaldehyde solution

10X TBE:

216 g Tris

110 g Boric acid

14.89 g EDTA

Bring to a final volume of 2L with dH_2O

1X TBE:

100 ml 10X TBE

Bring to a final volume of 1L with dH_2O

LB medium:

10g Bacto®-tryptone

5g Bacto®-yeast extract

5g NaCl

Bring to final volume of 1L and adjust pH to 7.5.

Autoclave

LB Agar Amp plates:

10g Tryptone

5g Yeast Extract

5g NaCl

15g Agar

Bring to volume (1L) with dH2O

Autoclave, allow to cool, add 2ml [50mg/ml] Amp and pour plates

APPENDIX 6.3

Protocols

Protocol for extraction of genomic DNA from whole blood

(Adapted from the protocol by Miller *et al.*, 1988)

- Place ~10 ml blood in a 50 ml Falcon tube, add 30 ml cold Cell Lysis Buffer and place on ice for 30 min, shaking every 10 min interval
- Centrifuge @ 1500 rpm for 15 min
- Pour off supernatant, keeping pellet and add 10 ml PBS and shake
- Centrifuge @ 1500 rpm for 15 min
- Pour off supernatant, keeping pellet and dissolve pellet in 3 ml Nuclear Lysis Buffer, 30 µl 10 mg/ml Proteinase K, 300 µl 10% SDS
- Mix well and incubate overnight in 55˚C waterbath
- Add 1 ml NaCl and shake for 1 min
- Centrifuge @ 3500 rpm for 30 min
- Transfer supernatant to new tube and shake for 15 s
- Centrifuge @ 2500 rpm for 15 min
- Transfer supernatant to new tube and add 2 volumes ice cold 100% EtOH
- Put on ice for 1hr
- Scoop out DNA and place in eppi with 500 µl 70% EtOH
- Centrifuge @ 14000 rpm for 10 min @ 4˚C
- Dissolve pellet in 200-800 µl TE Buffer (depending on pellet size)

Protocol for mutation detection by Multiphor gel electrophoresis

Preparation of the gel plates:

- Clean plates 4 X with 70% ethanol
- Clean back plate with 80 µl plate glue and 8 µl 10% glacial acetic acid
- Clean well plate with 80 µl repelcote
- Clean plates 2 X with 70% ethanol
- Clean spacers with 70% ethanol
- Clamp spacers and plates in place
- Prepare gel mix, pour quickly and allow to polymerize at room temperature
- Separate plates
- Prepare gel on Multiphor apparatus
- Soak buffer strips in TRIS-Borate buffer
- Place strips on both sides of the gel, close to wells

Preparation of PCR products:

- Add 3 µl SSCP loading dye to 3-5 µl PCR product
- Denature at 95°C for 5 min
- Quench on ice immediately for 3 min
- Load 3 µl onto the gel
- Run at optimized temperature and time at 355 V

Visualization of resolved DNA fragments:

- Remove gel from apparatus
- Rinse in dH_2O
- Incubate for 10 min in Solution I
- Rinse in dH_2O
- Incubate for 10 min in Solution II
- Rinse in dH_2O
- Blot dry

SureClean Protocol (Bioline)

- Add an equal volume of SureClean to nucleic acid solution and mix thoroughly
- Incubate at room temperature for at least 10 mins
- Centrifuge at maximum speed in a bench-top centrifuge for 10 minutes
- Carefully remove supernatant by aspiration
- Add a volume of 70% Ethanol equal to 2x original sample volume and vortex for 10 seconds
- Centrifuge at maximum speed in a bench-top centrifuge for 10 min
- Remove supernatant and air-dry to ensure complete removal of ethanol
- Resuspend pellet in desired volume of TE, water or any other appropriate buffer for downstream procedures.

Protocol for Wizard® SV Gel and PCR Clean-Up System (Promega)

Load and run the gel using an established protocol. Weigh a 1.5 ml microcentrifuge tube for each DNA fragment to be isolated and record the weight. Visualize and photograph the DNA using a long-wavelength UV lamp and an intercalating dye such as ethidium bromide. Excise the DNA fragment of interest in a minimal volume of agarose using a clean scalpel or razor blade. Transfer the gel slice to the weighed microcentrifuge tube and record the weight. Subtract the weight of the empty tube from the total weight to obtain the weight of the gel slice.

- Add Membrane Binding Solution at a ratio of 10 µl of solution per 10 mg of agarose gel slice
- Vortex the mixture and incubate at 50.65°C for 10 minutes or until the gel slice is completely dissolved.
- Place one SV Minicolumn in a Collection Tube for each dissolved gel slice
- Transfer the dissolved gel mixture or prepared PCR product to the SV Minicolumn assembly and incubate for 1 minute at room temperature
- Centrifuge the SV Minicolumn assembly in a microcentrifuge at 14,000 rpm for 1 minute. Remove the SV Minicolumn from the Spin Column assembly and discard the liquid in the Collection Tube. Return the SV Minicolumn to the Collection Tube.
- Wash the column by adding 700µl of Membrane Wash Solution, previously diluted with 95% ethanol, to the SV Minicolumn
- Centrifuge the SV Minicolumn assembly for 1 minute at 14,000 rpm. Empty the Collection Tube as before and place the SV Minicolumn back in the Collection Tube. Repeat the wash with 500µl of Membrane Wash Solution and centrifuge the SV Minicolumn assembly for 5 minutes at 14,000 rpm.
- Empty the Collection Tube and recentrifuge the column assembly for 1 minute with the microcentrifuge lid open (or off) to allow evaporation of any residual ethanol.
- Carefully transfer the SV Minicolumn to a clean 1.5 ml microcentrifuge tube.

- Apply 50µl of Nuclease-Free Water directly to the center of the column without touching the membrane with the pipette tip. Incubate at room temperature for 1 minute.

- Centrifuge for 1 minute at 14,000 rpm

- Discard the SV Minicolumn and store the microcentrifuge tube containing the eluted DNA at 4°C or .20°C.

Transformation of E.cloni® Chemically Competent Cells (Lucigen)

Preparation for transformation:

- For best results the ligation reaction MUST be purified or heat killed at 70˚C for 15 minutes before transformation (Failure to do so may prevent transformation)
- All microcentrifuge tubes must be thoroughly pre-chilled on ice before use
- The cells must be completely thawed on ice before use
- For highest transformation efficiency, use the provided Recovery Medium to resuspend the cells after transformation

Transformation Protocol:

- Chill 3 sterile tubes on ice (one tube for each transformation reaction - pGL4:558, pGL4:132, pGL4 vector as positive control)
- Remove E.cloni cells from -80˚C freezer and thaw completely on wet ice (10-20 minutes)
- Purified / heat kill ligation reaction at 70˚C for 15 minutes before transformation
- Add 40 µl cells to the chilled culture tube
- Add 5 µl ligation reaction or DNA sample to the cells on ice. Stir briefly with pipette tip. DO NOT pipette up and down to mix as this can introduce air bubbles and warm the cells
- Incubate on ice for 30 minutes
- Heat shock the cells by placing them in a 42˚C waterbath for 45 seconds
- Return the cells to ice for 2 minutes
- Add 960 µl of room temperature Recovery Medium to the cells in the culture tube
- Place the tubes in a shaking incubator at 250rpm for 1 hour at 37˚C
- Plate 300 µl transformed cells onto LB agar plates containing Amp.
- Incubate the plates overnight at 37˚C

GenElute Plasmid Mini-Prep Kit Protocol (Sigma-Aldrich)

(All spins at 12,000 x g, except as noted.)

