PLACENTAL TAURINE TRANSPORT IN PRE-ECLAMPSIA

A thesis submitted to the University of Manchester for the degree of PhD

in the Faculty of Medical and Human Sciences

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SCHOOL OF MEDICINE
Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>List of Figures</td>
<td>9</td>
</tr>
<tr>
<td>List of Tables</td>
<td>11</td>
</tr>
<tr>
<td>Abstract</td>
<td>12</td>
</tr>
<tr>
<td>Declaration</td>
<td>13</td>
</tr>
<tr>
<td>Copyright statement</td>
<td>13</td>
</tr>
<tr>
<td>List of abbreviations</td>
<td>14</td>
</tr>
<tr>
<td>Publications arising from this work</td>
<td>16</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>17</td>
</tr>
</tbody>
</table>

Chapter 1: Introduction ................................................................. 18

1.1 Introduction ................................................................................. 18

1.2 Overview of pre-eclampsia ...................................................... 19

1.2.1 Diagnosis, consequences and treatment .................................. 19

1.2.2 Risk factors for pre-eclampsia ............................................ 20

1.3 The placenta in normal pregnancy ........................................... 23

1.3.1 Placental development ........................................................ 23

1.3.2 Placental structure at term ............................................... 24

1.3.3 Syncytiotrophoblast renewal ............................................... 26

1.3.4 Syncytiotrophoblast function .............................................. 31

1.4 The placenta in pre-eclampsia ................................................. 34

1.4.1 Abnormal spiral artery remodelling ...................................... 34

1.5 Syncytiotrophoblast turnover in pre-eclampsia .......................... 36

1.5.1 Abnormal syncytiotrophoblast turnover associated with pre-eclampsia 36

1.5.2 Causes of dysregulated syncytiotrophoblast renewal .................. 38

1.5.3 Consequences of dysregulated syncytiotrophoblast turnover .......... 38

1.6 Taurine ......................................................................................... 40

1.6.1 Properties and synthesis of taurine ....................................... 40
1.6.2 Functions of taurine ................................................................. 41

1.7 Taurine in pregnancy .................................................................. 49

1.7.1 Taurine is required for normal fetal growth and development .... 49

1.7.2 Syncytiotrophoblast taurine transport in normal and compromised pregnancy 50

1.7.3 Regulation of TauT .................................................................. 55

1.8 Placental oxidative and nitrative stress in pre-eclampsia ............... 58

1.8.1 Important reactive oxygen/nitrogen species in the placenta ......... 58

1.8.2 Consequences of elevated reactive oxygen/nitrogen species in the placenta .... 62

1.8.3 Evidence for increased placental oxidative/nitrative stress in pre-eclampsia .... 62

1.9 Summary ................................................................................. 64

1.10 Hypothesis and aims ................................................................. 66

Chapter 2: Materials and methods .................................................. 67

2.1 Source of reagents .................................................................... 67

2.2 Study groups and placental collection ....................................... 67

Studies on Fresh Placental Tissue .................................................... 68

2.3 Measurement of TauT activity .................................................. 68

2.3.1 Placental dissection ............................................................... 68

2.3.2 $^3$H-taurine uptake assay ................................................... 68

2.3.3 Protein assay .......................................................................... 72

2.3.4 Calculation of $^3$H-taurine uptake .......................................... 72

2.4 Assessment of TauT protein expression and tyrosine nitration in placenta from normal pregnancy and pre-eclampsia .................................................. 73

2.4.1 Placental dissection ............................................................... 73

2.4.2 Preparation of membrane enriched placental villous homogenates .......... 73

2.4.3 Preparation of microvillous membranes ................................... 74

2.4.4 Protein assay of microvillous membranes .................................... 74

2.4.5 Alkaline phosphatase enrichment assay .................................... 75

2.4.6 Preparation of reagents for Western blotting .............................. 76
2.4.7 Gel electrophoresis ................................................................. 78
2.4.8 Gel transfer ............................................................................. 78
2.4.9 Antibody probing ................................................................. 78
2.4.10 Enhanced chemiluminescence (ECL) development ................. 79
2.4.11 Re-probing of nitrocellulose membranes ............................... 79
2.4.12 Densitometry ........................................................................ 79
2.5 Assessment of TauT nitration by immunoprecipitation ............... 80
  2.5.1 Sample preparation ............................................................. 80
  2.5.2 TauT immunoprecipitation ................................................... 80
  2.5.3 Western blot analysis of immunoprecipitated samples .......... 80

Placental Explant Studies .................................................................. 82
2.6 Placental explant culture preparation ...................................... 82
  2.6.1 Placental dissection ............................................................. 82
  2.6.2 Experimental procedure ...................................................... 82
2.7 Assessment of nitrotyrosine formation and syncytiotrophoblast renewal by immunohistochemistry ............................................ 83
  2.7.1 Tissue dissection and processing ........................................ 83
  2.7.2 Immunohistochemistry for nitrotyrosine, cytokeratin 7 and M30 ............................... 84
  2.7.3 Colour development and mounting ....................................... 85
  2.7.4 Dual staining for Ki67 and E-cadherin ................................... 85
  2.7.5 Liquid permanent red colour development .......................... 86
  2.7.6 Analysis of staining ............................................................ 87
2.8 Expression of results and statistics ............................................ 91

Chapter 3: Placental TauT activity in normal pregnancy and pre-eclampsia ................................................. 92
3.1 Introduction ............................................................................... 92
3.2 Methods .................................................................................. 94
  3.2.1 Measuring TauT activity in placental villous fragments .......... 94
  3.2.2 Presentation of results and statistical analysis ...................... 94
3.3 Results.........................................................................................................................96

