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The protein expression of the nicotinic acetylcholine receptors in the human placenta, and effects of cigarette smoking and pre-eclampsia.

EMMA GHAZAVI
BMedSc (Hons)

A thesis submitted for the degree of Masters of Medical Science
In Medicine

Central Clinical School of Medicine
The University of Sydney
NSW Australia

February 2014
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Declaration

This thesis is submitted in fulfillment of the requirements of the degree of Masters of Philosophy at the University of Sydney Medical School, Central Clinical School within the Faculty of Medicine.

I hereby declare that this thesis was completed solely on my own, with guidance and supervision from Dr Rita Machaalani and Dr Tina Hinton and support from the Bosch Facility, University of Sydney as well as the Electron Microscopy Unit, University of Sydney.

The presented material has not, to the best of my knowledge, previously been published or written by another individual, unless it has been fully cited and referenced, and has not been submitted for a higher degree in any university.

Emma Ghazavi
Abstract

Smoking during pregnancy is associated with low birth weight, premature delivery, neonatal morbidity and mortality. Nicotine is a major pathogenic compound of cigarette smoke, and binds to the nicotinic acetylcholine receptors (nAChR). To date, a total of 16 nAChR subunits have been identified in mammals including 9 α subunits, 4 β subunits and 1 δ, ε and γ subunit. The effect of smoking on these subunits in the human placenta has not yet been determined. Smoking is also associated with a reduced pre-eclampsia (PE) risk and its protective effects may occur via changes in nAChRs.

Using western blot and immunohistochemistry (IHC) experimentation, this study investigated nine of the sixteen mammalian nAChR subunits (α2, α3, α4, α5, α7, α9, β1, β2 and δ) in the normal healthy human placenta, and compared protein expression of placentas from smokers (n=8), PE (n=8) and controls (n=8).

The western blot data from this study showed that when compared to the control group, α9 and δ were increased in both the smoker and PE groups. An increase in α7 was additionally seen in the PE group. Consistent with the western blot findings, a change in α7, α9 and δ subunit expression was observed at the cellular level using IHC, with additional, changes for α2 and β1 subunit. Specifically, for α7, the percentage of positive endothelial cells per villous was decreased in the smokers compared to both control and PE groups. For α9, a decrease was observed for the percentage of villi with positive cells in PE compared to controls, while a lower percentage of endothelial cells per villous in PE was evident compared to smokers. δ subunit expression was increased in the decidual cells of smokers compared to controls. For α2, the percentage of positive endothelial cells per villous and the villous cells was greater in the smoker group compared to the PE group. Finally, β1 was increased in villous and decidual cells of smokers compared to control and PE.

Therefore, both exogenous regulation of placental nAChRs by cigarette smoke exposure and endogenous regulation, via ACh, are likely to alter nAChR subunit expression, and ultimately function, in the human placenta, impacting on infant outcomes. When comparing the nAChR subunits between smoking and PE groups, the possibility of a protective mechanism becomes an apparent possibility whereby alterations in certain subunits as a result of PE, can be counteracted by either exogenously agonizing or antagonizing the same subunits to reverse the effects.
Acknowledgements

This year, as a masters student, has been arduous and challenging yet a satisfying and enriching experience. Without the help and support of an overwhelming number of people it would have been far less enjoyable and fruitful. I would like to take the opportunity to thank all those people that assisted me and made this year possible.

Firstly, I would like to acknowledge my supervisor Dr. Rita Machaalani who has been a source of strength and persistence. Her calm, collected nature has grounded me during more challenging times and her wise words and resilience have inspired me to persevere.

I would also like to acknowledge Dr. Tina Hinton who has continually provided me with insight and always offered an alternative perspective during conflicting times. Her warm, friendly nature has provided me with comfort and the much needed support to encourage me to be resilient.

Importantly, I would like to thank Mu Yao and Moumita Paul who I was extremely lucky to meet through the Bosch Facility. Both Mu and Moumita were overwhelmingly generous with their time, advice, resources and efforts to help make my year of research possible and enjoyable.

I would like to acknowledge two research students I have worked closely with over the last year; Nicholas Hunt and Man Kuen Du. I thank Nick for his continual support and the positive energy he brings to our work environment. I thank Maggie for being selfless with her time and very kindly helping me manage my time and experiments.
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<th>Full Form</th>
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</thead>
<tbody>
<tr>
<td>Acetylcholine</td>
<td>(ACh)</td>
</tr>
<tr>
<td>Ammonium Persulfate</td>
<td>(APS)</td>
</tr>
<tr>
<td>Avidin-biotin complex</td>
<td>(ABC)</td>
</tr>
<tr>
<td>Calcium</td>
<td>(Ca²⁺)</td>
</tr>
<tr>
<td>Central nervous system</td>
<td>(CNS)</td>
</tr>
<tr>
<td>Decidua cells</td>
<td>(DC)</td>
</tr>
<tr>
<td>Diaminobenzidine</td>
<td>(DAB)</td>
</tr>
<tr>
<td>Endothelial cells</td>
<td>(EC)</td>
</tr>
<tr>
<td>Enhanced chemiluminescence</td>
<td>(ECL)</td>
</tr>
<tr>
<td>Ethylenediaminetetraacetic acid</td>
<td>(EDTA)</td>
</tr>
<tr>
<td>Formalin fixed and paraffin embedded</td>
<td>(FFPE)</td>
</tr>
<tr>
<td>Haeme oxygenase-2</td>
<td>(HO-2)</td>
</tr>
<tr>
<td>Heme oxygenase 1</td>
<td>(HO-1)</td>
</tr>
<tr>
<td>Histocompatibility antigen class 1 G</td>
<td>(HLA-G)</td>
</tr>
<tr>
<td>Human placental lactogen</td>
<td>(hPL)</td>
</tr>
<tr>
<td>Immunohistochemistry</td>
<td>(IHC)</td>
</tr>
<tr>
<td>Interleukin 6</td>
<td>(IL-6)</td>
</tr>
<tr>
<td>Intra uterine growth restriction</td>
<td>(IUGR)</td>
</tr>
<tr>
<td>Natural killer cells</td>
<td>(NK)</td>
</tr>
<tr>
<td>Nicotinic acetylcholine receptors</td>
<td>(nAChRs)</td>
</tr>
<tr>
<td>Nitric oxide</td>
<td>(NO)</td>
</tr>
<tr>
<td>Non-competitive inhibitors</td>
<td>(NCIs)</td>
</tr>
<tr>
<td>Normal horse serum</td>
<td>(NHS)</td>
</tr>
<tr>
<td>Nuclear transcription factor kappa B</td>
<td>(NF-kB)</td>
</tr>
<tr>
<td>Optical density</td>
<td>(OD)</td>
</tr>
<tr>
<td>Phosphate buffered saline</td>
<td>(PBS)</td>
</tr>
<tr>
<td>Placenta growth factor</td>
<td>(PlGF)</td>
</tr>
<tr>
<td>Polyvinylidene fluoride</td>
<td>(PVDF)</td>
</tr>
<tr>
<td>Pre-eclampsia</td>
<td>(PE)</td>
</tr>
<tr>
<td>Room temperature</td>
<td>(RT)</td>
</tr>
<tr>
<td>Royal Prince Alfred Hospital</td>
<td>(RPAH)</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>(NaCl)</td>
</tr>
<tr>
<td>Sodium dodecyl sulphate</td>
<td>(SDS)</td>
</tr>
<tr>
<td>Soluble endoglin</td>
<td>(sEng)</td>
</tr>
<tr>
<td>Soluble fms-like tyrosine kinase-1</td>
<td>(sFlt-1)</td>
</tr>
<tr>
<td>Standard error of the mean</td>
<td>(SEM)</td>
</tr>
<tr>
<td>Sudden infant death syndrome</td>
<td>(SIDS)</td>
</tr>
<tr>
<td>Tetramethylethylenediamine</td>
<td>(TEMED)</td>
</tr>
<tr>
<td>Transforming growth factor- beta</td>
<td>(TGF-β)</td>
</tr>
<tr>
<td>Tris(hydroxymethyl)aminomethane</td>
<td>(Tris)</td>
</tr>
<tr>
<td>Tumour necrosis factor- alpha</td>
<td>(TGF-α)</td>
</tr>
<tr>
<td>Vascular endothelial growth factor</td>
<td>(VEGF)</td>
</tr>
<tr>
<td>Western blot</td>
<td>(WB)</td>
</tr>
</tbody>
</table>
Chapter 1

Literature Review

1.0 Introduction

An estimated 20% of Australian women continue to smoke during pregnancy despite knowledge of the adverse effects smoking has during pregnancy (Australian Institute of Health and Welfare, 2006), including placenta previa (Oyelese & Smulian, 2006), abruption placenta (Oyelese & Ananth, 2006), ectopic pregnancy and preterm premature rupture of the membranes (Lee & Silver, 2001). Smoking also has detrimental effects on the foetus including low birth weight (Ventura et al., 2003; Fantuzzi et al., 2008), preterm birth (Kolas et al., 2000), foetal and neonatal death, and increases the risk of sudden infant death syndrome (SIDS) during infancy (Shah et al., 2006).

Despite the adverse effects of smoking during normal pregnancy, a systematic review of 48 epidemiology studies found that cigarette smoking is consistently associated with a reduced prevalence of pre-eclampsia (PE) (England & Zhang, 2007). PE is described as a pregnancy-specific and multi-system disorder. PE affects approximately 3-5% of pregnancies worldwide (Sibai et al., 2005; Redman et al., 2005), while in Australia, it is reported to occur in 2-5% of all pregnancies (Roberts et al., 2011). PE is characterised by the new onset of hypertension and proteinuria after 20 weeks of gestation (Muttar & Sibai, 1999; Sibai, 2008; Mutter & Karumanchi, 2008). The exact mechanism(s) involved in the development of PE are still not understood. However, it is apparent that the placenta plays a major role in the pathophysiology of PE, as its delivery resolves all clinical symptoms (Myatt & Webster, 2009; Young et al., 2010; Andrus & Wolfson, 2010). The leading hypothesis postulates that PE occurs in two stages: 1) abnormal placentation, resulting in an ischaemic placenta secreting factors that lead to 2) the clinical manifestations of the syndrome (Roberts & Redman, 1993; Roberts & Cooper, 2001).
The mechanism(s) explaining how cigarette smoking is protective against PE remain unknown. Nicotine is the main pathogenic compound in cigarettes and it has been suggested that its protective effects are induced through the activation of nicotinic acetylcholine receptors (nAChRs) in the placenta (Sharentuya et al., 2012), of which there are 16 mammalian subunits (α1-10; β1-4, δ, ε and γ subunits) (Fryer et al., 1998). Currently, there are only 2 studies concerning the expression of nAChR subunits in the placenta (Kwon et al., 2007; Lips et al., 2005) however, they do not investigate how cigarette smoke affects nAChR subunit expression or how they are regulated by PE, with the exception of the α7 subunit (Kwon et al., 2007).

Previously in our laboratory, we studied the mRNA expression of the 16 nAChR subunits in the placenta from normal, PE, and cigarette smoking women (David, 2012; Ghazavi, 2012). Our results showed that all 16 subunits are expressed at the mRNA level in the normal healthy placenta, and that in the placentas of smoking women the α9 subunit is increased and the δ subunit is decreased, while in the PE placenta, α2, α9 and β2 subunits are increased. Despite this novel data, understanding the changes in subunit expression at the protein level would provide a more precise indication of changes to receptor functionality, given it is at the protein level that functional receptors occur. Thus, through the application of western blot and immunohistochemistry, the studies described in the present thesis aimed to determine the effects of smoking and PE on nAChR subunit expression in the human placenta, at the protein level. We hypothesise that the same subunits with altered expression at the mRNA level will have altered expression at the protein level.

1.1 Cigarette smoke and nicotine
According to the American Lung Association (Abbott et al., 2012), cigarette smoke contains over 4800 identified chemicals. Besides nitrogen and oxygen, major components in cigarette smoke include carbon monoxide, methane and nicotine, with lesser but still substantial amounts of acetone, acetylene, formaldehyde, propane, hydrogen cyanide and toluene (Seaton et al., 1993).
While thousands of compounds are found in cigarette smoke, nicotine is the major psychoactive component (Rollema et al., 2007). Nicotine is a poisonous alkaloid with a short half-life of approximately two hours, and can rapidly perfuse through the human placenta due to a low molecular weight and high lipid solubility (Lambers et al., 1996). Cotinine is the major metabolite of nicotine. It is the preferred biological marker for measuring precise cigarette smoke exposure as it has a much longer half-life (15–20 hours) and provides serum concentrations that are 10-fold higher than nicotine (Zevin et al., 1998).

1.1.2 Cigarette smoking during pregnancy
Chronic exposure of the foetus to the effects of tobacco smoke is recognised as the most important preventable risk factor for a complicated pregnancy outcome in all developed, and an increasing number of developing, countries (Jauniaux et al., 2007). Despite this, it is estimated that one in five Australian women smoke during pregnancy (Australian Institute of Health & Welfare, 2006) and this is associated with several negative outcomes for both the mother and the foetus. For the mother, negative outcomes include an increased risk of ectopic pregnancy, abruptio placentae, placenta praevia and preterm prelabour rupture of membranes (Ananth et al., 1999). For the foetus, prematurity and intrauterine growth restriction (IUGR) are two major adverse consequences. Both of these result in decreased birth weight of the infant by an average of 170 to 250 g (Hardy & Mellits, 1972; Horta et al., 1997). Lower infant birth weight is strongly associated with infant morbidity and mortality, including an increased risk of heart, breathing and brain abnormalities (Huang et al., 2007), and increased risk of Sudden Infant Death Syndrome (SIDS) (Adgent, 2004; Shah et al., 2006).

1.2 Pre-eclampsia (PE)
1.2.1 Definition and characterization of PE
Pre-eclampsia (PE) is a multi-system disorder, which is characterized by proteinuria developing after 20 weeks of gestation as well as hypertension. Specifically, in Australia the definition of hypertension constitutes systolic blood pressure $\geq$140 mmHg and diastolic blood pressure $\geq$90 mmHg (Douglas et al., 2001). Additional symptoms (summarized in Table 1.1) are associated for consideration of diagnosis within Australia. PE affects 2-5% of
pregnancies (Roberts et al., 2011) and causes substantial maternal and neonatal morbidity and mortality (Redman et al., 2005; Sibai et al., 2005).