Harvest & lyse bacteria
- Pellet cells from 1-5 ml overnight culture for 1 min, discard supernatant by aspiration
- Resuspend cells in 200 µl Resuspension Solution, pipette up and down or vortex
- Add 200 µl Lysis Solution. Invert gently to mix – don't vortex. Allow to clear for no longer than 5 minutes.

Prepare cleared lysate
- Add 350 µl Neutralization Solution and invert 4-6 times to mix
- Pellet debris at max speed for 10 minutes

Prepare binding column
- Add 500 ml Column Preparation Solution to binding column in a collection tube
- Spin at 12,000 x g for 1 minute. Discard flow-through

Bind plasmid DNA to column
- Transfer cleared lysate into binding column by aspiration
- Spin for 1 minute, discard flow-through

Wash to remove contaminants
- Add 750ml Wash Solution to column,
- Spin for 1 minute, discard flow-through
- Spin for 1 minute to dry, discard flow-through

Elute purified plasmid DNA
- Transfer column to a new collection tube
- Add 50 µl Elution Solution, spin for 1 minute

EndoFree® Plasmid Purification Maxi Kit (QIAGEN)

Preparation:

- Add RNAase A solution to Buffer P1
- Add 40 ml of 100% EtOH to the endotoxin-free water supplied with the kit
- Add LyseBlue reagent to Buffer P1
- Check Buffer P2 for SDS precipitation
- Pre-chill Buffer P3 to 4°C
- Prepare bacterial culture

Protocol for plasmid DNA extraction:

Bacterial culture, harvest, lysis

- Pellet 100 ml overnight LB culture at 6000 x g for 15 min at 4°C
- Resuspend the bacterial pellet in 10 ml Buffer P1
- Add 10 ml Buffer P2, mix thoroughly by vigorously inverting 4-6 times, and incubate at room temperature for 5 min
- During the incubation prepare the QIAfilter cartridge: Screw the cap onto the outlet nozzle of the QIAfilter Maxi Cartridge. Place the Cartridge into a convenient tube
- Add 10 ml chilled Buffer P3, mix thoroughly inverting 4-6 times

Bacterial lysate clearing

- Pour the lysate into the barrel of the QIAfilter cartridge, incubate at room temperature for 10 min. Remove the cap from the cartridge, gently insert the plunger and filter the cell lysate into a 50 ml tube
- Add 2.5 ml Buffer ER to the filtered lysate, mix by inverting the tube approximately 10 times and incubate on ice for 20 min
- Bind, wash and elute plasmid DNA on QIAGEN-tip
- Equilibrate a QIAGEN-tip 500 by applying 10 ml Buffer QBT and allow the column to empty by gravity flow
- Apply the filtered lysate to the QIAGEN-tip and allow it to enter the resin by gravity flow

- Wash the QIAGEN-tip with 2 x 30 ml Buffer QC

Precipitate, wash and redissolve plasmid DNA

- Precipitate DNA by adding 10.5 ml room temperature isopropanol to the eluted DNA and mix. Centrifuge at 15,000 x g for 30 min at 4°C. Carefully decant the supernatant
- Wash DNA pellet with 5 ml of endotoxin-free room temperature 70% EtOH and centrifuge at 15,000 x g for 10 min. Carefully decant the supernatant without disturbing the pellet
- Air-dry the pellet for 5-10 min and redissolve the DNA in a suitable volume of endotoxin-free Buffer TE

APPENDIX 6.4

LGALS13 Gene Annotation

(5' 1500 bp, 3' 600 bp)

Exons are represented by the shaded yellow areas
5' UTR primers - for amplification of 700 bp fragment
Exon 1 F & R primers
Exon 2 F & R primers
Exon 3.1 F & R primers
Exon 3.2 F & R primers
Exon 4.1 F & R primers
Exon 4.2 F & R primers

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   1 AAGAGAGTAAACAGACAGCCTAAGGAATGGGAGAAAATGTTCTCAAACTATGTATCTGAA   60
  61 GAAGGTCTAATATCCGAGCATTTATAAGAAATTTCAACAACTTCACGAGAAAGAAACAAA   120
 121 CAAACCCATAAAAAAGTGGGTAAAGAGCTTGACACAGTGGCTCATGCCTGTAATCCCAGC   180
 181 GCTTTGGGCAACCAAGGCTGGTGGATCACTTGAGCCCAGGAATTTGAGAACAGGCTGGGT   240
 241 AACATGGCAAAAACCCATCTCTACTAAAAATAGAAAAAAAAAATAGCCTAGCATGGAGAC   300
 301 ATGCACCTGTAGTCCCAGCTACTCAGGAGACTGAGCTTAGAAAAATCACCTGAGCCCCGG   360
 361 AGCCAGAGGTTACAGTGAGCCAAGATGGTGCCACATCACTCCAGCCTGAATGTCAGAGTG   420
 421 AGACCTGTTTTAAAACAACAACAAAAAAGTTGAGCAAAGGACACGAACAGACACTTTTCA   480
 481 AAAGTAAGGATACAGGTGGCCAAAAAACATATAAAAAAGCTCAATATCACTGAACATTAG   540
 541 AGAAATGCAAATCAAAACCACAGTGAGATACCATCTCACACCAGTCAGAATGGCTGTTAT   600
 601 TAAAAAGTCAAAAAAGGACAGATGCTTCAAGGTTGTGGAGAAAAGGGAGAACTCACACAT   660
 661 TGTTGGTGACAGTGTAAATTAGTTCACCCATTGTGGAAAGCAGTGTGGTGATTCCTCAAA   720
 721 GAGCTAACAATAGATCTACCATTCAACCCAGCAATCCCATCACTGGGTGTATACCCAGAA   780
 781 GAAAAGAAATCATTCTGCCATAAAGACATATGCACATGAATGTTCATTGCAACACTATGC   840
 841 ACAATAGCGAAGACACAGAATGAACCCAAATGCCAATCAATGACAGATCGGTTACAGAAA   900
 901 ATGTGGTACATGTACACAATGAAATACTATGCAGTCCTAAAAAAGAATGATATTATGTCT   960
 961 TTTGCAGGAACATGGATGGAACTGGAGGCCATTATTCTTAGCAAACTAATGCAGGAACAG   1020
1021 AAAACCAAATACTGCATGTTCTCACTTATCAGCGTGAACTAAATGAGGAGGCTCATGGAC   1080
1081 ATCAAGAGGGAAACAACACACACTGGGGCCTCCTTGAGGGTGGAGGATGAAAGGTGGAAG   1140
1141 AGGAGAAAAAAAATAACTTTTGAGTACTACACTTAGTAACCAGGTGAAGAAATCATATAC   1200
1201 ACCACAAATCCCATGACACGACTTTATCTGCATATGTACCCCGAACCTAAAATAAAATGA   1260
1261 AAGAAAAATCAAATAAAAACAACAATCTCACCTTCCTCCCAGAATCTGCAGGAGAAACAG   1320
1321 TGTCTCTGAAACTTTTATTTACATGGACACCAAGAGAAAGGGAGGGTGTAGCTAGAGCC   1380
1381 CAGGTGTGTGACAGTCAGGTGAGAGTGGCCCTTACCTGGCAGAGCCAAGCAGCCTAGTGT   1440
1441 GGAATAGGTTCATGCAAGAAATTTCTCTGCTTCTCTCCACAGATCCTCCTGAGGATCAAG   1500
1501 GTCAGTGCTCTCCCAGAACGTTCAAAAGCAGAGAGAATCATTACCTTCTAGGGCTAATGT   1560
1561 ATTTCCCCAAAATCTCCAACTTTTTTCTTTTCAGCCGTTGTACAAATTGGGAAAATGTAC   1620
1621 ACTTTCTGGGTATATTCTGCATAGATGAGAAAAGGCTTGAGAGGCATAAAGGCTGGGCTT   1680
1681 TACAACCACTACCGCTCACTCCATCATCTTTATCTATGCATTTCCCTGGTAACCCAATCC   1740
1741 ACAGCCTGGTAACTCCCCCACTGTCACTTCTGCTGCATTACAGATACAGACCTGCAAATA   1800
1801 GCTATGGTTGTGACTGAGTTTCTTCCTAACAACTAAACCTGTCTAAAGCTGCAAGGAAAT   1860
1861 CTTGCTGGGAGGAGCTTGGAATCTGGAATGAAGCCCGAGGGCAAGGCTGAAGTGGGTCAT   1920
1921 TTAAATGCTGCAACTCAGAGATTCACTCAGAAGACTGGACTCAATTCTGAAGGTCGCCAA   1980
1981 GAAGGAGAGAACAATGTCTTCTTTACCCGTGAGTTGAAAAGGCACAGCCTTCAAAAATTT   2040
2041 CGTGTCACACAAACCAAGAAAGAAATGGGAGATTTTATGAGATGAAAATATGAGCATTTT   2100
2101 TGCTGTGAATGCTTTACTTAGAGCTATTGAGGTGTGGAATAGAAACCCTGAGGCTATGGT   2160
2161 ATCTGAGATGCTTGTGGGGATTGCGGTGTGGACCCTGGACCAGTGTAACCTTCGGTGAGT   2220
2221 GTGAGGTGGTGTCTGATGAGCATAAACTCTGCGGAGATAATGTGACTTTAAGTGGGAGGT   2280
2281 GGATTACCCACCAGGACGAGCAGGTGTGTGAATGGAGAGTAAAGGGGTGAGTCGGCTTTG   2340
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2341 TTTCCTGTCTATGATGTGAGTATGTGTGTGAGCACAGCCAGACCAGTGCATGAGCATCGT 2400
2401 TATGTGCACAGTGAGCAGGTGTGTGTGACGCAGTGAGTGGTGTGGCTCTGTGTGACTGTG 2460
2461 AGTGTGTGTGCTGGGATGTGACTGTATGTTGAAGTGGATCATGCATGTGGCTTTCTTCA 2520
2521 CATGTGAATGTTTCTCCCCACACAGCAGCACCTCTGATGTGAATCCCATGTGGCTTAACT 2580
2581 GGTGGGGATCTTGGGGATTGTGAGTTACCAAGGACTTATTTCAGATCACTTGTGAGAGCA 2640
2641 GAATGTATGATCAGTAGATGTCTCTTCCCAGTAAGAGTGGGGAAGAGGAATGCAGACTAT 2700
2701 GGGGGTGATGGTTCCTCAGGTGGGGTCTGCACAGAGTGACCACTGTCTCTTGCCTCCCAG 2760
2761 GCTCAGCTTGCCTGTCCCTAGCCCATCCAGTTCTTCCCACTGTGTGACAGGAGGGTGTGC 2820
2821 AGATACTGGTTTGTTAGGGAAGGACATTAGAGGCCGTGATTTCAGAGACTGAATCACACA 2880
2881 AAATATCCACTGTAAGCTCTGTCTGAACTGAGACTAAACTTGTTCTACACCCATGAAAGA 2940
2941 ACAAGTTAAGTCTCAGTTCAGAAGGAGCTTACAGTGGATATTAATCAACCAAGCAGCTGG 3000
3001 GTTCACTGGCTTATGGCTATAATCCCACCAATTTTGGAGGCAAAGATGAGAGGATAACGA 3060
3061 GGCCAGAAGTTTGAGACCAGTCTGGGTATCAGAGTGAGACCCTTTCTTTAAAAAACTTTA 3120
3121 AATATTTGACTGGCAGAGAGGCAGGTACCTTTCTTCCCAGCTATTCAAGAGGGAGCAGAT 3180
3181 GTGTGAGATTCACTGCAGCCCAGGAGTCTGAGGTTTCAGTGAACCATGATTGCATCCCTG 3240
3241 CATGTCAGCCTGGGTGACAGAGCAAGAACCTATCTCAAAAGTACAGAAAAATCATCTCAT 3300
3301 CTACTTGTAGTCATCATAGAAATCAATCATTCCCTCCAGTTATGTCCCTGACCCACAAGC 3360
3361 TTCATTTGTGCAAGTACTGGGGCTGTGCTCTCAGTAGTGTGTGCCCCTTCTTGGAAGGAT 3420
3421 GTCCATGGCCCTTGATGATAGTGATGCATGTGCATCCCACACACAGGGGTTTCCTGTTCT 3480
3481 TTCATCATTTCCCTCTCCCTTTCAACAAGTGTTGTTTCTCACTGGAGTGAATGTTTAAAT 3540
3541 TCTTACATTTACAAGTCTTGTTTTAATTTTCAGAGTTGAAAATGAAGGCAGGTCAGTCTT 3600
3601 TCAAGAGGACTCTCCTGTCCTGTTAGGTCACCCTAAGAGGCCCTACGCATGACAGGGATT 3660
3661 AGGGTAACACTTTTACATGTATTAACTCCCTATTACGGAGAACATCCTACAAAGTAGAAA 3720
3721 TTCCTGATAATCCTCGTTGTCCTAGGGAAAATGAGGGAGAGTTCAAGTAGCAAATCTAAG 3780
3781 ACTGCACTGCTAGTAAAAGGCAGAATGAGGACTGGAATCCAGGCATCCTGGCTCCTGAAC 3840
3841 CCTTGCTCTAACTTATGGGTCCGCCATATCTTCAGGAATATGGGGCCCTGAATGCGGTAG 3900
3901 GGTTAAAGAGGAGAGTCCACAGAGTCTGCCCTTTCATCTCCAACCTCCTGCACCATGAGA 3960
3961 ATATGTTACAGGAGGGGAGACTGCACCTGACCCTGCACCTCTCACTTACTCTCAATACTC 4020
4021 TGGCAGGTGCCATACAAACTGCCTGTGTCTTTGTCTGTTGGTTCCTGCGTGATAATCAAA 4080
4081 GGGACACCAATCCACTCTTTTATGTGAGTACTCCATGGTCCAATGGAGGGGTTGGAGAAG 4140
4141 AAGGGAGAATATTTGCGAAGATTTGACCTTACATGTGGGTGATGTGGAAATGTCTAGTTG 4200
4201 GCAGAATGGAGGCCCCATGCAGGTGCAGGTCTTGGAGACCCTCCAGCACCAGGCATGAGA 4260
4261 CCCACAGCAACTGCATGTGGGCCAGCCATGGGGCCTACAGGAGGAGAACACTCAGTGGGT 4320
4321 TGGGGCTGTGGCTCTTTATCAGGGGGCATAGAATTTTCGGGAATGAACAAGTCATAGGCC 4380
4381 CAGGTCAGTGACTGTGGCTCAGTTTTCATCTGGGGATGAGGAGCACAGAATCTCCCTGCC 4440
4441 TGGGGGCATGAGGAGCTGAAGCATCCCCACAGGGACCTGGCCATCAGTATTATCTGGGAG 4500
4501 ACTTTTTCCCTAGGTAAATGGGGGAAGGGATTTGTGTGTGTCGAGTGTGTGTCTGCGC 4560
4561 AAGGGAGGGACCTGCCCAACATTCTGCGTGCTTCACCCTCAGCAATGACCCACAGCTGCA 4620
4621 GGTGGATTTCTACACTGACATGGATGAGGATTCAGATATTGCCTTCCGTTTCCGAGTGCA 4680
4681 CTTTGGCAATCATGTGGTCATGAACAGGCGTGAGTTTGGGATATGGATGTTGGAGGAGAC 4740
4741 AACAGACTACGTGCCCTTTGAGGATGGCAAACAATTTGAGCTGTGCATCTACGTACATTA 4800
4801 CAATGAGTATGAGGTGAGCATCCCAGGAGCTCCCAGCACCCAGGCTCTGTGGGCTCCCAA 4860
4861 AACAGGAGGCAGCTCTCATTGATCTGGCCTCAGTAGTCCGTCAGGGTCCATCTCCCATAA 4920
4921 CTACTCCTGCCCCTGGTTTTCATCACAGAGCACCCCCTGCTTGCACTGCCATCCTCAGCT 4980
4981 CTTTCCCAAATCTGACCAATGTCAAGGTCAGCTCACCTGACATTTCCCCCAAAGTAGATA 5040
5041 ATCTCCTCTGTCTTTTCACATACTGCTCATTTCTACTTATGCCATTATTTAAATTTTCAT 5100
5101 TTAGCTGAGTGAATACAATATACTTCAGGAAAAAAAGTTTAAAAAAAAAAGGTTGTTTT 5160
5161 AATCAGTCACAAATTAAGTCTTTGTCTCATTTTGGGGCCCGAATTCTACCATAGGAAAAA 5220
5221 CATTACCAGAGCTCTGCTTTCATTCAAAGAGGTTTTGTTTGTTTGTTTGTTTTTGTTTGT 5280
5281 TTGTTTTCTTGAGATAGAGTCTCATTTTTCATCCAGGTTGGAGTGCAGTGGCATGATATC 5340
5341 AGCTCACTGCAACCTCCACCTCCTGGATTCAAACAATTCTCTCGCCTCAGCCTCCCAAGT 5400
5401 AGCTGGGACTACAGGTGTGTGCCAGCACACCTGGCTAATTTTTGTATTTTTAGGGGAGAC 5460
5461 GGGGGTTCACCACTTTGGCCAGGCTGGTCTTGAACTCCCCACCTCAAGTTATCTGCCCAA 5520
5521 TTTGGCCTCTCAATATAATGGGATTACAGGCTTAAGCCACCATGCCCAGCCCTTCAGAGG 5580
5581 AAATTTTTGCTTATGGAAGCATATAGATATGTCTATGATTTCATTTGTTTTTAAGCAAAT 5640
5641 CCTGGTATAAGTTGCTCTACACATTACGCTTCAAGAACTAAGTTGATTTGTTGCCAAAGA 5700
5701 ATATATATTGAAGACATTATCAATCTGTCGTGCTGCTTCAGTTTTTAAAGATTGCATAAT 5760
5761 ATTGTGTTAAGAAGTTTACCCAAAGTTGTGGAACTTGGCCCTTTTGGGGGCACTAAATTT 5820
5821 CCTTCCAGTATTTTGCTCTCACAAATGATGTCGTATTAAGTTTTCTAATCAATCCTTTTG 5880
5881 TCATTACCAACCTCCCAATACTTGTACTAGAAGATAATTACAAAAAAATGGAAAACTGG 5940
5941 CTCAAAAATGTGTGCATTTTAAATTCGAAAAATTAATACTAATAAGGTTACTTTCTCTAT 6000
6001 AGCCTCAATATAAAGAATACATCCATACTTTCTGGCTTTCTCTAACGAGTAGGCCAAAAC 6060
6061 ATTCACATCTTATTCAGATTCAAGCGAGGGATCTTTTCCTATGCTTAGAAGTTTTTGAAG 6120
6121 GTAATATCCTGTTTCACAAGAACAGTTCTCCTTTCATTCTATAAGAACCCTGGGTTTCCT 6180
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130