**Table 1.1: Clinical symptoms associated with PE as additional considerations for diagnosis used in Australia**

<table>
<thead>
<tr>
<th>System involvement</th>
<th>Clinical symptoms</th>
</tr>
</thead>
<tbody>
<tr>
<td>Renal involvement</td>
<td>Significant proteinuria – dipstick proteinuria subsequently confirmed by spot urine protein/creatinine ratio $\geq 30 \text{ mg/mmol}$. Serum or plasma creatinine $&gt; 90 \mu\text{mol/L}$. Oliguria</td>
</tr>
<tr>
<td>Hematological involvement</td>
<td>Thrombocytopenia</td>
</tr>
<tr>
<td></td>
<td>Hemolysis</td>
</tr>
<tr>
<td></td>
<td>Disseminated intravascular coagulation</td>
</tr>
<tr>
<td>Liver involvement</td>
<td>Raised serum transaminases</td>
</tr>
<tr>
<td></td>
<td>Severe epigastric or right upper quadrant pain</td>
</tr>
<tr>
<td>Neurological involvement</td>
<td>Convulsions (eclampsia)</td>
</tr>
<tr>
<td></td>
<td>Hypereflexia with sustained clonus</td>
</tr>
<tr>
<td></td>
<td>Severe headache</td>
</tr>
<tr>
<td></td>
<td>Persistent visual disturbances (photopsia, scotomata, cortical blindness, retinal vasospasm)</td>
</tr>
<tr>
<td></td>
<td>Stroke</td>
</tr>
<tr>
<td>Other</td>
<td>Pulmonary edema</td>
</tr>
<tr>
<td></td>
<td>Foetal growth restriction</td>
</tr>
<tr>
<td></td>
<td>Placental abruption</td>
</tr>
</tbody>
</table>

(Table derived from Douglas et al., 2001)
1.2.2 Risk factors associated with PE

There are a number of known prior risk factors for PE separated into pre-conception and pregnancy related, as summarized in Table 1.2.

Table 1.2. Risk factors for pre-eclampsia

<table>
<thead>
<tr>
<th>Pre-conception and/or chronic risk factors</th>
<th>Partner-related risk factors</th>
<th>Non partner related risk factors</th>
<th>Presence of specific underlying disorders</th>
<th>Exogenous factors</th>
<th>Pregnancy-associated risk factors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nullparity/primiparity/teenage pregnancy</td>
<td>Limited sperm exposure, donor insemination, oocyte donation</td>
<td>History of previous PE</td>
<td>Chronic hypertension and renal disease, obesity, insulin resistance, low birth weight, gestational diabetes, type I diabetes mellitus, activated protein C resistance, protein S deficiency, antiphospholipid antibodies, hyperhomocysteinemia, sickle cell disease, sickle cell trait</td>
<td>Stress, work-related psychosocial strain</td>
<td>Multiple pregnancy</td>
</tr>
<tr>
<td>Partner who fathered a PE pregnancy in another woman</td>
<td></td>
<td>Age, interval between pregnancies</td>
<td></td>
<td>In utero diethylstilbestrol exposure</td>
<td>Structural congenital abnormalities</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Family history</td>
<td></td>
<td></td>
<td>Hydrops fetalis</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Chromosomal anomalies (trisomy 12, triploidy)</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Hydatidiform mole</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Urinary tract infection</td>
</tr>
</tbody>
</table>

(Table derived from Dekker, 1999)

1.2.3 PE management and prophylaxis

Serious morbidity associated with PE can occur from 20 weeks gestation to after delivery. PE onset before 32 weeks leads to the most severe outcome, and on average the interval between diagnosis and delivery is 14 days (0-62 days) with a substantial number of women delivering within 72 hours (Sibai et al., 1994). In fact, 15% of all preterm births are a consequence of PE.
About one third of the foetuses from women with severe PE suffer from IUGR (Shibata et al., 2008). Prenatal identification of IUGR is important as these foetuses are at increased risk of short term adverse outcomes such as: foetal distress, intrauterine demise, cerebral bleeding and seizures, respiratory distress, sepsis and infant death (Fang, 2005). Also, later in life babies born too small are at increased risk of type 2 diabetes, hypertension and attention deficit disorder (Bele et al., 2006).

Currently, the only “cure” for PE is the delivery of the placenta (Myatt & Webster, 2009; Young et al., 2010; Andrus & Wolfson, 2010). Therefore, current management aims to lower high blood pressure through the use of antihypertensive agents. In Australia, the common antihypertensive medication administered includes labetalol, hydralazine and diazoxide (Xu et al., 2006). In severe cases of PE, the protocol to prevent seizures is the administration of magnesium sulfate. Aspirin has also been used as a prophylactic treatment. This drug potentially inhibits platelet production of thromboxane, a vasoconstrictor and stimulant of platelet aggregation. The overall aim of PE management is to prolong gestation to improve foetal prognosis although it is understood that this increases the risk of potential harm to the mother.

1.3 The human placenta

1.3.1 Anatomy

The human placenta is the most important foetal accessory structure. It brings maternal and foetal circulations and tissues into close relationship (Figure 1.1). Morphologically, its origin is part foetal (the trophoblast) and part maternal (from a transformation of the uterine mucosa). The highly proliferative, migratory, and invasive extra-villous trophoblast cells of the placenta migrate and invade the uterus and its vasculature, to provide a vital link between the mother and the developing foetus (Spencer et al., 1994). It is therefore, ideally suited to perform life-sustaining functions such as the exchange of nutrients, respiratory gases and metabolic wastes, with the maternal supply. It also plays a central role in the maintenance of the immunologically privileged status of the foetus while allowing anchorage to the maternal uterine wall (Donnelly and Campling, 2011), and serves as a special endocrine organ by its
ability to elaborate a myriad of protein, peptide and steroid hormones (Vause and Saroya, 2005).

Figure 1.1. Diagram of the placental circulation.
Maternal blood enters the placental circulation through the endometrial arteries, and circulates around the chorionic villi for the exchange to occur. The deoxygenated blood exits the placenta through the endometrial veins. From the foetal side, the foetal blood enters the placenta through the umbilical arteries, which branch out into the chorionic villi for the exchange to occur. The oxygenated foetal blood returns to the foetus through the umbilical vein. (Figure taken from Kumar et al., 2009).

1.3.2 Histology and Microscopic features
As described by Benirschke (1998), the trophoblast can be thought of as the working end of the placenta. It covers all villi and also surrounds the membranes, the chorion laeve. The trophoblast in turn, is surrounded by maternal blood and for this reason the human placenta is recognized as being haemochorial in type. Two principal types of trophoblast can be distinguished: cytotrophoblasts and unicellular extravillous trophoblast. The cytotrophoblast has proliferative ability sending nuclei and cytoplasm into the syncytiun. The
cytotrophoblastic cells, existing as a single Langhans layer over the connective tissue of the villi, are known only to add cellular components to the syncytium. Little endocrine or synthetic activity has been ascribed to this tissue, but its continued reproduction relentlessly adds new cytoplasm, organelles, and nuclei to the syncytiotrophoblast (Benirschke, 1998). This syncytial sheet of contiguous cytoplasm, nearly 10 m² in size, is known to regulate the exchange between mother and foetus, and is additionally responsible for placental hormone and enzyme production. Its surface provides the major haemal contact with the intervillous blood and possesses a plethora of microvilli for enhanced exchange. As the intervillous circulation is hampered occasionally by disease states (e.g. PE), there is less fluid transfer to the villous circulation; the consequence of this is foetal growth retardation. The villi also suffer this malnutrition and shrink, allowing the syncytial epithelium to buckle. They thus produce an excessive number of knots, the so-called Tenney-Parker change, named after the first description of this feature (Tenney and Parker, 1940) but now commonly referred to as syncitial knots (Huppertz et al., 1998) (Figure 1.2C).

The other principal type of trophoblast is known as the unicellular extravillous trophoblast that occurs primarily beneath the chorionic plate and, as anchoring cell columns, at the site of placental villous attachment to the decidua basalis. Because of its initially disputed origin (maternal or foetal), the cells were originally called the X-cells (Scipiades and Burg, 1930). These cells are now recognized as being of foetal origin and were recently misnamed the intermediate trophoblast, although they are not a precursor to any other cell type. They produce various interesting substances whose physiological function in pregnancy is unknown including being the source of large quantities of major basic protein [MBP] (Wasmoen et al., 1985), a protein that is essentially similar to the eosinophilic granular protein of leukocytes (Benirschke, 1998). There is a remarkable increase of this toxic protein in the maternal blood during pregnancy, only to disappear immediately after delivery (Maddox et al., 1983). Likewise, much of the human placental lactogen (hPL) is produced by these X-cells for reasons that are also obscure, and the cells may be stained for cytokeratin. Slightly better understood now is their production of a variety of basement membrane proteins that impose as fibrinoid in the evolution of the placenta. They open the maternal blood vessels and alter their structure by replacing the muscular wall of spiral arterioles with
a stiff eosinophilic matrix. They also lay down some of the fibrinoid in the decidua basalis (Benirschke and Kaufmann, 1995).

Hofbauer cells are also considered a microscopic component of the placenta. They are the macrophages within the villous stroma and chorio-amnionic membranes. Hofbauer cells are often more abundant in immature placentas. Naturally, these cells are often engaged in phagocytosis (e.g., meconium, hemosiderin) and originally derive from the mononuclear phagocyte system (Castellucci et al., 2000). From the occasional hemosiderin content of Hofbauer cells, the occurrence of focal hemorrhage in certain villi can be determined, usually after congenital infection with cytomegalovirus, thrombosis and destruction of capillaries. The Hofbauer cells may well represent an important sentinel population that accumulates unwanted products during disturbed gestations. In the superficial membranes, Hofbauer-like macrophages are best known from their ability to ingest meconium pigment after its discharge into the amnionic sac.

This thesis will focus its study on three particular cellular layers of the placenta being 1) endothelial cells, 2) villous cells and 3) decidual cells. These are discussed in detail below and are summarized in Table 1.3.
1.3.2.1 Endothelial trophoblast cells

The endothelial trophoblast cells are embedded within the connective tissue of the maternal villous (Allaire et al., 2000; Zhou et al., 2002). EC constitute microvessels where angiogenesis occurs. Vascular endothelial growth factor (VEGF) is expressed in EC and is up-regulated under hypoxia, which is the principal inducer of angiogenesis (Nomura et al., 1995, Yonekura et al., 1999). Endothelial cells express several angiostatic factors such as sFlt-1, soluble neuropilin-1, thrombospondin and endostatin (Wu et al., 2001, Li et al., 2004). VEGF binds to sFlt-1, a potent anti-angiogenic factor, where it regulates various physiological processes in tissues including maintaining avascularity (Ambati et al., 2006), guiding vessel sprouting, and morphogenesis in developing blood vessels (Chappell et al., 2009). Excess circulating sFlt-1 has been implicated in the aetiology of endothelial dysfunction, hypertension and proteinuria in PE (Maynard et al., 2003), and sFlt-1 over
expression has been reported to inhibit tumour growth and angiogenesis (Goldman et al., 1998). Thus EC play an important role in vascular regulation and angiogenesis, both of which are essential for embryogenesis and blood vessel development.

1.3.2.2 Villous cells

Foetal growth and development depends on a continuous nutrient supply from maternal blood (Battaglia et al., 1986, Morriss et al., 1988). In the human placenta the supply process includes uptake from maternal blood and transfer across the syncytiotrophoblast and cytotrophoblast layers, the underlying basal lamina, the foetal connective tissue space, and the foetal capillary endothelium. The placental syncytiotrophoblast is a continuous epithelial layer. It covers the maternal surface of the human placenta (Sideri et al., 1983, Williams et al., 1989) and forms by fusion and terminal differentiation of the underlying cytotrophoblast. The microvillous membrane of the syncytiotrophoblast is in contact with the maternal blood while the basal membrane faces the foetal circulation. For the purpose of this thesis, analysis of the villous cells will be for the combination of syncytio- and cyto- trophoblasts. Flux across the syncytiotrophoblast and its plasma membranes represent the rate-limiting steps in maternal-foetal transfer of most important nutrients. Most sites of known mechanisms of cellular transport are localized to the maternal- and foetal-facing plasma membrane surfaces of the syncytiotrophoblast (Sideri et al., 1983). Regulation of syncytiotrophoblast transport mechanisms is potentially of great importance in the control of foetal nutrient supply. Regulation of transport may be affected either by intrinsic mechanisms, in which substrate or cellular proteins interact with transporters, or by extracellular or circulating hormones or effectors (Kilberg et al., 1985) and their receptors in the trophoblast (Brunette et al., 1988). In addition to physiologic regulators, considerable evidence suggests that environmental substances such as ethanol, cannabinoids and nicotine can alter transport of various nutrients (Fisher et al., 1987).

1.3.2.3 Decidual cells

The endometrium undergoes a remarkable transformation and remodeling response to implantation of the blastocyst, collectively referred to as decidualisation. During this process, the spindle-shaped endometrial stromal fibroblasts are formed into large, secretory decidual
cells. Thus, the decidual cells are found embedded within the decidual connective tissue (Benirschke et al., 2012) (Figure 1.2D). Decidual and trophoblast cells are likely to provide the signaling system that coordinates the activities of the maternal compartment. Decidual cells are modified uterine endometrial stromal cells. The differentiation of decidual cells is one of the earliest uterine adaptations to pregnancy (DeFeo, 1967; Parr and Parr, 1989; Aplin, 2000). Decidual cell differentiation is exquisitely sensitive to the regulatory actions of progesterone, interleukin-11, and activators of cyclic AMP/protein kinase A (Tang et al., 1994; Lydon et al., 1995; Brar et al., 1997; Dimitriadis et al., 2005; Brosens and Gellersen, 2006). During gestation, decidual cells are located at the interface separating invading trophoblast cells from the maternal environment (Figure 1.2 A & D). A number of important functions have been attributed to decidua (Bell, 1983; Aplin, 2000; Brosens and Gellersen, 2006): (1) a protective role in controlling trophoblast cell invasion; (2) a nutritive role for the developing embryo; (3) a role in preventing immunological rejection of genetically disparate embryonic/foetal tissues; and (4) an endocrine/paracrine role in controlling maternal adaptations required for the establishment and maintenance of pregnancy. Pregnancy is dependent upon decidual cell acquisition of each of these specialized functions. Disruptions in decidual cell development are not compatible with pregnancy (Lydon et al., 1995; Bilinski et al., 1998; Robb et al., 1998; Mantena et al., 2006). Progress in understanding specialized decidual cell functions has been limited.
Table 1.3. Function of the various cell types found in the human placenta. Namely the decidual cells, villous cells and endothelial cells.

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Function</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Decidual cells</td>
<td>Contribute to regulation of vascular remodeling</td>
<td>Nomura et al., 1995,</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Yonekura et al., 1999</td>
</tr>
<tr>
<td>Villous cells (encompassing</td>
<td>Facilitate oxygen and nutrient exchange</td>
<td>Sideri et al., 1983</td>
</tr>
<tr>
<td>syncytiotrophoblasts)</td>
<td>Endocrine role which supports and regulates placental and foetal</td>
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<tr>
<td>Endothelial cells</td>
<td>Provide an intelligent semipermeable barrier for transcytosis and delivery of macromolecules such as hormones, growth factors, and immunoglobulins</td>
<td>Alam et al., 2007</td>
</tr>
</tbody>
</table>

1.3.3 Effect of cigarette smoking on the human placenta

Most tobacco toxins have a low molecular weight and high water solubility, and therefore readily cross the placenta. In particular, nicotine and cotinine pass freely across the placenta to the foetus, which as a result is exposed to approximately 15% higher nicotine concentrations than its mother (Eskenazi et al., 1995). Many changes have been reported in the placentas of smoking mothers (a selection of these have been summarized in Table 1.4). Histologically, placenta samples from smoking mothers have increased apoptosis (Erel et al., 2001; Jauniaux et al., 2007), reduced placental weight and atrophic placental villi compared to gestational age-matched non-smoking mothers (Naeye, 1987). The differences have mainly been observed in perivillous fibrin deposition and in the syncytiotrophoblast layer, which is the outermost foetal component of the placenta involved in nutrient exchange between mother and foetus (Naeye, 1987). Functionally, at twenty weeks of gestation, women who smoke exhibit significantly higher umbilical artery resistance, which is interpreted to be a surrogate measure for abnormal placental vascular formation as well as reduced estimated foetal weights (Kho et al., 2009). Other changes include calcification (Klesges et al., 1998), a reduction in the diameter of chorionic villi, and depression of active amino acid uptake (Sastry et al., 1991).
1.3.4 Effects of PE on the human placenta

The etiology of PE remains unknown, however, a shallow cytotrophoblast invasion of maternal spiral arterioles (Figure 1.3), resulting in placental insufficiency is considered central to the pathogenesis (Redman et al., 2005). The oxidatively stressed placenta, releases factors into the maternal circulation, which induces a maternal systemic inflammatory response and generalized endothelial dysfunction that leads to the clinical signs of PE, including hypertension, glomerular endotheliosis, and proteinuria (Redman et al., 2005).