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6181 TCTTTGCCACTAACACCTCAGATGTAGGTTACTCCAAAGAGAAACTGTGCCATCAGTAGT  6240
6241 GAAAAACAATCACAGTTCATGGAACTGAAAAGTATGCATTCAACGAACATGGTCTAGCAC  6300
6301 TAACCCTGTGGCAGGTCCTGTGCGAGATGCAGGGTCTCAAGTTCCTGAGACACAGTCCCT  6360
6361 GGCGATGGGGATCTTCCAGGTTAGAAGGGAGGCTGAGTAAGCAAAGACTTTGTGACACAG  6420
6421 AGTATAGAACTTCGCTAGAGGAATGAGTGGAAACATTAGAAATAAAGTCGGGTACTGTCT  6480
6481 CAAATAGGCACAAAACGTCATCTGTAAACATAAGTGTATCTAATACGTTAACTTGTATAA  6540
6541 CTAGGAATTTTCTTGGGGAATGTTATTTGTACCAGGACAGAGTGGAGAGGAGGCCGAAAA  6600
6601 CTTGTTTGGTGGCATGCTTTCTTTCTGATGCATTTTTCCTCTTGTAGATAAAGGTCAATG  6660
6661 GCATACGCATTTACGGCTTTGTCCATCGAATCCCGCCATCATTTGTGAAGATGGTGCAAG  6720
6721 TGTCGAGAGATATCTCCCTGACCTCAGTGTGTGTCTGCAATTGAGGGAGATGATCACACT  6780
6781 CCTCATTGTTGAGGAATCCCTCTTTCTACCTGACCATGGGATTCCCAGAACCTGCTAACA  6840
6841 GAATAATCCCTGCTCACATTTTCCCCTACACTTTGTCATTAAAACAGCACGAAAACTCAC  6900
6901 ATGATTTGGTTCTTGCTTTCAGAGGGGAAAAGAGGAAGTTGTCATCCCCAAGGGGGGCCA  6960
6961 GGGCATTCTATGGGAGGCATCAGGAAATCAAAGGGGATAAACCTTCCTGTGACAAAGGGA  7020
7021 GTGAGTGACAAGGTCCGTGGAATGTCTGAGAAGACATTAGAAACAGCATCCTTCTATAGC  7080
7081 ACGTAGTTGACCTCAGACAGTCTGAGCTAAAATACTTACCCAATCTCAAATGACTCAAGG  7140
7141 CACTTAAATGTTAGGTAGCTGTTTACAGCCACGCAAGTGTACATGGTGGCTTTGGGGAAA  7200
7201 ATCTGTAACTCGAGGAAAATTATGTGGAATTTCTTAATTTTTTTCCATTGTTCTCAGGAT  7260
7261 GCTTACTGTGTATGAACACTGTGCCCGGCTTTGTGCGGGCTGCTGTGAAGGAACTGAAGA  7320
7321 AAAAGATGACAAGTTTGCATGTGAAGTTTTCAATCCAAGTAATAGTTTAGAGAATCACTG  7380
7381 GAGCCCAGTGATTTCCCTTAAAGAATCCCAATTATTCTTATTTTCAAATATTTGAAATT   7440
7441 GCTTCATTTTATGTGGGGAGAATCCAGGGGTTTCTCTTTGGAAATGAATGGTCATGAT    7498
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APPENDIX 6.5

Vector Maps and Sequences

pGL4.23 [luc2/minP] vector map and sequence
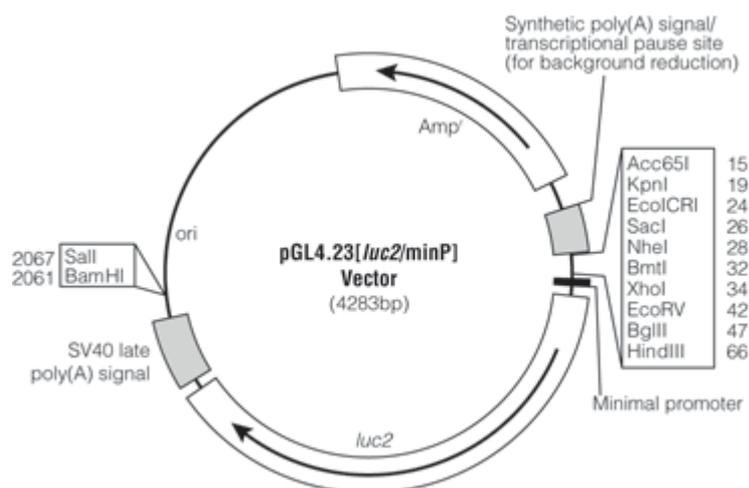

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pGL4.23[luc2/minP] Vector sequence reference points:

Base pairs                                               4283
Multiple cloning region                                  1-70
Minimal promoter                                         78-108
luc2 reporter gene                                       141-1793
SV40 late poly(A) signal                                 1828-2049
Reporter Vector primer 4 (RVprimer4) binding region      2117-2136
ColE1-derived plasmid replication origin                 2374
Synthetic Beta-lactamase (Ampr) coding region            3165-4025
Synthetic poly(A) signal/transcriptional pause site      4130-4283
Reporter Vector primer 3 (RVprimer3) binding region      4232-4251
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   1  GGCCTAACTG GCCGGTACCT GAGCTCGCTA GCCTCGAGGA TATCAAGATC
  51  TGGCCTCGGC GGCCAAGCTT AGACACTAGA GGGTATATAA TGGAAGCTCG
 101  ACTTCCAGCT TGGCAATCCG GTACTGTTGG TAAAGCCACC ATGGAAGATG
 151  CCAAAAACAT TAAGAAGGGC CCAGCGCCAT TCTACCCACT CGAAGACGGG
 201  ACCGCCGGCG AGCAGCTGCA CAAAGCCATG AAGCGCTACG CCCTGGTGCC
 251  CGGCACCATC GCCTTTACCG ACGCACATAT CGAGGTGGAC ATTACCTACG
 301  CCGAGTACTT CGAGATGAGC GTTCGGCTGG CAGAAGCTAT GAAGCGCTAT
 351  GGGCTGAATA CAAACCATCG GATCGTGGTG TGCAGCGAGA ATAGCTTGCA
 401  GTTCTTCATG CCCGTGTTGG GTGCCCTGTT CATCGGTGTG GCTGTGGCCC
 451  CAGCTAACGA CATCTACAAC GAGCGCGAGC TGCTGAACAG CATGGGCATC
 501  AGCCAGCCCA CCGTCGTATT CGTGAGCAAG AAAGGGCTGC AAAAGATCCT
 551  CAACGTGCAA AAGAAGCTAC CGATCATACA AAAGATCATC ATCATGGATA
 601  GCAAGACCGA CTACCAGGGC TTCCAAAGCA TGTACACCTT CGTGACTTCC
 651  CATTTGCCAC CCGGCTTCAA CGAGTACGAC TTCGTGCCCG AGAGCTTCGA
 701  CCGGGACAAA ACCATCGCCC TGATCATGAA CAGTAGTGGC AGTACCGGAT
 751  TGCCCAAGGG CGTAGCCCTA CCGCACCGCA CCGCTTGTGT CCGATTCAGT
 801  CATGCCCGCG ACCCCATCTT CGGCAACCAG ATCATCCCCG ACACCGCTAT
 851  CCTCAGCGTG GTGCCATTTC ACCACGGCTT CGGCATGTTC ACCACGCTGG
 901  GCTACTTGAT CTGCGGCTTT CGGGTCGTGC TCATGTACCG CTTCGAGGAG
 951  GAGCTATTCT TGCGCAGCTT GCAAGACTAT AAGATTCAAT CTGCCCTGCT
1001  GGTGCCCACA CTATTTAGCT TCTTCGCTAA GAGCACTCTC ATCGACAAGT
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132