The implication of this theory is that PE is mainly a disease of the maternal vascular endothelium (Roberts et al., 1998; Wang et al., 2007). Normal pregnancy is characterized by a mild systemic inflammatory response with evidence for an acute phase reaction and activation of multiple components of the inflammatory network (Borzychowski et al., 2006). As pregnancy advances, the systemic inflammatory response strengthens, peaking during the second half of pregnancy. In PE, this systemic inflammatory response is reported to be more intense than in normal pregnancy (Borzychowski et al., 2006). Although the precise mechanisms remain undetermined, endothelial cells and unknown circulating factors from placenta are considered to play a key role in this systemic inflammatory response (Redman et al., 2009).

Morphological and pathological changes reported in the PE placenta include increased apoptosis and calcification and reduced amino acid uptake (summarised in Table 1.4). Modification in chorionic villi size seen in PE is not yet known to date. The rate of apoptosis is thought to be increased in PE as a result of placental oxidative stress, which is frequently, although not universally, increased in the condition (Hung et al., 2002). Other gross abnormalities also noted are placental infarcts, retroplacental haematoma and calcification (Sodhi et al., 1990). Furthermore, women with PE are reported to have a higher levels of ACh in their placenta (Murthy et al., 1977). However, ACh synthesis in advanced syncytial degeneration, such as PE, is significantly reduced suggesting that in the placenta, ACh synthesis reflects the state of the syncytiotrophoblast (Satyanarayana, 1986). Moreover, the output of ACh by the PE placenta is greatly reduced (Krishna et al., 1987; Murthy et al.,
1985) including through the foetal vessels (King et al., 1991). Thus, it seems that although maternal surface ACh levels are increased in PE, the transport to the foetal circulation is reduced, which may affect amino acid transport, and could result in fetal intrauterine growth restriction (IUGR) (Brennecke et al., 1988) (Table 1.4).

Figure 1.3. Normal placental development (top) and placental development in pre-eclampsia (bottom). The bottom figure shows the shallow invasion of the cytotrophoblasts, leading to the failure to complete vascular remodeling: vessels retain features of low capacitance and high resistance. Thus, resulting in the reduced blood flow to the placenta, compared to normal placental development. (Figure taken from Lam et al., 2005)
Table 1.4. Smoking and PE induced morphological changes and implications in the human placenta

<table>
<thead>
<tr>
<th>Placental Pathology</th>
<th>Smoking</th>
<th>PE</th>
<th>Implications</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcification</td>
<td>↑</td>
<td>↑</td>
<td>Reflection of cell death and degradation</td>
<td>Klesses, 1998</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Sodhi, 1990</td>
</tr>
<tr>
<td>Size of chorionic villi</td>
<td>↓</td>
<td></td>
<td>Compromised exchange of nutrients, gases and wastes</td>
<td>Mayhew, 1996</td>
</tr>
<tr>
<td>Transport of amino acids</td>
<td>↓</td>
<td>↓</td>
<td>Intrauterine growth restriction (IUGR)</td>
<td>Battaglia et al., 2001</td>
</tr>
<tr>
<td>Apoptosis</td>
<td>↑</td>
<td>↑</td>
<td>Cell death</td>
<td>Sastry, 1991</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Hung et al., 2002</td>
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1.4 Hypotheses of the pathophysiology of PE

There are a number of theories that attempt to explain the exact mechanisms involved in the development of PE. The leading theory of the disorder is that PE occurs in two distinct stages. Stage one depends on defective placental development, resulting in placental ischaemia and hypoxia, which leads to the second stage of maternal symptoms. Although the placenta may play a role in the development of PE, the maternal vascular endothelium appears to be responsible for the clinical manifestations observed (Roberts & Redman, 1993; Roberts & Cooper, 2001). Endothelial disruption in PE results in increased capillary permeability, vasoconstriction and end-organ ischaemia (Roberts & Redman, 1993).

1.4.1 Circulating angiogenic factors implicated in the pathogenesis of PE

Vascular endothelial growth factor (VEGF), and placenta-like growth factor (PIGF), promote angiogenesis, acting through the tyrosine kinase receptors, VEGF receptor 1 (VEGFR-1) and VEGF receptor 2 (VEGFR-2). Soluble fms-like tyrosine kinase 1 (sFlt-1), is an anti-angiogenic factor and a splice variant of VEGFR-1 (Levine et al., 2009). sFlt-1 binds to circulating VEGF and PIGF preventing their interaction with endothelial cell receptors, in particular inhibiting VEGF stimulation of the nitric oxide system through VEGFR2 (Venkatesha et al., 2006). sFlt-1 has been shown to be elevated in pregnant women with PE. Levine et al. (2004) have reported elevated levels in maternal serum five weeks before clinical symptoms appeared. Typically, PIGF is lower in pre-eclamptic women before the sflt-1 levels change with less consistent findings for VEGF (Ohkuchi et al., 2007).
PlGF is expressed at high levels by trophoblast cells in the placenta. Low levels of free PlGF have been reported in the 1st and 2nd trimester in women developing PE (Akolekar et al., 2010; Poon et al., 2009; Romero et al., 2008). Vatten et al. suggest that a low increase in PlGF concentration in early pregnancy, independent of change in sFlt-1, is associated with highly increased risk for preterm PE (Vatten et al., 2007). Also, lower urinary levels of PIGF have been reported at pregnancy weeks 25-28 following later development of PE.

Soluble endoglin (sEng) is another anti-angiogenic factor that has been implicated in the pathogenesis of PE showing higher plasma sEng levels after pregnancy week 23-26 or approximately 8 weeks before the onset of clinical preterm PE. TGF-β interacts with endoglin receptor, which has been shown to be upregulated in the placenta in PE leading to increased secretion of the soluble form into maternal circulation. Placenta specific sEng appears to play a role in trophoblast invasion and differentiation (Levine et al., 2006; Romero et al., 2008; Venkatesha et al., 2006).

At present it is unclear which factors trigger the excessive upregulation of placenta sFlt-1 production. One proposed model has been the hypoxic condition of PE causing endothelial dysfunction and impaired angiogenesis. However, Savvidou et al. (2003) suggest that there is no direct causal effect between decreased level of second trimester maternal serum PIGF, increased level of sEng and the endothelial dysfunction assessed by flow-mediated dilation of the brachial artery.

Combined, the above changes support the hypothesis that PE is a result of an exaggerated immunological response, which can disrupt trophoblast invasion leading to a reduced placental perfusion (Hennessy et al., 1999; Hung et al., 2004; Orange et al., 2003). PE is also associated with an up-regulation of inflammatory factors such as, tumour necrosis factor-alpha (TNF-α), interleukins (IL) IL-1, -6, -8, -18, natural killer cells (NK), and oxygen free radicals, which contribute to the maternal endothelial dysfunction seen in PE (Hennessy et al., 1999; Hung et al., 2004; Orange et al., 2003). A common inducer of these changes is thought to be increased hypoxia.
1.5 Hypoxia and the placenta

During in vivo placental development and before the opening of the intervillus space, placentation is known to occur in a low-oxygen environment, which is the stimulus of cytotrophoblast proliferation (Genbacev et al., 1996; Genbacev et al., 1997; Jaffe et al., 1997). The amniotic cavity in normal healthy pregnancy, is normoxic, with oxygen levels at 20% (Wang et al., 2010). In smoking and PE, this becomes hypoxic with estimated oxygen levels being 14% for smoking (calculated as a 30% decreased based on the findings of Benowitz et al., 1988, Jensen et al., 1991 and Socol et al., 1982) and 2% for PE (Genbacev et al., 1997). The mechanisms by which smoking and PE lead to the hypoxic levels are thought to differ.

1.5.1 Cigarette smoke related hypoxia

In addition to the vascular changes induced as a result of cigarette smoking, other secondary changes are also likely to occur. For example, smoking decreases the flow of uterine blood to the placenta (Castro et al., 1993) through mechanisms that include vasoconstriction. Conducive to this, placentas of mothers who smoke show morphological signs of reduced perfusion (Jauniaux et al., 1992). Furthermore, a key observation made by Shiverick and Salafia (1999) in the study of chorionic villous samples from early gestation was that the formation of trophoblast cell columns was markedly reduced in smokers. In this study, when chorionic villi from non smokers were exposed to nicotine in vitro, there was a dose related inhibition of cell column formation and reduced invasion of basement membrane, apparently related to decreased cytotrophoblast synthesis and activation of the 93 kDa Type IV collagenase (Genbacev et al., 1995). Recent studies with cultured trophoblasts demonstrated that hypoxic conditions inhibit the ability of cytotrophoblasts to differentiate along the invasive pathway (Genbacev et al., 1997). Given evidence that oxygen tension determines whether cells proliferate or invade, it is likely that smoking-induced hypoxia may be a factor in altered trophoblast differentiation. Alterations in trophoblast differentiation along invasive or proliferative pathways may explain the changes in endocrine function as well as vascular morphology that are observed in smokers (summarised in Table 1.4).
Signs of adaptive angiogenesis have also been reported in the foetal blood vessels within the mesenchymal cores of chorionic villi (Pfarrer et al., 1999), indicative of a compensatory mechanism that helps to increase blood flow to the foetus to overcome the induced hypoxic condition (Genbacev et al., 2003).

1.5.2 PE related hypoxia

After the opening of the intervillous space, the oxygen level within the placenta is ~6-8% (Jauniaux et al., 2000). In PE, decreased uteroplacental perfusion is the main pathophysiological mechanism behind increased hypoxic levels whereby it is decreased by 50-70% (Lunell et al., 1982). Indeed failure of differentiation to the invasive phenotype is thought to compromise remodelling of maternal myometrial spiral arteries and may predispose the developing placenta to a state of chronic intermittent hypoxia and/or oxidative stress, as is the case in PE (Hung et al., 2002). Consequently, this may result in an intervillous oxygen concentration of ~2-4% (Zamudio et al., 2003).

Typically, placentation is carried out in a hypoxic milieu. Existing dogma states that initially the spiral arterioles are plugged by extravillous trophoblasts allowing for early villous development and placental angiogenesis. Specifically, these processes are reported to occur at low oxygen levels of approximately 3%, similar to that which is necessary for VEGF induced nephrogenesis (Jauniaux et al., 2000). Despite understanding the necessity of a hypoxic environment in creating a viable environment for the foetus, Fisher et al. (2006), found that hypoxia blocks cytotrophoblasts from differentiating into cells with an ‘invasive’ phenotype. Specifically, culturing the 6–8 week old villous explants in either 20% or reduced 2% oxygen conditions, they noticed that both integrin α1 and histocompatibility antigen, class 1, G (HLA-G) expressions (two markers of invasive phenotype) were upregulated in 20% oxygen, but failed to express integrin α1 in 2% oxygen (Genbacev et al., 1997). These data suggest that at least some aspects of cytotrophoblast differentiation/invasion are arrested by hypoxia. Zhou et al. (1997) also reported that vascular endothelial (VE)-cadherin, an endothelial junction molecule, may help regulate the invasion process by allowing the cytotrophoblasts to “mimic” the adhesion molecule expression associated with endothelial cells. However, during experimental hypoxia, VE-cadherin is not expressed by
cytotrophoblasts in vitro. This finding is supported by an apparent lack of VE-cadherin expression in cytotrophoblasts in the placenta from cases of PE (Genbacev et al., 1996). As a result of these events, the failure to remodel the decidual spiral arterioles would limit placental vascular development, reduce the placental blood supply, and increase placental vascular resistance. Consequently, these events promote a further sustained hypoxic environment. Based on this it can be suggested that in pre-eclampsia, abnormally shallow cytotrophoblast invasion and faulty differentiation lead to inadequate vascular transformation (Genbacev et al., 1997; Zho et al., 1997). These observations collectively suggest that abnormal placental adaptation or insufficient trophoblast function contributes to the pathogenesis of PE.

1.6 Suggested association between smoking and PE

The biological mechanism underlying the relationship between smoking and reduced risk of PE, has not been established, however, recent research has implicated nicotine and carbon monoxide as the major mediators of this effect (Bainbridge et al., 2005).

Nicotine, has been extensively studied for its pro-angiogenic properties (Heeschen et al., 2001; Cooke et al., 2007) at concentrations found in the plasma of smokers (10^{-8} – 10^{-7} M) (Darby et al., 1984; Hill et al., 1983). A study conducted by Mimura et al. (2010), demonstrated that nicotine restored endothelial function damaged by excess sFlt-1 and/or sEng. As mentioned previously, PE is considered to be characterised by an anti-angiogenic state and maternal vascular endothelial dysfunction, which is caused by the release of excessive quantities of two major circulating anti-angiogenic proteins (sFlt-1 and sEng) into maternal blood (Venkatesha et al., 2006). Angiogenesis involves a variety of coordinated events, including matrix degradation, migration, proliferation, and morphogenesis. As mentioned previously, binding of nicotine to nAChRs on endothelial cells is considered to stimulate secretion of several pro-angiogenic growth factors, especially VEGF, which contribute to endothelial cell motility, proliferation, and morphogenesis (Jain et al., 2001; Conklin et al., 2002; Heeschen et al., 2002). Mimura et al. (2010) further showed in their study that sFlt-1 and sEng suppressed endothelial fundamental functions for angiogenesis, and suggested that nicotine restores these functions probably by its stimulatory effects on
secretion of growth factors. Therefore, it has been speculated that nicotine restores endothelial function damaged by excess sFlt-1 and/or sEng possibly through increased secretion of PlGF. For these aforementioned reasons it is anticipated that nicotine, or other nAChR agonists may have some therapeutic potential for PE via its pro-angiogenic properties.

In addition to pro-angiogenic properties, nicotine also has anti-inflammatory properties (Wang et al., 2003; Wang et al., 2004). Nicotine blocks pro-inflammatory cytokine production induced by macrophages via nAChRs. It does this by preventing the activation of the nuclear transcription factor kappa B (NF-κB) (Wang et al., 2004) and inhibiting endothelial cell activation or adhesion molecule expression (Saied et al., 2005; Speer et al., 2002). Normal pregnancy is characterised by a mild systemic inflammatory response with evidence for an acute phase reaction and activation of multiple components of the inflammatory network (Borzychowski et al., 2006). In PE, the systemic inflammatory response is exaggerated (Borzychowski et al., 2006). Recently, a study conducted by Mimura et al. (2010) found that nicotine suppresses interleukin-6 production from vascular endothelial cells through the NF-κB pathway (Mimura et al., 2010).

The protective effect of smoking on PE is likely multifactorial and summarised in Table 1.5. Although this thesis hypothesises the protective effects of smoking are via the nicotine component of cigarettes, we cannot exclude the effects of carbon monoxide. Carbon monoxide, although typically considered a toxic by-product of smoking, possesses important physiological functions (Bilban et al., 2006), including inhibition of pro-inflammatory cytokines and chemokines (Otterbein et al., 2000), prevention of vascular constriction (Zhang et al., 2001), inhibition of platelet aggregation and plasminogen activation (Fujita et al., 2001), inhibition and decrease of sFlt-1 (Powers et al., 2005), increase in heme oxygenase 1 (HO-1, an anti-oxidant) (Sidle et al 2007), and inhibition of apoptosis (Brouard et al., 2000; Liu et al., 2002). Additionally, carbon monoxide has been found to inhibit formation of reactive oxygen species (Wang et al., 2007) and inhibit apoptosis in the differentiated syncytiotrophoblast layer of the placenta (Bainbridge et al., 2006), predominantly attributed to its vasodilatory effects (Bainbridge et al., 2002).
Table 1.5. Smoking and PE induced biological changes of certain markers and implications in the human placenta

<table>
<thead>
<tr>
<th>Marker</th>
<th>PE</th>
<th>Smoking</th>
<th>Smoking + PE</th>
<th>Implications</th>
</tr>
</thead>
<tbody>
<tr>
<td>sFlt-1</td>
<td>↑</td>
<td>↓</td>
<td>↓</td>
<td>Reversed endothelial dysfunction (Mimura et al., 2010)</td>
</tr>
<tr>
<td>PlGF</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>Stimulates angiogenesis under conditions of ischemia, inflammation, and wound healing (Carmeliet et al., 2001)</td>
</tr>
<tr>
<td>VEGF</td>
<td>↓</td>
<td>↑</td>
<td>↑</td>
<td>Promoted angiogenesis, induction of NO and vasodilatory prostacyclins in endothelial cells → ↓ vascular tone and blood pressure (He et al., 1999)</td>
</tr>
<tr>
<td>IL-6</td>
<td>↑</td>
<td>↓</td>
<td>↓</td>
<td>Reduced inflammatory response (Wang et al., 2003)</td>
</tr>
<tr>
<td>Nitric Oxide</td>
<td>↑</td>
<td>↓</td>
<td>↓</td>
<td>Endothelial dysfunction with reduction of dilatory capacity of vessels → retarded foetal growth (Anderson et al., 2004)</td>
</tr>
</tbody>
</table>

1.7 Nicotinic acetylcholine receptors (nAChRs)
The nicotinic acetylcholinergic receptors (nAChRs) belong to the cys-loop family of ligand gated ion channels. All nAChRs form as pentamers of subunits, arranged symmetrically around a central pore (Cooper et al., 1991) (Figure 1.4). Genes encoding a total of seventeen subunits (α1-10, β1-4, δ, γ and ε) have been identified. All subunits are of mammalian origin with the exception of α8 (avian) (Papke et al., 2008). nAChRs are found at skeletal neuromuscular junctions and autonomic ganglia as either homopentamers or heteropentamers. The predominant conformation of these subunits within the central nervous system (CNS) is heteromeric, although α7 and α9 homopentamers exist (Cohen et al., 2005). The conformation of nAChRs in the placenta currently remains unknown. The α1, β1, γ, δ and ε subunits are classified as muscle type (Ke et al., 1998) while the remaining are classed as neuronal. In general, neuronal heteromeric nAChRs have higher affinities for nicotine than α7 homomeric nAChRs (Abbott et al., 2012). The composition of subunits in the receptor determines ligand specificity and affinity, cation permeability and channel kinetics. Classically, nAChRs are activated by the binding of endogenous acetylcholine (Ach).
However, as suggested by the receptor name, nicotine can also bind at this same site and with greater affinity.

![General structure of a nicotinic acetylcholine receptor](image)

**Figure 1.4. General structure of a nicotinic acetylcholine receptor.** (Figure taken from Wrightson Biomedical Publications, Petersfield, UK).

### 1.7.1 Activation of the nAChRs

Endogenously, the nAChRs are activated by acetylcholine (Ach). Exogenously, nAChRs are activated by nicotine and other agonists summarized in Table 1.6. The placental cholinergic system develops during the first half of pregnancy, and the highest Ach concentration is found at 20 weeks of gestation (Sastry *et al.*, 1997; Sastry *et al.*, 1976). Endogenously released Ach in the placenta is reported to regulate blood flow by dilating the placental vasculature and decreasing the resistance to foetal blood flow (Sastry *et al.*, 1991). In addition, the contractile properties of placental myofibroblasts and active amino acid uptake are thought to be related to Ach release (Sastry *et al.*, 1997). Early electrophysiology studies indicated that there are two Ach binding sites per nAChR. The binding sites reside at the interface between two adjacent subunits (Karlin *et al.*, 2002; Sine *et al.*, 2002). In the case of the muscle receptors, the two Ach molecules bind at the interface between the α-γ subunits (or α-ε in the adult form) and between the α-δ subunits (Osaka *et al.*, 1999).
Nicotine stimulates nAChRs by binding to the two α-subunits. Exogenous agonists, such as nicotine, have different affinities for the different types of nAChRs (Lips et al., 2005). It was found in the Lips et al. (2005) study that nicotine binds with the highest affinity to α4β2 nAChR and with 2-3 orders of magnitude lower affinity at α7 nAChR. It also binds with an intermediate affinity to receptors containing the subunit α3 (Lips et al., 2005).
Table 1.6. Binding affinities (K) and functional potencies for nicotinic agonists at native or recombinant α4β2 and α7 nAChRs and recombinant α3β4 nAChRs.

<table>
<thead>
<tr>
<th>nAChR subtype/compound</th>
<th>Binding affinity, K (nM)</th>
<th>Functional potency, EC50 (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>α4β2*</td>
<td>α7</td>
</tr>
<tr>
<td>Acetylcholine</td>
<td>8.8-57</td>
<td>4000-10830</td>
</tr>
<tr>
<td>Carbachol</td>
<td>207-562</td>
<td>1800-5600000</td>
</tr>
<tr>
<td>Mcc</td>
<td>3.8 (47)</td>
<td>10580 (64)</td>
</tr>
<tr>
<td>(−)-Nicotine</td>
<td>1.1-1</td>
<td>400-8000</td>
</tr>
<tr>
<td>Anabasine</td>
<td>76-260</td>
<td>56 (85)</td>
</tr>
<tr>
<td>(−)-Cytisine</td>
<td>0.14-2.7</td>
<td>1400-3883</td>
</tr>
<tr>
<td>(+)-Anatoxin A</td>
<td>3.5 (165)</td>
<td>63.3-380</td>
</tr>
<tr>
<td>(−)-Epibatidine</td>
<td>0.019-0.041</td>
<td>9.9-590</td>
</tr>
<tr>
<td>(−)-Epibatidine</td>
<td>0.01-0.06</td>
<td>3.1-350</td>
</tr>
<tr>
<td>(5) Epibatidine</td>
<td>0.01-0.05</td>
<td>20.8-233</td>
</tr>
<tr>
<td>(2) JU-165</td>
<td>0.27-0.44</td>
<td>2790 (87)</td>
</tr>
<tr>
<td>RJR-2429</td>
<td>1 (98)</td>
<td>n.d.</td>
</tr>
<tr>
<td>ABT-418</td>
<td>3-44 (74, 121)</td>
<td>&gt;100000</td>
</tr>
<tr>
<td>RJR-2403</td>
<td>26 (121)</td>
<td>36000 (125)</td>
</tr>
<tr>
<td>SIB-1508Y</td>
<td>4.6 (72)</td>
<td>n.d.</td>
</tr>
<tr>
<td>ABT-594</td>
<td>0.04 (76)</td>
<td>1580-13860</td>
</tr>
<tr>
<td>A-85380</td>
<td>0.05</td>
<td>148 (184)</td>
</tr>
<tr>
<td>GTS-21 (DMX3)</td>
<td>85 (86)</td>
<td>212-2652</td>
</tr>
<tr>
<td>Anabasine</td>
<td>32-75 (161)</td>
<td>56-759</td>
</tr>
<tr>
<td>(±)-AR-R17779</td>
<td>1600 (95)</td>
<td>190 (150)</td>
</tr>
<tr>
<td>(±)-AR-R17779</td>
<td>1600 (56)</td>
<td>92 (76)</td>
</tr>
<tr>
<td>Choline</td>
<td>112000 (121)</td>
<td>2380000</td>
</tr>
<tr>
<td>DMPP</td>
<td>9.4-400</td>
<td>160-2300</td>
</tr>
<tr>
<td>Lobeline</td>
<td>4.50 (171)</td>
<td>11000-13100</td>
</tr>
</tbody>
</table>

Values are expressed as a range determined by the references cited below each entry. Binding affinities reported for competition binding assays of [H]-agonist binding (except [H]-epibatidine) to brain membranes (α4β2* nAChRs) and [H]-agonist binding to heterologously expressed nAChRs, [I]-Bgt or [H]-MLA binding to brain membranes or heterologously expressed α7 nAChR, [H]-epibatidine binding to heterologously expressed α4 nAChRs. Functional potencies reported at brain thalamic synaptosomes (*nAChRs) or heterologously expressed nAChRs. (Table taken from Sharples and Wonnacott, 2001).
1.7.2 nAChR expression and function in the human placenta

Currently, the function of nAChRs in the placenta has been based on Ach binding only. It has been postulated that the placental cholinergic system develops during the first half of pregnancy, and the highest Ach concentration is found at 22 weeks of gestation (Sastry et al., 1997). Ach is endogenously synthesized in the syncytiotrophoblast, after which it is released into both the foetal and maternal circulation of the placenta and is suggested to be an important placental signaling molecule that, through stimulation of nAChRs, controls the uptake of nutrients, blood flow and fluid volume in placental vessels, and the vascularization during placental development (Sastry et al., 1997).

Despite the well known adverse effects of smoking during pregnancy, there has been no investigation of how it affects the expression or function of the nAChRs in the placenta.

There are only two published reports to date concerning nAChR expression in the placenta (Kwon et al., 2006; Lips et al., 2005). Lips et al., (2005) reported the protein and mRNA expressions of eight nAChR subunits (α2-7, 9 & 10) in normal human and rat placenta, while Kwon et al., (2006) compared α7 expression between PE and normal placentas. These studies showed that the α2-7, 9 & 10 nAChR subunits are present in the placenta and that their level of expression varies according to cell type (Table 1.7).

### Table 1.7. Expression of nAChR subunits in the different cellular levels of the human placenta

<table>
<thead>
<tr>
<th>Subunit</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>α2</td>
<td>Villous endothelial cells&lt;br&gt;- Syncytiotrophoblast (much less intense)</td>
</tr>
<tr>
<td>α3</td>
<td>Villous endothelial cells&lt;br&gt;- Syncytiotrophoblast (much less intense)</td>
</tr>
<tr>
<td>α4</td>
<td>Syncytiotrophoblast&lt;br&gt;- Decidual cells</td>
</tr>
<tr>
<td>α5</td>
<td>Syncytiotrophoblast&lt;br&gt;- Endothelial cells&lt;br&gt;- Decidual cells</td>
</tr>
<tr>
<td>α7</td>
<td>Syncytiotrophoblast&lt;br&gt;- Endothelial cells</td>
</tr>
<tr>
<td>α9</td>
<td>Enriched in basal membrane of the syncytiotrophoblast&lt;br&gt;- Decidual cells&lt;br&gt;- Endothelial cells of capillaries in terminal villi (weak immunolabelling)</td>
</tr>
</tbody>
</table>

Data summarized from Lips et al., 2004.
In our laboratory, we have studied the mRNA expression of 16 nAChR subunits in the normal healthy placenta and subsequently compared the expression of 8 subunits (α2, α3, α5, α6, α7, α9, β1, and δ) in placentas of both smoking and PE (non smoking) mothers. We found increased expression of the α9 subunit and decreased expression of the δ subunit in placentas of smoking mothers, while in PE samples we found increased expression of the α2, α9 and β2 subunits. Changes at the protein level remain to be determined.

1.8 Aim and hypotheses of this project

1.8.1 Aim

Using the experimental methods of western blotting (WB) & immunohistochemistry (IHC), the aims of the present investigation were to:

1- determine normal protein expression (WB), localisation and distribution (IHC), of 10 subunits (α2, α3, α4, α5, α7, α9, β1, β2, β4, δ) in the human placenta;

2- determine which of the 10 subunits have changes in WB & IHC* expression when comparing:

   a) placentas from maternal cigarette smokers to non-smokers

   b) pre-eclamptic placentas to non-pre-eclamptics

* the IHC data will narrow down which cellular layer expresses these changes providing more precise mechanistic input.

1.8.2 Hypotheses

1- In the normal healthy placenta:

   a) based on previous placenta studies from our laboratory conducted at the mRNA level, it is expected that normal protein expression of the 10 nAChR subunits in the placenta will follow the same pattern observed in the decreasing order of mRNA concentration α3, α9, α2 > α5, β1 > δ, α7, α4, β2.

   b) based on the study conducted by Lips et al. (2005), it is expected that the protein expression of different nAChR subunits will vary at each cellular level, with those subunits being of a higher expression, more likely to be present in more cell types than those of low expression.
2- Comparing the smoking and PE placenta to the normal placenta in light of our previous mRNA findings:
   a) in the placentas of smoking women, protein expression of the $\alpha_9$ and $\alpha_2$ subunits will increase while $\delta$ subunit will decrease;
   b) in the PE placenta; protein expression of the $\alpha_2$, $\alpha_9$ and $\beta_1$ subunit will increase.
Chapter 2

Methodology

2.1 Patient recruitment and Placental tissue collection and fixation

With the approval of the Sydney Local Health District Ethics Committee (Royal Prince Alfred Hospital (RPAH) zone) and that of the University of Sydney, after providing written informed consent, placental samples were obtained from women presenting to both RPAH and Campbelltown Hospital from April 2012 to July 2012. Women were recruited into three study groups: 1- those who had a healthy pregnancy and no report of smoking (normal group), 2- those who had a healthy pregnancy and did report smoking during their pregnancy (smoking group) and 3- those diagnosed with pre-eclampsia (PE group).

Upon recruitment, clinical data pertaining to the mother and subsequently the baby when delivered, was obtained from the patient records at the hospital by our associate research midwife. History as to whether the mother smoked during pregnancy was also available in the records and further verified by measuring cotinine levels in either serum or placenta as recently reported (Ghazavi 2013). Cotinine a breakdown product of nicotine was measured. It is the preferred biological marker for measuring precise cigarette smoke exposure as it has a much longer half-life (15–20 hours) and provides serum concentrations that are 10-fold higher than nicotine (Zevin et al., 1998).