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1051   ACGACCTAAG CAACTTGCAC GAGATCGCCA GCGGCGGGGC GCCGCTCAGC
1101   AAGGAGGTAG GTGAGGCCGT GGCCAAACGC TTCCACCTAC CAGGCATCCG
1151   CCAGGGCTAC GGCCTGACAG AAACAACCAG CGCCATTCTG ATCACCCCCG
1201   AAGGGGACGA CAAGCCTGGC GCAGTAGGCA AGGTGGTGCC CTTCTTCGAG
1251   GCTAAGGTGG TGGACTTGGA CACCGGTAAG ACACTGGGTG TGAACCAGCG
1301   CGGCGAGCTG TGCGTCCGTG GCCCCATGAT CATGAGCGGC TACGTTAACA
1351   ACCCCGAGGC TACAAACGCT CTCATCGACA AGGACGGCTG GCTGCACAGC
1401   GGCGACATCG CCTACTGGGA CGAGGACGAG CACTTCTTCA TCGTGGACCG
1451   GCTGAAGAGC CTGATCAAAT ACAAGGGCTA CCAGGTAGCC CCAGCCGAAC
1501   TGGAGAGCAT CCTGCTGCAA CACCCCAACA TCTTCGACGC CGGGGGTCGCC
1551   GGCCTGCCCG ACGACGATGC CGGCGAGCTG CCCGCCGCAG TCGTCGTGCT
1601   GGAACACGGT AAAACCATGA CCGAGAAGGA GATCGTGGAC TATGTGGCCA
1651   GCCAGGTTAC AACCGCCAAG AAGCTGCGCG GTGGTGTTGT GTTCGTGGAC
1701   GAGGTGCCTA AAGGACTGAC CGGCAAGTTG GACGCCCGCA AGATCCGCGA
1751   GATTCTCATT AAGGCCAAGA AGGGCGGCAA GATCGCCGTG TAATAATTCT
1801   AGAGTCGGGG CGGCCGGCCG CTTCGAGCAG ACATGATAAG ATACATTGAT
1851   GAGTTTGGAC AAACCACAAC TAGAATGCAG TGAAAAAAAT GCTTTATTTG
1901   TGAAATTTGT GATGCTATTG CTTTATTTGT AACCATTATA AGCTGCAATA
1951   AACAAGTTAA CAACAACAAT TGCATTCATT TTATGTTTCA GGTTCAGGGG
2001   GAGGTGTGGG AGGTTTTTTA AAGCAAGTAA AACCTCTACA AATGTGGTAA
2051   AATCGATAAG GATCCGTCGA CCGATGCCCT TGAGAGCCTT CAACCCAGTC
2101   AGCTCCTTCC GGTGGGCGCG GGGCATGACT ATCGTCGCCG CACTTATGAC
2151   TGTCTTCTTT ATCATGCAAC TCGTAGGACA GGTGCCGGCA GCGCTCTTCC
2201   GCTTCCTCGC TCACTGACTC GCTGCGCTCG GTCGTTCGGC TGCGGCGAGC
2251   GGTATCAGCT CACTCAAAGG CGGTAATACG GTTATCCACA GAATCAGGGG
2301   ATAACGCAGG AAAGAACATG TGAGCAAAAG GCCAGCAAAA GGCCAGGAAC
2351   CGTAAAAAGG CCGCGTTGCT GGCGTTTTTC CATAGGCTCC GCCCCCCTGA
2401   CGAGCATCAC AAAAATCGAC GCTCAAGTCA GAGGTGGCGA AACCCGACAG
2451   GACTATAAAG ATACCAGGCG TTTCCCCCTG GAAGCTCCCT CGTGCGCTCT
2501   CCTGTTCCGA CCCTGCCGCT TACCGGATAC CTGTCCGCCT TTCTCCCTTC
2551   GGGAAGCGTG GCGCTTTCTC ATAGCTCACG CTGTAGGTAT CTCAGTTCGG
2601   TGTAGGTCGT TCGCTCCAAG CTGGGCTGTG TGCACGAACC CCCCGTTCAG
2651   CCCGACCGCT GCGCCTTATC CGGTAACTAT CGTCTTGAGT CCAACCCGGT
2701   AAGACACGAC TTATCGCCAC TGGCAGCAGC CACTGGTAAC AGGATTAGCA
2751   GAGCGAGGTA TGTAGGCGGT GCTACAGAGT TCTTGAAGTG GTGGCCTAAC
2801   TACGGCTACA CTAGAAGAAC AGTATTTGGT ATCTGCGCTC TGCTGAAGCC
2851   AGTTACCTTC GGAAAAAGAG TTGGTAGCTC TTGATCCGGC AAACAAACCA
2901   CCGCTGGTAG CGGTGGTTTT TTTGTTTGCA AGCAGCAGAT TACGCGCAGA
2951   AAAAAAGGAT CTCAAGAAGA TCCTTTGATC TTTTCTACGG GGTCTGACGC
3001   TCAGTGGAAC GAAAACTCAC GTTAAGGGAT TTTGGTCATG AGATTATCAA
3051   AAAGGATCTT CACCTAGATC CTTTTAAATT AAAAATGAAG TTTTAAATCA
3101   ATCTAAAGTA TATATGAGTA AACTTGGTCT GACAGCGGCC GCAAATGCTA
3151   AACCACTGCA GTGGTTACCA GTGCTTGATC AGTGAGGCAC CGATCTCAGC
3201   GATCTGCCTA TTTCGTTCGT CCATAGTGGC CTGACTCCCC GTCGTGTAGA
3251   TCACTACGAT TCGTGAGGGC TTACCATCAG GCCCCAGCGC AGCAATGATG
3301   CCGCGAGAGC CGCGTTCACC GGCCCCCGAT TTGTCAGCAA TGAACCAGCC
3351   AGCAGGGAGG GCCGAGCGAA GAAGTGGTCC TGCTACTTTG TCCGCCTCCA
3401   TCCAGTCTAT GAGCTGCTGT CGTGATGCTA GAGTAAGAAG TTCGCCAGTG
3451   AGTAGTTTCC GAAGAGTTGT GGCCATTGCT ACTGGCATCG TGGTATCACG
3501   CTCGTCGTTC GGTATGGCTT CGTTCAACTC TGGTTCCCAG CGGTCAAGCC
3551   GGGTCACATG ATCACCCATA TTATGAAGAA ATGCAGTCAG CTCCTTAGGG
3601   CCTCCGATCG TTGTCAGAAG TAAGTTGGCC GCGGTGTTGT CGCTCATGGT
3651   AATGGCAGCA CTACACAATT CTCTTACCGT CATGCCATCC GTAAGATGCT
3701   TTTCCGTGAC CGGCGAGTAC TCAACCAAGT CGTTTTGTGA GTAGTGTATA
3751   CGGCGACCAA GCTGCTCTTG CCCGGCGTCT ATACGGGACA CACCGCGCC
3801   ACATAGCAGT ACTTTGAAAG TGCTCATCAT CGGGAATCGT TCTTCGGGGC
3851   GGAAAGACTC AAGGATCTTG CCGCTATTGA GATCCAGTTC GATATAGCCC
3901   ACTCTTGCAC CCAGTTGATC TTCAGCATCT TTTACTTTCA CCAGCGTTTC
3951   GGGGTGTGCA AAAACAGGCA AGCAAAATGC CGCAAAGAAG GGAATGAGTG
4001   CGACACGAAA ATGTTGGATG CTCATACTCG TCCTTTTTCA ATATTATTGA
4051   AGCATTTATC AGGGTTACTA GTACGTCTCT CAAGGATAAG TAAGTAATAT
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4101   TAAGGTACGG GAGGTATTGG ACAGGCCGCA ATAAAATATC TTTATTTTCA
4151   TTACATCTGT GTGTTGGTTT TTTGTGTGAA TCGATAGTAC TAACATACGC
4201   TCTCCATCAA AACAAAACGA AACAAAACAA ACTAGCAAAA TAGGCTGTCC
4251   CCAGTGCAAG TGCAGGTGCC AGAACATTTC TCT
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pGL4.73 [hRluc2/SV40] vector map and sequence
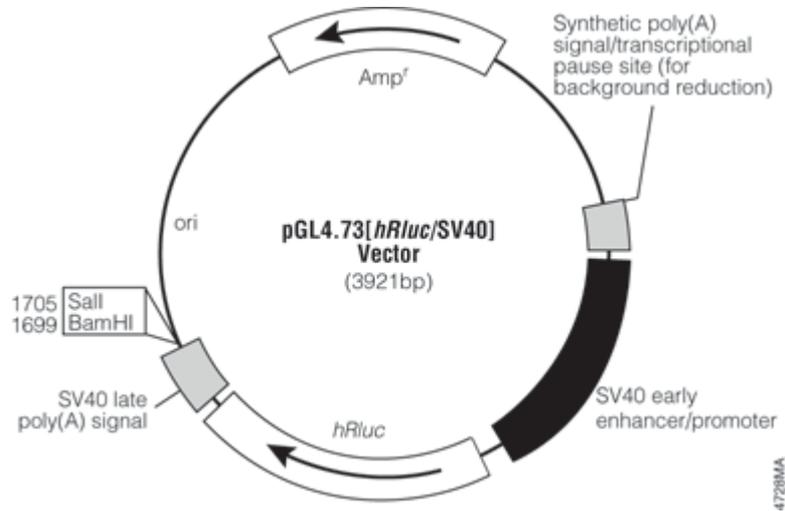