The placentas were collected from the operating theatre within 30 min of delivery. Four small (2 x 2 cm) separate samples were obtained systematically from different areas of the placenta. Samples were collected from the maternal side consisting of endothelial, villous and decidual cells. The samples were taken from the centre and one from each quadrant to avoid sampler bias. Two samples were immediately snap frozen in liquid nitrogen and stored at -80°C. The other two samples were washed in Dulbecco’s phosphate buffered saline (PBS) and were fixed for one day in 10% formalin. These samples were then processed for
paraffin embedding, which was carried out by the Department of Histology, RPAH and subsequently stored at room temperature.

The frozen tissue blocks were used for the Western blot (WB) aspect of this study, while the formalin fixed and paraffin embedded (FFPE) blocks were used for the immunohistochemistry (IHC) part of this study.

Due to a lack of time for recruitment, three cases in the PE group were sourced from our larger dataset which was developed in 2008. Additionally, these cases did not have FFPE tissue. As such, the dataset characteristics of the PE group in the WB and IHC studies are slightly varied.

### 2.2 Sourcing and verification of nAChR antibodies

The specificity and properties of the nAChR antibodies selected for this study have been substantiated by the published authors listed in Table 2.1. The ultimate test of antibody specificity is by its study in knockout tissue - this was only found for (α4, α5, β2, β4) (Table 2.1). On our part, we further verified specificity by examining the antibody sequences using BLAST search (UNIPROT).
<table>
<thead>
<tr>
<th>Antibody</th>
<th>Host</th>
<th>Company &amp; Cat#</th>
<th>Antibody sequence</th>
<th>Specificity determined by (reference)</th>
<th>Dilution in WE</th>
<th>Dilution in IHC</th>
</tr>
</thead>
<tbody>
<tr>
<td>nAChR α2</td>
<td>Rabbit Polyclonal</td>
<td>Santa Cruz, sc-5589</td>
<td>HWVRGALLGCYPRWLLMNPRPPFVELCHPLRLKPSYHWLESVDVAAERFVVEEDERWACAGHVA5PVGTLCSIHGHLHSCAGPFAQAEIIQEGELPLLSPHQKAQGAYH</td>
<td>Di Angelantonio et al., 2003</td>
<td>1:1000</td>
<td>1:600</td>
</tr>
<tr>
<td>nAChR α3</td>
<td>Rabbit Polyclonal</td>
<td>Santa Cruz, Sc-5590</td>
<td>RTPFTTHTMPSWVKTYFNLINLPKPRFMTKYTPSNEQNAKPRPRLPGCAELSLHDCFAERKGSKCEKCGPQCGDGCMGCYCRRHRK15NSFSANLTSSSSSVEVDALVSLSALSPEIKEAIQSVKYGIAEINMKAQNEAKEIDQDWDKYVAM</td>
<td>Jie Liu, 2011</td>
<td>1:1000</td>
<td>1:200</td>
</tr>
<tr>
<td>nAChR α4</td>
<td>Goat Polyclonal</td>
<td>Santa Cruz, sc-1772</td>
<td></td>
<td>Govind, 2012</td>
<td>1:1000</td>
<td>1:200</td>
</tr>
<tr>
<td>nAChR α5</td>
<td>Rabbit Polyclonal</td>
<td>Santa Cruz, sc-28795</td>
<td>AQRLSFPSSIAKHEDSSLKDLDQPYERWVPVHELNLDKIKKFGLAIA</td>
<td>Maruko, 1999</td>
<td>1:1000</td>
<td>1:200</td>
</tr>
<tr>
<td>nAChR α6</td>
<td>Rabbit Polyclonal</td>
<td>Abcam, ab10096</td>
<td>LYKELVENVNPRLERPVANDQPLTVYFSLSLLQMDVDENQVTLTNWLQMSTWDRHYLQWNSYEPQKVTVFVPDGQIKWDPLLNYSADE</td>
<td>Paulo et al., 2009; Mielke and Mermel, 2009.</td>
<td>1:2000</td>
<td>1:500</td>
</tr>
<tr>
<td>nAChR α9</td>
<td>Goat Polyclonal</td>
<td>Santa Cruz, sc-13806</td>
<td>MNWWSIESFCWIFFAFASRLRAAETAQDFKGYAQLNDFEDYNSNLARFVE</td>
<td>Colomer, 2010</td>
<td>1:1000</td>
<td>1:300</td>
</tr>
<tr>
<td>nAChR β1</td>
<td>Rabbit Polyclonal</td>
<td>Santa Cruz, sc-11371</td>
<td>LYLRLKKEPPERTDLMPPEPHCSSFGSNGWGRGTDEYIRKPPSDLFKPNFQFELSAPDLRRIGFDFNRMLVPTELEVGSSUYAQLQjugSHDIAL</td>
<td>Venfind from website – tested for WEC and IHC in whole cell lysate</td>
<td>1:2000</td>
<td>1:500</td>
</tr>
<tr>
<td>nAChR β2</td>
<td>Rabbit Polyclonal</td>
<td>Santa Cruz, sc-11372</td>
<td>KFALLPQQPQRRHCAQRLQELRRQEREAGALFLPEAPQGADSCTCFVMRASVQGLAGAFPAAPVAGPGKSUEPQCSCGLREAVDGYRF</td>
<td>Quataram et al. 2005; Pollock et al. 2007, Kabbani and Leveson 2007.</td>
<td>1:1000</td>
<td>1:300</td>
</tr>
<tr>
<td>^B4</td>
<td>Mouse Monoclonal</td>
<td>mAb337</td>
<td></td>
<td>Jon Lindstrom</td>
<td>1:50</td>
<td>1:25</td>
</tr>
<tr>
<td>nAChR δ</td>
<td>Rabbit Polyclonal</td>
<td>Abcam, ab26095</td>
<td>LTGILAAALAVGSGWGLNEEELHRFQEGYNKELRPVHKEE SVVDA</td>
<td>Soeda, 2012</td>
<td>1:2000</td>
<td>1:700</td>
</tr>
</tbody>
</table>

^A kind gift from Jon Lindstrom, Medical School of the University of Pennsylvania
2.3 Western blotting

2.3.1 Protein extraction

Protein extraction and immunoblotting were modified slightly from our previous protocol (Tang et al., 2009). Tissue samples (50 mg) were manually homogenized on ice in a lysis buffer (0.1 M EDTA, 1 M Tris HCL (Tris(hydroxymethyl)aminomethane hydrochloride), Triton (10%), 5 M NaCl, Protease Inhibitor) by pipetting and sonication. Samples were sonicated three times at 70% intensity in 10 second cycles. Samples were kept on ice for 20 minutes and then centrifuged at 16 000g for 30 minutes at 4°C. Supernatant was collected and protein concentration was measured using Direct Detect Spectrophotometer (Millipore, Germany).

2.3.2 SDS Polyacrylamide gel electrophoresis

10% SDS polyacrylamide gels were used for electrophoresis (SDS, TEMED, 30% acrylamide, 10% APS).

Placental tissue samples (10 µg/lane) were denatured at 90°C for 5 minutes and transferred to ice immediately. Prior to loading, samples were centrifuged for 10 seconds. Placental samples and pre-stained molecular mass markers (Precision Plus Protein Dual Colour 161-0374, BioRad and Broad Range Markers sc-2361, Santa Cruz) were loaded into the gel for electrophoresis. Gels were run using a BioRad power pack cell at a running power of 90V for 100 minutes.

2.3.3 Protein transfer and antibody incubation

Once proteins were separated within the gel, proteins were transferred to a polyvinylidene fluoride (PVDF) membrane (Bio Rad; cat#170-4156) using Trans-Blot Turbo (Bio Rad, cat#170-4155). Following transfer, membranes were blocked in 5% skim milk in 0.1% tween 20-phosphate buffered saline (PBS) for 30 minutes before the membranes were incubated with the respective dilution of the primary antibody (Table 2.1) overnight at room temperature (RT). The membranes were washed 3 x 10minutes in 0.1%TweenPBS and then incubated with the respective horseradish peroxidase-
conjugated secondary antibody (anti-goat, anti-rabbit or anti-mouse IgG; 1:2000) for 2 hours at RT.

2.3.4 Chemifluorescence and analysis
Immunoreactive bands were detected using enhanced chemiluminescence (ECL) (Bio-Rad Clarity™ Western ECL Substrate, Cat. # 170-5060) and visualized in Bio Rad ChemiDoc machine. ImageJ software (java based image processing program, developed by the National Institutes of Health, USA) was used for optical density (OD) measurement of the protein bands. Results were calculated and expressed as a ratio relative to beta actin loading control gene (Abcam 8226, 1:5000 dilution). Beta-actin was chosen as the loading control due to its stable expression in placental tissue (Wang et al., 2000).

2.4 Immunohistochemistry (IHC)

2.4.1 Tissue sectioning
Placental sections were cut at 7 µm from the FFPE blocks using a rotary microtome (Shandon Finesse 325, Thermo Fischer Scientific Inc, Massachusetts, USA). Sections were mounted on silanized superfrost slides ready for staining.

2.4.2 IHC staining
All reactions occurred at room temperature unless otherwise stated. Sections were deparaffinised in 2 changes of xylene and brought to water by a graded series of ethanol (100%, 100%, 95%, 95%, 70%, distilled H_2O).

Protein cross-linking occurs through formalin fixation (Shi et al., 1991). Thus, antigen retrieval was required to uncover antigenic sites. Our antigen retrieval method involved microwaving (Black and Decker, 700W, USA) sections in a 10% TRIS-EDTA buffer (1 mM EDTA, 1 mM sodium citrate, 2 mM Tris; pH 9.0) for 13 minutes on high. This method was developed and optimized recently for use on placental tissue in our laboratory (Machaalani et al., 2008). The sections were brought to RT, rinsed with distilled H_2O, and washed in PBS. A hydrophobic barrier was drawn around the sections
and then sections were quenched of endogenous peroxidase activity by immersing the slides in 3% H$_2$O$_2$, 50% methanol and PBS for 25 minutes. After washing in PBS, sections were blocked against proteins within the tissue that may cross-react with the secondary antibody and produce false positive staining. This was performed with 10% normal horse serum (NHS) in PBS for a 30 minute incubation period. Sections were then incubated with the respective antibodies, at dilutions listed in Table 2.1, at 4°C, overnight.

After the overnight stage the sections were rinsed in 2 changes of PBS and then incubated with the respective biotinylated secondary anti-rabbit, anti-goat or anti-mouse antibody (VEBA-1100, VEBA-9500, VEBA-1400 Vector Laboratories Inc, California, USA) for 40 minutes.

After PBS washing, sections were incubated with avidin-biotin complex (ABC) labelled with peroxidase (VEPH4000, Vector Laboratories Inc, California, USA) for 40 minutes. The colour labelling was subsequently developed with 3, 3’-diaminobenzidine (DAB) (VESK3100, Vector Laboratories Inc, California, USA) for 7 minutes. Optimal incubation time with DAB was determined empirically.

Sections were counterstained with Harris’s Haematoxylin for 25 seconds, washed in H$_2$O, dipped 3 times in acid alcohol to remove excess stain, washed in H$_2$O and stained in Scott’s Blueing solution for 2 minutes. Following H$_2$O washing, sections were dehydrated through graded ethanol (70%, 95%, 95%, 100% and 100%; 1 minute each) cleared through xylene (2 changes, 5 minutes each), mounted and coverslipped with DPX.

Negative control sections were treated as above but were incubated with 1% NHS instead of the nAChR subunit antibodies. A minimum of 10% of cases had a duplicate section stained to ensure reproducibility of staining. Sections from all cases of the study were stained within the same day for each antibody to avoid day-to-day variation in staining.
2.5 Quantitative analysis for IHC

2.5.1 Image Capture
Images of the decidual, villous and endothelial cells of the human placenta were captured using a DFC400 camera (Leica Microsystems Ltd. Heerbrugg, Switzerland) mounted on a Nikon Eclipse e800 (Nikon Corporation, Tokyo, Japan) at 10x magnification. Five images of the decidual cells and 10 images of the villous cells were captured per section (at random but dispersed along the entire length of the tissue section) using leica application suite software (LAS V3.8, Leica Microsystems Ltd. Heerbrugg, Switzerland). Endothelial cells were observed within the villous cells.

2.5.2 Quantitative Analysis
Images were analysed using ImageJ software. Using the cell counter functions, the numbers of positive and negative trophoblasts were counted manually. These values were then exported to Microsoft Excel and calculated to express the percentage of villi with positive endothelial cells staining (EC), the percentage of positive stained endothelial cells per villous cell (% +ve EC), and decidua cells (%+DC). The intensity of the villous cells was manually determined and given a H Score ranging from 1-3 which accounts for staining intensity in conjunction with percentage of cells stained positively, with 3 indicating greatest intensity (Demir et al., 2004). Scoring was performed by one observer only and the observer was blinded to diagnosis. A minimum of 10% of sections were double scored to ensure reproducibility of scoring.

2.6 Statistical Analysis
The patient characteristics of the control, smoker and PE groups were compared using one way analysis of variance (ANOVA) and data presented as mean and standard deviation (SD).

For western blot analysis, the mean value of optical density (OD) was determined for each nAChR subunit, in each group. The results express this value ± the standard error of
the mean (SEM). For IHC analysis the mean value was determined for each nAChR subunit, in each group and in each studied cellular layer. The results are expressed as mean ± SEM.

Statistical analysis was performed using SPSS for Windows (V21; SPSS Inc., Illinois, USA). The results were compared among groups using multivariate general linear modelling with bonferroni’s adjustment. A p-value of <0.05 was considered statistically significant. Optical density (WB), the percentage of villi with positive endothelial cells staining (EC), % +ve EC and %+DC (IHC) were selected as the dependent variables, while the three diagnostic groups i.e. control, smokers and PE were the independent variables selected for the general linear model.
Chapter 3

Results

3.1 Patient characteristics

A total of 23 women were studied and their group characteristics are shown in Table 3.1. The control, PE and smoker groups consisted of 8, 7 and 8 subjects, respectively for WB and 8, 8 and 8 subjects, respectively for IHC. Due to a lack of recruiting sufficient numbers of PE subjects during our collection period, three PE cases were derived from our larger dataset. The variation in n value for the PE group during WB and IHC experimentation is due to unavailability of FFPE tissue for these subjects and as such, two varying datasets were derived for WB and IHC.

When comparing the control and PE groups, the weight of the newborn and the women’s blood pressures differed, with PE newborns being of a smaller weight (p=0.001), and PE women having a higher peak diastolic (p=0.0002) and peak systolic (p=0.001) blood pressure (Table 3.1). The mode of delivery, age, gestational age, booking systolic (first consultation) and diastolic blood pressures, Apgar scores at 1 and 5 min and birth weight centile were comparable with no significant difference between the two groups.