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pGL4.73[hRluc/SV40] Vector sequence reference points:

Base pairs                                               3921
SV40 early enhancer/promoter                             51-469
hRluc reporter gene                                      499-1434
SV40 late poly(A) region                                 1466-1687
Reporter Vector primer 4 (RVprimer4) binding region      1755-1774
ColE1-derived plasmid replication origin                 2012
Synthetic beta-lactamase (Ampr) coding region            2803-3663
Synthetic poly(A) signal/transcriptional pause region    3768-3921
Reporter Vector primer 3 (RVprimer3) binding region      3870-3889
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  51   GCGCAGCACC ATGGCCTGAA ATAACCTCTG AAAGAGGAAC TTGGTTAGGT
 101   ACCTTCTGAG GCGGAAAGAA CCAGCTGTGG AATGTGTGTC AGTTAGGGTG
 151   TGGAAAGTCC CCAGGCTCCC CAGCAGGCAG AAGTATGCAA AGCATGCATC
 201   TCAATTAGTC AGCAACCAGG TGTGGAAAGT CCCCAGGCTC CCCAGCAGGC
 251   AGAAGTATGC AAAGCATGCA TCTCAATTAG TCAGCAACCA TAGTCCCGCC
 301   CCTAACTCCG CCCATCCCGC CCCTAACTCC GCCCAGTTCC GCCCATTCTC
 351   CGCCCCATGG CTGACTAATT TTTTTTATTT ATGCAGAGGC CGAGGCCGCC
 401   TCGGCCTCTG AGCTATTCCA GAAGTAGTGA GGAGGCTTTT TTGGAGGCCT
 451   AGGCTTTTGC AAAAAGCTTG GCAATCCGGT ACTGTTGGTA AAGCCACCAT
 501   GGCTTCCAAG GTGTACGACC CCGAGCAACG CAAACGCATG ATCACTGGGC
 551   CTCAGTGGTG GGCTCGCTGC AAGCAAATGA ACGTGCTGGA CTCCTTCATC
 601   AACTACTATG ATTCCGAGAA GCACGCCGAG AACGCCGTGA TTTTTCTGCA
 651   TGGTAACGCT GCCTCCAGCT ACCTGTGGAG GCACGTCGTG CCTCACATCG
 701   AGCCCGTGGC TAGATGCATC ATCCCTGATC TGATCGGAAT GGGTAAGTCC
 751   GGCAAGAGCG GGAATGGCTC ATATCGCCTC CTGGATCACT ACAAGTACCT
 801   CACCGCTTGG TTCGAGCTGC TGAACCTTCC AAAGAAAATC ATCTTTGTGG
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1001   GCGAAGAGGG CGAGAAAATG GTGCTTGAGA ATAACTTCTT CGTCGAGACC
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1301    AGGGAGCTAA GAAGTTCCCT AACACCGAGT TCGTGAAGGT GAAGGGCCTC
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1451    GCCGGCCGCT TCGAGCAGAC ATGATAAGAT ACATTGATGA GTTTGGACAA
1501    ACCACAACTA GAATGCAGTG AAAAAAATGC TTTATTTGTG AAATTTGTGA
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1701    TCCGTCGACC GATGCCCTTG AGAGCCTTCA ACCCAGTCAG CTCCTTCCGG
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1901    CTCAAAGGCG GTAATACGGT TATCCACAGA ATCAGGGGAT AACGCAGGAA
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3001    CGAGCGAAGA AGTGGTCCTG CTACTTTGTC CGCCTCCATC CAGTCTATGA
3051    GCTGCTGTCG TGATGCTAGA GTAAGAAGTT CGCCAGTGAG TAGTTTCCGA
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3151    TATGGCTTCG TTCAACTCTG GTTCCCAGCG GTCAAGCCGG GTCACATGAT
3201    CACCCATATT ATGAAGAAAT GCAGTCAGCT CCTTAGGGCC TCCGATCGTT
3251    GTCAGAAGTA AGTTGGCCGC GGTGTTGTCG CTCATGGTAA TGGCAGCACT
3301    ACACAATTCT CTTACCGTCA TGCCATCCGT AAGATGCTTT TCCGTGACCG
3351    GCGAGTACTC AACCAAGTCG TTTTGTGAGT AGTGTATACG GCGACCAAGC
3401    TGCTCTTGCC CGGCGTCTAT ACGGGACAAC ACCGCGCCAC ATAGCAGTAC
3451    TTTGAAAGTG CTCATCATCG GGAATCGTTC TTCGGGGCGG AAAGACTCAA
3501    GGATCTTGCC GCTATTGAGA TCCAGTTCGA TATAGCCCAC TCTTGCACCC
3551    AGTTGATCTT CAGCATCTTT TACTTTCACC AGCGTTTCGG GGTGTGCAAA
3601    AACAGGCAAG CAAAATGCCG CAAAGAAGGG AATGAGTGCG ACACGAAAAT
3651    GTTGGATGCT CATACTCGTC CTTTTTCAAT ATTATTGAAG CATTTATCAG
3701    GGTTACTAGT ACGTCTCTCA AGGATAAGTA AGTAATATTA AGGTACGGGA
3751    GGTATTGGAC AGGCCGCAAT AAAATATCTT TATTTTCATT ACATCTGTGT
3801    GTTGGTTTTT TGTGTGAATC GATAGTACTA ACATACGCTC TCCATCAAAA
3851    CAAAACGAAA CAAAACAAAC TAGCAAAATA GGCTGTCCCC AGTGCAAGTG
3901    CAGGTGCCAG AACATTTCTC T
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APPENDIX 6.5