When comparing the control and smoker groups, patient characteristics including age, gestation, mode of delivery, peak systolic BP, peak diastolic BP, booking systolic BP, booking diastolic BP, Apgar scores at 1 and 5 min, birth weight and birth weight centile were comparable with no significant difference between the two groups. However, the reported use of tobacco and tissue cotinine concentration was statistically significant between groups (p = <0.001 and p = <0.001, respectively) as represented in Table 3.1.
Table 3.1 Patient characteristics of the Control, PE, and smoker groups.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Controls</th>
<th>PE WB</th>
<th>PE IHC</th>
<th>Smokers</th>
<th>P value PE WB vs Control</th>
<th>P value PE IHC vs Control</th>
<th>P value Smokers vs Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of cases</td>
<td>8</td>
<td>7</td>
<td>8</td>
<td>8</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Age (years)</td>
<td>34.0 ± 6.6</td>
<td>34.0 ± 4.8</td>
<td>35.0 ± 6.7</td>
<td>31.0 ± 2.0</td>
<td>1.0</td>
<td>0.6</td>
<td>0.3</td>
</tr>
<tr>
<td>Gestation (weeks)</td>
<td>38.0 ± 0.5</td>
<td>35.0 ± 4.1</td>
<td>35.0 ± 2.5</td>
<td>39.0 ± 0.4</td>
<td>0.1</td>
<td>0.9</td>
<td>0.3</td>
</tr>
<tr>
<td>Mode of delivery (vaginal/caesarian)</td>
<td>0.8</td>
<td>0.7</td>
<td>0.5</td>
<td>4/4</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Peak Systolic BP (mmHg)</td>
<td>112.5 ± 11.0</td>
<td>150.0 ± 16.2</td>
<td>150.1 ± 24.8</td>
<td>110.0 ± 13.5</td>
<td>0.001</td>
<td>&lt;0.001</td>
<td>0.9</td>
</tr>
<tr>
<td>Peak Diastolic BP (mmHg)</td>
<td>77.0 ± 8.4</td>
<td>100.0 ± 6.9</td>
<td>100.0 ± 15.1</td>
<td>71.4 ± 3.8</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>0.8</td>
</tr>
<tr>
<td>Bookings Systolic BP (mmHg)</td>
<td>111.5 ± 10.2</td>
<td>120.0 ±10.4</td>
<td>119.3 ± 9.2</td>
<td>112.4 ± 8.3</td>
<td>0.2</td>
<td>0.07</td>
<td>0.6</td>
</tr>
<tr>
<td>Bookings Diastolic BP (mmHg)</td>
<td>60.0 ± 6.3</td>
<td>70.0 ± 10.9</td>
<td>71.8 ± 3.6</td>
<td>66.0 ± 5.7</td>
<td>0.2</td>
<td>0.01</td>
<td>0.2</td>
</tr>
<tr>
<td>Apgar 1 min</td>
<td>9.0 ± 0.4</td>
<td>9.0 ± 1.7</td>
<td>8.6 ± 1.5</td>
<td>8.4 ± 1.8</td>
<td>0.2</td>
<td>0.6</td>
<td>0.5</td>
</tr>
<tr>
<td>Apgar 5 min</td>
<td>9.0 ± 0.0</td>
<td>9.0 ± 1.0</td>
<td>9.5 ± 0.5</td>
<td>9.0 ± 0.0</td>
<td>0.4</td>
<td>0.02</td>
<td>0.3</td>
</tr>
<tr>
<td>Birth weight (g)</td>
<td>3613 ± 615</td>
<td>2636 ± 847</td>
<td>2778 ± 125</td>
<td>3600 ± 145</td>
<td>0.001</td>
<td>0.002</td>
<td>0.5</td>
</tr>
<tr>
<td>Birth weight centile</td>
<td>77.0 ± 45.6</td>
<td>25.0 ± 22.6</td>
<td>19.1 ± 11.7</td>
<td>66.0 ± 29.0</td>
<td>0.08</td>
<td>0.03</td>
<td>0.9</td>
</tr>
<tr>
<td>Cigarettes/day</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>13.0 ± 3.1</td>
<td>NA</td>
<td>NA</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Placental Cotinine (ng/ml)</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>183.0 ± 6.2</td>
<td>NA</td>
<td>NA</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Note the 2 columns of PE- one representing the characteristics of the WB dataset, the other for the IHC dataset for the reason indicated in Methods Section 2.1. Data presented as mean ± SD.

* Placental Cotinine levels were measured in our previous study (Ghazavi 2013)
3.2 Western Blot

3.2.1 Antibody specificity

From the western blot experimentation, we were able to verify antibody specificity according to the manufacturers’ specifications. The predicted size of subunits α2, α3, α4, α7, α9, β1, β2 and δ, obtained from either Santa Cruz or Abcam, ranged between 50-78 kDa. Respectively, their predicted sizes are 70 kDa, 55 kDa, 78 kDa, 53 kDa, 56 kDa, 50 kDa, 55 kDa, 50 kDa and 59 kDa and these were mostly observed for all subunits except α2 (60 kDa instead of 70 kDa) and α4 (66 kDa instead of 78 kDa). For these subunits, the possible reasons for the inconsistent size may be due to dimeric or trimeric forms of the protein (Nicke et al., 1999). The product size of beta actin was consistently observed at 42 kDa which was as expected.

Unfortunately for β4, no signal was observed despite attempts of high concentrations of 1:25 dilution. As such, this antibody was removed from the study.

Figure 3.1. Antibody verification as compared to Santa Cruz Broad Range Marker. Note, in all images where 2 bands are observed, the bottom is the Beta-Actin.
3.2.2 Normal, control human placenta

Based on the control placentas, greatest protein expression was observed for the α4 subunit, while lowest expression was observed for the α7 subunit (Figure 3.2).

![Expression relative to Beta Actin (optical density)](image)

**Figure 3.2.** Protein expression of the nAChR subunits in the normal, control human placenta determined by western blot. The level of nAChR protein expression was measured as a function of optical density relative to beta actin.

3.2.3 Comparing smoker and PE groups to controls (Figure 3.3)

Comparing the expression of the subunits in smokers to controls, significantly increased expression was found for α9 (p=0.050) and δ (p=0.042). In PE compared to controls, significantly increased expression was found for α7 (p=0.004), α9 (p=0.024) and δ (p=0.003) subunits. Comparing the expression of the subunits between smokers and PE, significantly greater expression was found for β2 in the PE group (p=0.044) and a trend to decreasing β1 in the PE group (p=0.06) (Figure 3.3).
3.3 Immunohistochemistry of the nAChR subunits

3.3.1 The normal human placenta

Protein expression of the 9 nAChR subunits was observed at the cellular level via IHC experimentation. Expression of the subunits was quantified in three cellular layers including the endothelial cells, villous trophoblast and decidua cells. nAChR subunit staining was predominantly observed in the decidual cells (Figure 3.4), followed by the villous trophoblast cells and occasionally in endothelial cells (Figure 3.5). In the decidual cells, staining was primarily observed in the cytoplasm with the exception of staining observed for the β1 subunit which was mainly observed in the nucleus of the cells (Figure 3.4).
Figure 3.4 Staining in the decidual cells of normal, control human placenta. nAChR immunohistochemical staining in the normal placenta. Immunostaining in the human placenta showing positive staining for \( \alpha_2, \alpha_3, \alpha_4, \alpha_5, \alpha_7, \alpha_9, \beta_1, \beta_2 \) and \( \delta \) nAChR subunits in the decidual cells and negative staining for \( \alpha_2 \) and \( \alpha_7 \). Bar = 50 µm for each panel.
Figure 3.5 Staining of villous cells in normal, control human placenta. nAChR immunohistochemical staining in the normal placenta. Immunostaining in the human placenta showing positive staining for α2, α3, α4, α5, α7, α9, β1, β2 and δ nAChR subunits in the villous (Outer layer of connective cells) and endothelial cells (within the villi). Bar = 50 µm for each panel.
3.3.2 Comparing smoker and PE groups to controls.

Expression of the $\alpha_3$, $\alpha_4$, and $\alpha_5$, nAChR subunits in each of these cellular layers and between groups was comparable, with no significant differences.

**Control vs Smoker**
The $\alpha_9$ subunit expression in the EC of the control group was higher when compared to the smoker group ($p=0.020$). The $\beta_1$ subunit H score ($p=0.018$) and $%+DC$ ($p=0.030$) was significantly higher in the smoker group compared to the control group (Table 3.2)

**Control vs PE**
The $\beta_2$ subunit expression of EC was greater in the control group when compared to the PE group ($p=0.041$).

**Smoker vs PE**
The $%+EC$ and H score for the $\alpha_2$ subunit, were significantly higher in the smoker group when compared to the PE group ($p=0.007$, $p=0.032$ respectively). For the $\alpha_7$ subunit, the EC and the $%+EC$ was significantly higher in the PE group compared to the smoker group ($p=0.009$, $p=0.047$ respectively). It was found that the $\alpha_7$ subunit was more highly expressed in the decidual cells from placentas of smoking women compared to PE affected placentas ($p=0.005$).

For the $\alpha_9$ subunit, the $%+EC$ was significantly higher in the smoker group compared to the PE group ($p=0.038$).

For the $\beta_1$ subunit, the H score was found to be significantly higher in the smoker group compared to the PE group ($p=0.029$). Expression of the $\beta_1$ subunit was significantly greater in the decidual cells of the smoker group compared to the PE group ($p=0.037$).

Expression of the $\beta_2$ subunit in EC, the $%+EC$, H score and expression in the decidual cells was greater in the smoker group when compared to the PE group ($p=0.007$, $p=0.007$, $p=0.004$, $p=0.031$ respectively).
Table 3.2. nACHR ICH protein expression in multiple cellular layers of the human placenta comparing amongst controls, smokers and PE.

<table>
<thead>
<tr>
<th></th>
<th>α2</th>
<th></th>
<th>α3</th>
<th></th>
<th>α4</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Smoker</td>
<td>PE</td>
<td>Control</td>
<td>Smoker</td>
<td>PE</td>
</tr>
<tr>
<td>% villi with +ve EC</td>
<td>0.07 ± 0.03</td>
<td>0.09 ± 0.03</td>
<td>0.03 ± 0.03</td>
<td>0.07 ± 0.03</td>
<td>0.08 ± 0.03</td>
<td>0.09 ± 0.04</td>
</tr>
<tr>
<td>% +ve EC/ villous</td>
<td>0.07 ± 0.03</td>
<td>0.12 ± 0.03</td>
<td>0.01 ± 0.25</td>
<td>0.05 ± 0.02</td>
<td>0.05 ± 0.02</td>
<td>0.06 ± 0.02</td>
</tr>
<tr>
<td>H Score villous</td>
<td>62.9 ± 6.0</td>
<td>68.8 ± 5.6</td>
<td>50.6 ± 5.6</td>
<td>52.1 ± 3.3</td>
<td>57.5 ± 3.2</td>
<td>52.6 ± 5.6</td>
</tr>
<tr>
<td>Decidua</td>
<td>0.48 ± 0.16</td>
<td>0.46 ± 0.13</td>
<td>0.30 ± 0.12</td>
<td>0.34 ± 0.11</td>
<td>0.16 ± 0.11</td>
<td>0.21 ± 0.12</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>α5</th>
<th></th>
<th>α7</th>
<th></th>
<th>α9</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Smoker</td>
<td>PE</td>
<td>Control</td>
<td>Smoker</td>
<td>PE</td>
</tr>
<tr>
<td>% villi with +ve UC</td>
<td>0.10 ± 0.02</td>
<td>0.06 ± 0.02</td>
<td>0.07 ± 0.03</td>
<td>0.14 ± 0.05</td>
<td>0.07 ± 0.05</td>
<td>0.18 ± 0.05</td>
</tr>
<tr>
<td>% +ve UC villous</td>
<td>0.05 ± 0.01</td>
<td>0.04 ± 0.01</td>
<td>0.04 ± 0.01</td>
<td>0.07 ± 0.01</td>
<td>0.05 ± 0.01</td>
<td>0.12 ± 0.01</td>
</tr>
<tr>
<td>H Score villous</td>
<td>71.84 ± 4.6</td>
<td>58.78 ± 4.6</td>
<td>65.6 ± 4.9</td>
<td>67.1 ± 6.3</td>
<td>59.0 ± 6.3</td>
<td>54.3 ± 6.3</td>
</tr>
<tr>
<td>Decidua</td>
<td>0.39 ± 0.14</td>
<td>0.52 ± 0.14</td>
<td>0.55 ± 0.14</td>
<td>0.07 ± 0.07</td>
<td>0.22 ± 0.07</td>
<td>0.13 ± 0.07</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>β1</th>
<th></th>
<th>β2</th>
<th></th>
<th>δ</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C</td>
<td>S</td>
<td>PE</td>
<td>C</td>
<td>S</td>
<td>PE</td>
</tr>
<tr>
<td>% villi with +ve UC</td>
<td>0.16 ± 0.07</td>
<td>0.14 ± 0.05</td>
<td>0.03 ± 0.06</td>
<td>0.06 ± 0.03</td>
<td>0.10 ± 0.04</td>
<td>0.05 ± 0.01</td>
</tr>
<tr>
<td>% +ve UC villous</td>
<td>0.04 ± 0.02</td>
<td>0.07 ± 0.02</td>
<td>0.05 ± 0.02</td>
<td>0.05 ± 0.03</td>
<td>0.08 ± 0.03</td>
<td>0.01 ± 0.01</td>
</tr>
<tr>
<td>H Score villous</td>
<td>51.2 ± 8.5</td>
<td>50.4 ± 7.4</td>
<td>55.8 ± 7.4</td>
<td>52.5 ± 5.7</td>
<td>51.25 ± 5.3</td>
<td>37.50 ± 8.2</td>
</tr>
<tr>
<td>Decidua</td>
<td>0.40 ± 0.10</td>
<td>0.77 ± 0.11</td>
<td>0.46 ± 0.09</td>
<td>0.47 ± 0.13</td>
<td>0.42 ± 0.13</td>
<td>0.28 ± 0.07</td>
</tr>
</tbody>
</table>

*p<0.05 compared to controls; # p<0.05 compared to PE
Chapter 4

Discussion

4.1. Verifying smoking status

Self reported habits of cigarette smoking are often underestimated (Dietz et al., 2011), thus it is important to have biological verification of smoking status. Cotinine, the primary metabolite of nicotine, due to its longer half life (approximately 15–20 hours), is the preferred biomarker to measure the level of tobacco smoking, and in serum/plasma samples, a level >14 ng/ml is taken to indicate active smoking (Jarvis et al., 1987, Schluter, 2002) although a lower cut-off point of 4.5 ng/ml has recently been suggested, taking into consideration ethnic variability (Benowitz et al., 2009). In our dataset as previously reported (Ghazavi 2013), serum samples from 3 patients were not available since these were sourced from a previous dataset due to the time limit of this study. Thus, for the purpose of including a complete set of results, serum was directly extracted from the placental tissue and measured using ELISA analysis. However, to validate that this method is acceptable since at the time of our study, no reports of such a method were available in the literature, we compared cotinine levels between the venous serum and placenta-derived serum in the 5 patients from which both were available. We found that the mean of the two sample types was >85 ng/ml, categorising them in the high active smoking range. However, venous blood levels were on average 20 ng/ml higher than placental levels. Only recently has a study been reported comparing venous serum to placenta derived homogenate from a pregnant mouse model of cigarette smoke exposure (Vyhlidal et al., 2013). In support of our findings, these authors also showed a higher median level of cotinine in serum versus placenta and that this was positively correlated. Taken together, our findings indicate that measuring placenta-derived serum cotinine levels is a reliable indicator of cigarette smoking status.
4.2. nAChR subunits in the normal placenta

To date only two studies have looked at the expression of nAChRs in the human placenta, reporting the expression of the α2-7, α9 and α10 nAChR subunits in the normal healthy placenta (Lips et al., 2005) and α7 subunit in the normal placenta compared to PE placentas (Kwon et al., 2006).

Our findings, from a prior study, support those of Lips et al., (2005), and show additionally that the remaining (previously not studied) subunit mRNAs, including α1, β1, β3, β4, δ, ε and γ, are also expressed in the human placenta (Ghazavi, 2012). Among the 16 subunits studied, it was observed that mRNA levels of certain subunits were greater than others, in the order αβ4 > α3, α4, α9 > α2, α6, α10, β2, ε > α1, α5, α7, β1, β3, δ, and γ (Ghazavi, 2012). Findings from the current investigation demonstrate that at the protein level this pattern was retained, with the exception of α3 and α9, which were in the middle expression range rather than high expression range. Combined, mRNA and protein expression data suggest that in the non-diseased human placenta, ACh action on the nAChRs may occur predominantly via receptors containing α2, α3, α4, β2 and β4 subunits. All of these subunits are neuronal-type, based on the 4 subfamily divisions as reported by Le Novere and Changeux (1994) (summarized in Table 4.1).

Table 4.1. Subfamily division of the nAChRs.

<table>
<thead>
<tr>
<th>Neuronal-type</th>
<th>Muscle-type</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>II</td>
</tr>
<tr>
<td>α9, α10</td>
<td>α7, α8</td>
</tr>
<tr>
<td>α2, α3, α4, α6</td>
<td>β2, β4</td>
</tr>
</tbody>
</table>

The nAChRs have been divided into 4 subfamilies (I-IV) based on similarities in protein sequence (Le Novere and Changeux, 1994). In addition family 3 has been further divided into 3 tribes.

Most nAChR subtypes appear to exist as heteropentamers containing two or more different subunits. Heterologous expression studies suggest that α2, α3, α4, and α6 subunits can combine in binary fashion with β2 or β4 to form functional nAChRs, while α5 and β3 subunits are considered “wild-cards” not able to form nAChRs alone or with any other single type of subunit, but rather combine as trinary (e.g., α4β2α5, α3β4α5) or
quaternary (e.g., \( \alpha_4\delta_2\beta_3\beta_4\) or \( \alpha_3\beta_2\beta_4\alpha_5 \)) complexes (Wu and Lukas 2011). The muscle-type nAChRs are quaternary complexes composed of \( \alpha_1, \beta_1, \delta \) and either \( \gamma \) (foetal) or \( \epsilon \) (adult) subunits. The \( \alpha_7 \) and \( \alpha_9 \) subunits are unique in that they form functional homopentamers, although the function of the \( \alpha_9 \) subunit is enhanced when it co-assembles with the \( \alpha_{10} \) subunit (Sgard et al., 2002).

It is interesting to note that the \( \alpha_3, \alpha_4, \beta_2 \) and \( \beta_4 \) subunits were amongst the intermediate to higher expressing subunits in the normal healthy placenta. Functionally, the \( \alpha_3 \) subunit is important for body growth, bladder contractility, and pupil dilation, functions determined while examining \( \alpha_3 \) knockout mice (Xu et al., 1999a). Survival of these \( \alpha_3 \) knockout mice was also threatened, with >50% dying within the first postnatal week and most likely due to bladder and/or urinary tract infection (Xu et al., 1999a). Other functions of the \( \alpha_3 \) subunit are derived from expression studies. The \( \alpha_3 \) subunit is present on the resident macrophages (Hofbauer cells) in the full term human placenta (Lips et al., 2005), and in cultured human bronchial epithelial cells, the \( \alpha_3 \) subunit stimulates the release of granulocyte macrophage colony stimulation factor (Klapproth et al., 1998), thus indicating a potential role for the \( \alpha_3 \) containing nAChRs in regulating cytokine/chemokine secretion. In skin keratinocytes and bronchial epithelial cells the \( \alpha_3 \) subunit appeared to be involved in the maintenance of the flat shape of the cells (which is necessary to form a continuous epithelial lining) (Macklin et al., 1998; Maus et al., 1998). The role of the \( \alpha_4 \) subunit in the placenta remains in question given that the only role of the \( \alpha_4 \) subunit to date is in nociception as determined by \( \alpha_4 \) knock-out mice (Marubio et al., 1999).

The \( \beta_2 \) and \( \beta_4 \) subunits are integral for the formation of ACh binding sites and effective ion channels in nAChRs (Xu et al., 1999b). The \( \alpha_3\beta_4 \) and \( \alpha_4\beta_2 \) functional nAChRs are abundant on autonomic ganglia (Corriveau and Berg 1993). The \( \alpha_3\beta_4 \) assembly is important for ACh-induced fast excitatory postsynaptic transmission in the autonomic nervous system (ANS) (Wang et al., 1996; Nelson and Lindstrom, 1999), with a lower affinity for nicotine than the \( \alpha_4\beta_2 \) assembly (Corriveau and Berg 1993). Based on \( \beta_4 \) knockout studies, it was shown that animal survival was not at risk but the animals did
have an impaired heart rate response to cervical vagal stimulation, and greatly reduced ileal contractile responses, suggesting that $\beta_4$ subunits are critical in the ANS for the formation of functional receptors with $\alpha_3$ subunits, and that their deficiency adversely affects autonomic transmission, most probably by the loss of ACh binding sites in ganglia and by the alteration of properties of ion channels and affinities of some drugs (Wang et al., 2003).

Taken together, the above data indicate that nAChRs in the non-diseased human placenta, containing $\alpha_3$, $\alpha_4$, $\beta_2$ and $\beta_4$ subunits, which are requisite participants in the majority of functional ganglionic nAChRs (Xu et al., 1999b), may play the predominant role in facilitating the endogenous ACh mediated cellular growth, maintenance and survival, as well as nociception and contractility of the placenta.

**4.3. Effect of smoking and PE on nAChR subunit protein expression in the human placenta compared to controls**

This study was limited to nine of the sixteen mammalian nAChR subunits due to the availability of antibodies that are well characterised and have had their specificity determined. Although we were kindly provided with some mouse monoclonal antibody against the $\beta_4$ subunit (mAb337, Jon Lindstrom, Medical School of the University of Pennsylvania), we did not see any signal in the western blot and as such, this subunit was excluded.

IHC analysis encompassed 3 major cell/structure types: endothelial cells (EC), villous cells, and decidual cells. Each cell type has an important regulatory function as described in section 1.3.2. By undertaking the IHC study, we aimed to narrow down which of the cell types were showing the changes in expression to determine functionality. This could not be done with western blotting since tissue was derived from an arbitrary small block of placental tissue of the maternal interface where the constituent proteins derived were heterogeneous (of different cell types), and for some cases, protein derived from one cell type may have been greater than in other cases. Nevertheless, the western blot data from
this study showed that when compared to the control group, α9 and δ were increased in both the smoker and PE groups. An increase in α7 was additionally seen in the PE group.

Consistent with the western blot findings, a change in α7, α9 and δ subunit expression was observed at the cellular level using IHC, with additional, changes for α2 and β1 subunit. Specifically, for α7, the percentage of positive endothelial cells per villous was decreased in the smokers compared to both control and PE groups. For α9, a decrease was observed for the percentage of villi with positive cells in PE compared to controls while a lower percentage EC per villous in PE was evident compared to smokers. δ subunit expression was increased in the decidual cells of smokers compared to the control group. For α2, the percentage of positive EC per villous and the villous cells was greater in the smoker group compared to the PE group. Finally, β1 expression was increased in villous and decidual cells of smokers compared to control and PE.

4.3.1 Mechanism(s) leading to a change in α9 and δ expression
Our finding that the expression of α9 and δ subunits increased in both the smoking and PE groups compared to controls suggests a possible common mechanism causing this increase. Given both conditions induce hypoxia, we suggest this to be the common mechanism. At the cellular level, it was unexpected to see an opposite effect of decrease in α9 expression in the PE group, albeit only in the EC cell type. The implications of this finding will be discussed and summarized in Figure 4.1.

4.3.1.1 α9 subunit
Typically, nicotine acts as an agonist at nAChRs. However, nicotine has been shown to have a unique, antagonistic effect on α9-containing nAChRs. Recombinant α9α10 nAChRs (expressed in Xenopus laevis oocytes) were activated by ACh but inhibited by nicotine (Rothlin et al., 2003, Sgard et al., 2002, Verbitsky et al., 2000). Although the predominant role of α9 is reported to be in auditory regulation (Vetter et al., 1999), the α9-containing nAChRs are found mainly outside of the CNS (Colomer et al., 2010, Kummer et al., 2008, Lips et al., 2005) and play a role in keratinocyte adhesion (Nguyen et al., 2000) and re-epithelialization (Grando 2006), in mediating synaptic transmission
between the efferent olivocochlear fibres and cochlear hair cells (Maison et al., 2002, Vetter et al., 1999), and regulation of pain (McIntosh et al., 2009, Vincler et al., 2006).

The placenta of smoking women has shown increased trophoblastic apoptosis (Genbachev et al., 1995, Jauniaux and Burton 1992, Nelson et al., 1996), and this was found to induce a discontinuity in the trophoblastic layer of the villi (Nelson et al., 1996). A discontinuity in such a tissue layer (trophoblast) provides a matrix for re-epithelialization which is a mechanism of villous repair (Nelson et al., 1990). A role of α9-containing nAChRs in re-epithelialization has been reported whereby antagonism of α9 and RNA interference in keratinocyte cultures and null mutation in knockout mice delayed wound re-epithelialization in vitro and in vivo (Grando 2006). Thus, our finding of increased α9 nAChR expression in placentas from smoking women and those with PE may indicate an increase in its stimulation via ACh to increase the re-epithelialization of the trophoblast due to damage caused by smoking- and PE-induced apoptosis.

During pregnancy, the developing foetus is totally dependent on maternal amino acid supply, via the placenta, for protein synthesis and growth in size and weight (Pastrakuljic et al., 1999). Smoking- and PE-induced hypoxia, reduce ACh levels, in turn depressing active amino-acid uptake which is thought to be a major mechanism for IUGR of the foetus (Pastrakuljic et al., 1999, Sastry et al., 1991). Lips et al. (2005) found strong α9-immunolabelling localised in the syncytiotrophoblast, with a distinct enrichment in the basal membrane. Amino acids must cross both the maternal-facing microvillous plasma membrane and foetal-facing basal plasma membrane of the placental syncytiotrophoblast in order to reach the foetal circulation. Thus, regulation of amino acid uptake is a potential role for α9 containing nAChRs expressed in the syncytiotrophoblast. Given we found an increase in α9 expression in both the smoking and PE group, this may indicate a positive effect on active amino acid uptake via α9-containing nAChRs, potentially counteracting a decrease in amino acid uptake as a result of decreased ACh due to smoking and PE (Sastry et al., 1995).
Finally, both α9 and α10 are expressed in a variety of immune cells (Kawashima et al., 2007). McIntosh et al (2009) identified the α9 and α10 subunits in Jurkat, MT2 and CEM T-cell lines, purified populations of CD3+, CD4+ and CD8+ T-cells, CD19+ and CD80+ B cells, monocytes, macrophages and in tonsil (Lustig et al., 2001, Peng et al). The maternal-foetal interface is high in immune cells which cross-talk with hormonal, endocrine, and angiogenic regulators to program a normal pregnancy outcome. Among immune cell types, regulatory T cells play an important role in protecting the foetus by dampening harmful inflammatory immune responses at the maternal-foetal interface (Somerset et al., 2004). Placental hypoxia amplifies release of inflammatory stimuli into the maternal circulation. This might involve lipid peroxidation or other, undefined mechanisms. Hypoxia could also activate leukocytes (Scannell, 1996) in the intervillous space or stimulate proinflammatory cytokine production by the placenta (Benyo et al., 1997). Thus, it is possible that α9 expression is increased to dampen the inflammatory response caused by smoking- and PE- induced hypoxia.

Although our finding of decreased α9 immunostaining in the endothelial cells of PE samples is contradictory to the increase seen in the western blot data, it is not unusual. For example, haeme oxygenase-2 (HO-2), a regulator of carbon monoxide production and a potent vasodilator (McLean et al., 2000), was found to be unaffected in western blot experimentation of PE placentas, yet at the cellular level, it was found to be decreased in endothelial cells (Barber et al., 2001). Given PE has been ascribed to generalized maternal endothelial cell dysfunction (Redman et al., 1999), this specific finding of a decrease only in the ECs supports the role of the α9 subunit in the abnormal vascularisation seen in PE.

4.3.1.2 δ subunit
The δ subunit belongs to the muscle-type nAChRs which are pentameric complexes, consisting of four distinct protein subunits (α1, β1, δ, γ). The γ subunit is substituted for the ε subunit with age, altering the functional response of the receptor (Camacho et al., 1993; Mishina et al., 1986). The binding of two molecules of ACh to specific sites at the interface of the α subunits with the γ and δ subunits causes a conformational change in
the receptor protein allowing ion permeation (Blount and Merlie, 1989). Each subunit shares a characteristic configuration containing a large N-terminal hydrophilic sequence, followed by four putative transmembrane domains (Changeux et al., 1992). Previous studies have demonstrated that the second transmembrane domain (TM2) of each subunit lines the ion channel pore. This region plays a role in determining the divalent permeability of the receptor as well as determining pharmacological properties (Changeux et al., 1992).

Genetically, the δ subunit is linked with the muscle disorder of myasthenia gravis, where the level of the δ subunit mRNA coding for the adult nAChR is increased (Guyon et al., 1998). Thus, the role of the δ subunit seems to be specific in regulating synaptic activity and ion conduction pathways within muscle, particularly Ca$^{2+}$ (Carlisle et al., 2004, Zia et al., 2000). Although a direct action of this is via nicotine activating δ-containing nAChRs directly, it has also been reported to be via the role of the δ subunit in increasing affinity of nAChRs to non-competitive inhibitors (NCIs) (Francis and Papke, 1996). NCIs prevent receptor function by binding to sites which are believed to block the ion conduction pathway and which are distinct from the binding sites for agonist activation. Hence, our finding that the smoke exposed and PE affected placenta had an increase in the δ subunit, suggests this may have caused an increase in the affinity of NCI for the nAChR. By doing so, Ca$^{2+}$ permeability through these ion channels is decreased. As such, exaggerated energy expenditure, impaired energy production, initiation of cytoskeletal degradation, and ultimately cell death (apoptosis) are reduced (Nicotera et al., 1998). Therefore, this increase in δ by smoking and PE may reflect a compensatory protective physiological response.