Project Approval and Informed Consent Forms

UNIVERSITEIT·STELLENBOSCH·UNIVERSITY
jou kennisvennoot · your knowledge partner

6 December 2005

Dr R Hillermann
Dept of Genetics
Stellenbosch Campus

Dear Dr Hillermann

RESEARCH PROJECT: "PROFILING PLACENTAL PROTEIN 13/GALECTIN-13 IN PRE-ECLAMPSIA"
PROJECT NUMBER : N05/07/122

At a meeting of the Committee for Human Research that was held on 3 August 2005 the above project was approved on condition that further information that was required, be submitted.

This information was supplied and the project was finally approved on 5 December 2005 **for a period of one year from this date**. This project is therefore now registered and you can proceed with the work. Please quote the above-mentioned project number in all further correspondence.

Please note that a progress report (obtainable on the website of our Division) should be submitted to the Committee before the year has expired. The Committee will then consider the continuation of the project for a further year (if necessary).

Patients participating in a research project in Tygerberg Hospital will not be treated free of charge as the Provincial Government of the Western Cape does not support research financially.

Due to heavy workload the nursing corps of the Tygerberg Hospital cannot offer comprehensive nursing care in research projects. It may therefore be expected of a research worker to arrange for private nursing care.

Yours faithfully

CJ VAN TONDER
RESEARCH DEVELOPMENT AND SUPPORT (TYGERBERG)
Tel: +27 21 938 9207 / E-mail: cjvt@sun.ac.za

CJVT/ev

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Fakulteit Gesondheidswetenskappe · Faculty of Health Sciences

Verbind tot Optimale Gesondheid · Committed to Optimal Health
Afdeling Navorsingsontwikkeling en -steun · Research Development and Support Division
Posbus/PO Box 19063 · Tygerberg 7505 · Suid-Afrika/South Africa
Tel: +27 21 938 9677 · Faks/Fax: +27 21 931 3352

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INFORMATION AND INFORMED CONSENT DOCUMENT FOR DNA ANALYSIS AND STORAGE

TITLE OF THE RESEARCH PROJECT: *Profiling placental protein 13 / galectin-13 in pre-eclampsia.*

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: ……….……………]   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: Pre-eclampsia (high blood pressure in pregnancy) is a severe complication of pregnancy and it affects about 5% of all pregnancies. Currently there is no way to predict who will be affected. We are investigating a protein normally secreted by the placenta (the afterbirth) as it seems that this protein may predict the development of later problems.

2.1   **Procedures:**   I will be requested to provide information about my medical history.   Blood (10*ml*, about 2 teaspoonfuls) will be collected from me at delivery of my baby together with the routine blood samples taken at birth. In addition 6 small biopsies (pea-size) will be taken from the afterbirth (the placenta) after delivery and before the placenta is routinely destroyed.

*Delete where not applicable

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(s) 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 to have it and/*or the stored clinical data destroyed by contacting the investigator conducting the present study, dr GS Gebhardt at 938 9131 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.

2.5 **Voluntary participation: Participation** is voluntary and I may decline participation, or withdraw from the study at any time without any loss of

*Delete where not applicable

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;

- Biopsies of the placenta is not painful as it is taken after delivery before routine destruction of the placenta

- 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 material is used in further projects in the future, the written approval of the Research Subcommittee C/Ethics Committee, Faculty of Health Sciences, will be obtained.

3. The information conveyed above was explained to me by ……..……..………………………………….…………………….………..
(name) in English 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/*has 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

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

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

………………………………………………...…… ………

Signature or right thumb print of   …………………………………………
*participant/*representative of participant*   *Signature of witness*

DECLARATION BY OR ON BEHALF OF INVESTIGATOR(S):

I, …………………………………………………………………………….…*(name)*

declare that

- I explained the information in this document to ……………………………………. *(name of the patient/*participant)* and/or his/her representative …………………….………… *(name of the representative)*;

- she/*he was encouraged and afforded adequate time to ask me any questions;

- this conversation was conducted in Afrikaans/*English/*Xhosa/*Other …………….…………….. and no translator was used/*was translated into ……………….……………*(language)* by …………………….…………...……………….…… *(name).*

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

……………………………………………………………

……………………………………
…………

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

………………………………………………………

……………………………………
…….

              Signature *of translator*   *Signature of witness*

IMPORTANT MESSAGE TO PARTICIPANT/*REPRESENTATIVE OF PARTICIPANT:

Dear participant/*representative of 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 938-0131, 938-4707 (after hours) or 87 21 711 (all hours) or come to the labour ward on the second floor, Tygerberg Hospital.

*Delete where not applicable

APPENDIX 6.6

Research Outputs

<u>M Bosman</u>, N Bruiners, S Gebhardt, G Rebello and R Hillermann.Do LGALS*13* gene sequence variants impact on the risk of abruptio placentae ? *12th South African Society of Human Genetics (SASHG) Meeting*, Golden Gate, South Africa. March 1-3, 2007.

Bruiners N, <u>Bosman M</u>, Postma A, Gebhardt S, Rebello G, Sammar M, Meiri H, Hillermann R. Promoter variant -98A/C of the *LGALS13* gene and pre-eclampsia. *8th World Congress of Perinatal Medicine*, Florence, Italy. Sept 8-11, 2007.

<u>M Bosman</u>, G Rebello, M Sammar, H Meiri, S Gebhardt, R Hillermann.
An African origin for the PP13 truncation mutation 221delT ? *International Society for the Study of Hypertension in pregnancy (ISSHP) conference*, Washington, USA. 2-7 September 2008.

G Rebello, <u>M Bosman</u>, A Postma, N Bruiners, M Sammar, H Meiri, S Gebhardt, R Hillermann. A Genetic Change In *LGALS13 s*upports the Predictive Role Of PP13 In Pregnancy Outcome. *International Federation of Placental Associations (IFPA) conference*, Graz, Austria. September 11-14, 2008.

<u>M Bosman</u>, GS Gebhardt, H Meiri, S Marai, R Hillermann. Sequence variants in the *LGALS13* gene: a role in abruptio placentae? 13th *South African Society of Human Genetics (SASHG) Meeting*, Stellenbosch, South Africa. April 3-6, 2009 (Accepted).