Expression of δ subunit was increased in decidual cells of smokers compared to controls. The decidual cells provide nutrition to the developing embryo, protect the embryo from immunologic responses from the mother, and regulate trophoblast invasion into the uterine stroma (Kowaka et al., 1998). Smoking is known to reduce the exchange of nutrients from mother to foetus, depress active amino-acid uptake by human placental villi and trophoblast invasion (Jauniaux et al., 2007). It is therefore possible that
increased expression of δ in the decidual cells of smokers is mediating the adverse affects of smoking by inhibiting activation of the decidual cells and therefore their function.

4.3.2. Mechanism(s) leading to a change in α7 expression

Previously, our laboratory found no change in α7 nAChR expression at the mRNA level in either smoking (Ghazavi, 2012) or PE placentas (David, 2012). However, via western blot, an increase in the protein expression of the α7 subunit was observed in PE compared to control placentas. This is consistent with the findings of Kwon et al. (2006), the only other group to look at α7 nAChR expression in the PE placenta. An additional observation of this study was that at the cellular level, α7 expression is lower in the EC of smokers compared to both control and PE groups.

The α7 nAChR subunit was initially found in the nervous system (Flood et al., 1997). Its expression in non-neuronal cells, such as macrophages (Heeschen et al., 2002; Saeed et al., 2005), endothelial cells (Heeschen et al., 2002), alveolar type II cells (Sekhon et al., 1999), smooth muscle cells (Macklin et al., 2008), and the placenta (Lips et al., 2005), was only discovered recently.

Disruption of α7 nAChR subunit expression has been shown to significantly inhibit inflammatory angiogenesis and reduce ischaemia-induced angiogenesis, suggesting a role for the α7 nAChR subunit in mediating the angiogenic pathway (Heeschen et al., 2002). This is mediated by both ACh and nicotine. ACh-mediated anti-inflammatory activity via the α7 nAChRs has been reported by Shytle et al., (2004) and Wang et al., (2003). Nizri et al (2006; 2008) further showed the suppression of lymphocyte proliferation and the reduction of pro-inflammatory cytokine production upon treatment with inhibitors of acetylcholinesterase.

Evidence for nicotine-mediated anti-inflammatory effect of the α7 nAChR subunit is more diverse. Wang and colleagues (2003) observed that the amount of tissue necrosis factor (TNF) released from lipopolysachharide-stimulated human macrophages decreased when they were treated with nicotine and that this effect was absent when they were
pretreated with α-bungarotoxin, an α7 nAChR subunit blocker. Similarly, a study conducted by Saeed et al. (2005) showed that nicotine suppressed endothelial cell activation via the α7 subunit, whereby TNF induced adhesion molecule expression and chemokine production by endothelial cells was inhibited, and leukocyte migration during inflammation was blocked. Nicotine also prevented hypoxia-induced cell membrane disintegration and DNA fragmentation via the α7 nAChR subunit (Hejmadi et al., 2003; Tohgi et al., 2000). When cells over expressing the α7 nAChR subunit were pretreated with nicotine, the tolerance to G1 arrest and DNA fragmentation was increased (Utsugisawa et al., 2002). Considering these observations, a clear role of the α7 nAChR subunit in inflammation and endothelial cell activation in the placenta is evident and this difference may be involved in the pathophysiology of PE.

4.3.3 Mechanism(s) leading to a change in β1 expression

The greater expression of β1 in the villous and decidual cells of smokers compared to both control and PE groups, suggests a purely nicotine (rather than hypoxia-driven ACh) induced effect.

An analysis by Su et al. (2002) indicated that the β1 subunit is abundantly expressed in the neuromuscular junctions of muscles and components of muscular subtypes that cause excitatory effects on muscle leading to contraction. Commonly, β1 conforms with 3 other subunits α1, δ, and either γ (foetal) or ε (adult) (Giniatullin et al., 2005), this combination referred to as the muscle type nAChRs. The muscle type nAChRs are known to play a key role in ion channel permeability and increase Ca\(^{2+}\) influx at the nAChRs. Increases in Ca\(^{2+}\) cause apoptosis and lead to calcification. The placenta of smoking women has shown increased trophoblastic apoptosis (Genbachev et al., 1995, Jauniaux and Burton 1992, Nelson et al., 1996), and this was found to induce a discontinuity in the trophoblastic layer of the villi (Nelson et al., 1996). It is therefore possible that increased expression of β1, via nicotine from cigarette smoke, is implicated in the increased calcification and apoptosis seen in the trophoblast (Jauniaux et al., 2007) of placentas from smoking women although it is understood not all cellular calcium changes translate to clinically relevant calcification (Figure 4.1).
4.4 Differences between smokers and PE: $\alpha_2$ and $\beta_2$

This study aimed to recruit a PE+smokers group however given the study time-frame, we were unable to achieve this. Thus, the precise mechanism in this group remains outstanding and our analysis comparing smokers to PE is speculative. The discussion will propose a possible protective role of smoking in PE via the specific subunits where a difference in expression between the two groups was observed; these being $\alpha_2$ and $\beta_2$.

4.4.1 $\alpha_2$ subunit

Between the smoking and PE groups, we found the percentage of positive EC per villous and the villous cells containing $\alpha_2$ was greater in the smoker group compared to the PE group. The $\alpha_2$ subunit was among the first non-muscle nAChR subunits to be cloned and heterologously expressed in a functional subtype, with the $\beta_2$ subunit (Wada et al., 1989). In a study comparing the functional properties of rat nAChR $\alpha_2\beta_2$ channels to $\alpha_4\beta_2$ channels, it was found that low doses of nicotine and ACh were equally effective at activating $\alpha_4\beta_2$ channels, whereas nicotine was more potent than ACh at $\alpha_2\beta_2$ channels (Khirough et al., 2004; Luetje and Patrick, 1991). Thus it was not surprising to see greater expression of $\alpha_2$ in the smokers, presumably as an effect of nicotine from the cigarette, which is not present in the PE group. It is however understood that any observed changes can not be unequivocally attributed to nicotine alone as the comparison was between smokers and PE as opposed to the control group.

In the study conducted by Lips et al., (2005), weak to moderate $\alpha_2/4$-immunolabelling was detected intracellularly in the syncytiotrophoblast. The nAChR in placental vessels are suggested to be involved in the cholinergic regulation of blood flow and fluid volume (Sastry, 1997). In the present study, expression of the $\alpha_2$ subunit was found to be greater in the smokers compared to PE. Expression of the $\alpha_2$ subunit has been demonstrated within the syncytiotrophoblast where amino acid transporters can be found (Lips et al., 2005). Accumulative amino acid transporters and exchangers can account for the net uptake of amino acids from the maternal circulation across the microvillous membrane into the placenta (Cleal & Lewis, 2008). Although other subunits have been observed in
the syncytiotrophoblast, $\alpha_2$ is one of the most highly expressed subunits (David, 2012, Ghazavi, 2012). One may therefore speculate that greater expression of $\alpha_2$ subunit in smokers compared to PE may be occurring as a protective mechanism whereby a decrease in ACh, therefore reduced placental uptake of amino acids, caused by PE-induced hypoxia, is being counteracted by an increase in $\alpha_2$ subunit expression, as seen in smokers.

4.4.2 $\beta_2$ subunit

This study found that $\beta_2$ expression was less in the smokers compared to PE, as determined by western blot. A role of $\beta_2$ in inflammatory processes, regulating cytokine production (blocking or inhibiting), has been reported (Bencherife et al., 1996). Importantly, while the $\alpha_4\beta_2$-selective ligand (E)-metanicotine inhibits IL-8 and TNF- $\alpha$ production in human macrophages and in cells of the inflamed mucosa (Spoettl et al., 2007), nicotine down-regulates the production of proinflammatory cytokines in $\beta_2$-containing murine alveolar macrophage (MH-S) cells (Matsunaga et al. 2001). This effect was blocked by the antagonist d-tubocurarine (selective for $\beta_2$ containing receptors) but not the antagonist $\alpha$-bungarotoxin (selective for $\alpha_7$ containing receptors).

Furthermore, a recent paper shows that nicotine blocks cytokine production in mouse macrophages that lack $\alpha_7$ subunits but express $\alpha_4\beta_2$ receptors (van der Zanden et al. 2009). When studying $\alpha_4\beta_2$, Hosur et al. (2009) found that nicotine suppresses proinflammatory cytokines IL-1b and IL-6 in ha4b2 SH-EP1 cells in a time- and concentration-dependent manner. Further, selective antagonists dHbE and Mec reversed nicotine-induced suppression of proinflammatory cytokines suggesting that $\alpha_4\beta_2$ receptor activation is required to inhibit activation of the NFkB pathway and in turn attenuate cytokine synthesis.

Studies have demonstrated that up-regulation of $\alpha_4\beta_2$ receptors requires receptor occupation, but not necessarily ion flux through the receptors (Gopalakrishnan et al. 1996; Darsow et al. 2005). It is therefore possible that although $\beta_2$ expression was greater in the PE group, the functionality of receptors containing this subunit was not increased. Furthermore, given the heightened inflammatory response in PE and the anti-
inflammatory role of nicotine at the α4β2 nAChR, it is possible that nicotine may reduce the inflammation that is highly characteristic of the pathophysiology of PE.

Figure 4.1. Diagram summarizing nAChR subunit role in pathophysiology of smoking and PE. Both smoking and PE induce hypoxia. This study proposed that expression of δ and α9 was increased in both smokers and PE as a physiological response to minimize hypoxia induced apoptosis, calcification and inflammation due to the inherent properties of these subunits. Alone, smoking was found to increase expression of β1. This muscular type nAChR has a role in receptor excitability and when stimulated by nicotine causes Ca\(^{2+}\) influx. Subsequently, apoptosis and calcification result and therefore, this subunit may have a role in the adverse pathology seen in placentas from smoking mothers. Alone, α7 expression was found to increase in PE. This subunit has a reported role in inflammation and therefore may be implicated in the hallmark increase in inflammation experienced in PE. The change in α2 and β2 expression did not occur relative to controls and therefore the role of these subunits is speculative and not included in the above figure.
4.5 Limitations

As with all studies this one was limited by a number of factors:

1) Given the short time frame of the project only the minimum number of cases required per group was achieved thus resulting in a relatively small data set. The minimum required number of cases was determined by power calculations according to a previous study of nicotinic receptors in brain tissue from piglets exposed to nicotine in our laboratory where an n value of 7 per group resulted in p values <0.05 (Browne et al., 2010).

2) The amount of cigarettes smoked by the mother was derived from the patient records in the hospital. As such, bias may have been involved in underreporting this habit. This would have been a confounding factor. However, this was overcome by measuring cotinine levels (Ghazavi 2013) which allowed for biological verification of the extent of maternal smoking.

3) Although this study aimed to investigate the protective mechanism of smoking on PE a combined sample set for placentas from women who both smoked and had PE during pregnancy was unable to be recruited mostly because of the association of preeclamptic smokers being a rarity. Therefore, it has been acknowledged that any association between the two groups is speculative.

4) Experimentally, both the Western blot experimentation and IHC involved the use of antibodies. Although all antibodies were verified to the best of our ability from available literature, it is not until knock out models are developed for them that specificity can be ascertained.

4.6 Future directions

Some discordance was observed between the WB and IHC data. We presume this to be due to the origin of the final protein content in WB where we did not differentiate between the cell types of the placenta. Future studies using cell separation (Cervar et al., 1996) and measuring the amount of protein from each cell type would provide a more precise cellular analysis.
Although nicotine is the major pathogenic compound of cigarette smoke and one of the major agonists at the nAChRs, future studies may be better able to elucidate the effects of nicotine on nAChRs by looking at the specific effect of this compound via the collection of placentas from women who used nicotine patch therapy (NPT) during their pregnancy. This could help demonstrate whether nicotine has a specific effect on the development of the foetus by inducing a change in receptor subunit expression. Animal studies could also help elucidate the specific effect of nicotine on the nAChR subunits, whereby pregnant rats are exposed to nicotine during pregnancy and their placentas subsequently studied. Knock out models would also be an effective way of further investigating the role of each nAChR subunit at an individual level. Such models would allow the functional role of each subunit to be more precisely studied.

The recruitment and analysis of placental samples from women who both smoked and were diagnosed with PE (PE+smokers) during pregnancy would be a more significant method of elucidating the proposed protective role of smoking on PE whereby a more direct correlation could be drawn between these two factors.

Given we found that 6 of the 9 subunits had differences amongst the three groups, future receptor binding studies focusing on these receptor subtypes would be valid. This is indeed feasible given we have frozen placental tissue and radioactive markers are available on the market, although it may be time consuming and costly. Such experimentation would however, provide precise data on actual receptor functionality.

4.7 Conclusion

This study was novel in finding the additional protein expression of the β1, β2 and δ nAChR subunits in the normal, non-diseased human placenta. Of the nine subunits studied (α2, α3, α4, α5, α7, α9, β1, β2 and δ), α3, α5 and α9 were most highly expressed in the normal, non-diseased human placenta. These three subunits are known to have an important role in vasodilation which is necessary for healthy blood flow within the placenta, between the maternal and foetal circulation.
This study subsequently demonstrated, for the first time, changes in nAChR subunit expression in the placentas from women who smoked during pregnancy or had developed PE, and attempted to elucidate, albeit indirectly, the protective role of smoking on PE. Results showed that α9 and δ were increased in both the smoker and PE groups. It is most likely that this was a physiological response attributable to both smoking and PE-induced hypoxia rather than purely a response to one of these factors alone. An increase in α7 expression was additionally seen in the PE group and this can be implicated in the increased inflammatory response which characterizes PE. Expression of β2 was greater in the PE compared to smoker group and it is thought smoking may be inducing its protective role in PE via this subunit by eliciting an anti-inflammatory response. Additionally, a change in α2 and β1 subunit expression was observed at the cellular level. This thesis proposes that the greater expression of α2 in smokers compared to PE may be occurring as a protective mechanism via an increase in ACh signaling whereby amino acid uptake is increased. Furthermore, it is possible that the increased expression of β1, purely via nicotine from cigarette smoke, is implicated in the increased calcification and apoptosis seen in placentas from smoking women (Jauniaux et al., 2007) via an increase in ion channel permeability and subsequent Ca\(^{2+}\) influx.

Both exogenous regulation of placental nAChRs by cigarette smoke exposure and endogenous regulation, via ACh, are therefore likely to alter nAChR subunit expression, and ultimately function, in the human placenta, impacting on infant outcomes. When comparing the nAChR subunits between smoking and PE groups, the possibility of a protective mechanism becomes an apparent possibility whereby alterations in certain subunits as a result of PE, can be counteracted by either exogenously agonizing or antagonizing the same subunits to reverse the effects. Thus clinically, this study introduces the possibility of targeting the nAChR subunits via artificial nicotine mimetic(s) to elaborate protection against this pregnancy specific disease, a path worthy of further investigation.
Chapter 5

References


Douglas N, Robinson N, Fahy K (2001). Inquiry into Obstetric and Gynaecological Services at King Edward Memorial Hospital. *Government of Western Australia*


Francis MM and Papke RL (1996) Muscle-type nicotinic acetylcholine receptor delta subunit determines sensitivity to noncompetitive inhibitors, while gamma subunit regulates divalent permeability. *Neuropharmacology* **35**:1547-1556.