3.3.1 Demographics .........................................................................................................96

3.3.2 Placental TauT activity in normal pregnancy and pre-eclampsia...............98

3.3.3 Placental TauT activity in pre-eclampsia in relation to fetal weight and onset of
disease .............................................................................................................................100

3.3.4 Placental TauT activity in normal pregnancy and pre-eclampsia in relation to
maternal BMI ....................................................................................................................102

3.4 Discussion....................................................................................................................106

3.4.1 Methodological considerations................................................................................106

3.4.2 Placental TauT activity is significantly lower in pre-eclampsia compared to normal
pregnancy ..........................................................................................................................108

3.4.3 Placental TauT activity in pre-eclampsia: effect of maternal BMI ....................109

3.4.4 Implications of reduced TauT activity in pre-eclampsia .................................110

3.4.5 Summary .................................................................................................................110

Chapter 4: Investigating the mechanism of reduced placental TauT activity in pre-
eclampsia ...........................................................................................................................112

4.1 Introduction .................................................................................................................112

4.2 Methods ......................................................................................................................114

4.2.1 Western blot analysis of placental TauT expression .............................................114

4.2.2 Analysis of nitrotyrosine expression using Western blotting and
immunohistochemistry .....................................................................................................115

4.2.3 Expression of results and statistical analyses .......................................................115

4.2.4 Immunoprecipitation of TauT and Western blot analysis of nitrotyrosine ......115

4.2.5 Expression of results and statistical analyses .......................................................116

4.3 Results.........................................................................................................................117

4.3.1 Demographics .........................................................................................................117

4.3.2 Placental TauT protein expression in normal pregnancy and pre-eclampsia ....119

4.3.3 Placental TauT protein activity in relation to TauT protein expression in normal
pregnancy and pre-eclampsia .........................................................................................122
4.3.4 Assessment of nitrotyrosine in syncytiotrophoblast of normal pregnancy and pre-eclampsia by immunohisotchemistry ................................................................. 124

4.3.5 Assessment of nitrotyrosine protein modifications in normal pregnancy and pre-eclampsia by Western blotting ................................................................. 126

4.3.6 Evaluation of immunoprecipitation to compare placental nitration in normal pregnancy and pre-eclampsia ...................................................................................... 128

4.4 Discussion .......................................................................................................................................................... 130

4.4.1 Placental TauT expression is higher in pre-eclampsia compared to normal pregnancy ...................................................................................................................... 130

4.4.2 Placental TauT expression is unaltered in maternal obesity compared to normal pregnancy .................................................................................................................. 132

4.4.3 TauT activity is related to TauT expression in normal pregnancy ................................................................. 132

4.4.4 Placental nitrotyrosine in pre-eclampsia and normal pregnancy ................................................................. 133

4.4.5 Evaluation of immunoprecipitation methods to compare placental TauT nitration in pre-eclampsia and normal pregnancy ................................................................. 135

Chapter 5: Consequences of inducing nitrative stress on TauT nitration and syncytiotrophoblast turnover .............................................................................................................. 138

5.1 Introduction ..................................................................................................................................................... 138

5.2 Methods .......................................................................................................................................................... 140

5.2.1 The effect of RNS on TauT activity, syncytiotrophoblast nitrotyrosine and syncytiotrophoblast turnover ......................................................................................... 140

5.2.2 The effect of reducing intracellular taurine on syncytiotrophoblast turnover .............................................. 141

5.2.3 Expression of results and statistical analysis .................................................................................................. 143

5.3 Results ........................................................................................................................................................... 145

5.3.1 TauT activity in placental villous explants treated with ONOO⁻, SIN-1 and H₂O₂ .................................................. 145

5.3.2 Nitrotyrosine staining in placental villous explants treated with ONOO⁻ and SIN-1 ............................................. 146

5.3.3 Assessment of cell turnover in SIN-1 treated explants ..................................................................................... 149

5.3.4 Effect of β-alanine on intracellular taurine .................................................................................................... 154
5.3.5 Assessment of cell turnover in β-alanine treated explants .........................155

5.4 Discussion........................................................................................................160

5.4.1 Term placental villous explants .................................................................160

5.4.2 The effect of reactive nitrogen species on TauT activity ..........................161

5.4.3 Consequences of reduced TauT activity for syncytiotrophoblast turnover......164

5.4.4 Consequences of reduced intracellular taurine for syncytiotrophoblast renewal ........................................................................................................165

5.4.5 Summary ......................................................................................................167

Chapter 6: General Discussion ............................................................................170

6.1 Syncytiotrophoblast TauT activity but not protein expression is reduced in pre- eclampsia ........................................................................................................170

6.2 Downregulation of TauT activity by nitration in pre-eclampsia .................171

6.3 Decreased TauT activity is associated with dysregulated syncytiotrophoblast turnover .............................................................................................................173

6.4 Syncytiotrophoblast TauT activity but not expression is reduced in maternal obesity ........................................................................................................175

6.5 Further implications of reduced TauT activity in pre-eclampsia and maternal obesity ........................................................................................................175

6.5.1 Reduced intracellular taurine .....................................................................175

6.5.2 Reduced cytoprotection ............................................................................177

6.5.3 Reduced delivery of taurine to the fetus ..................................................178

6.6 Other potential down-regulators of placental TauT activity in pre-eclampsia/maternal obesity ............................................................................................179

6.7 Therapeutic application of taurine ................................................................181

6.8 Conclusions ....................................................................................................181

6.9 Future Directions ...........................................................................................182

References .............................................................................................................184

Appendix - Knockdown of TauT in placental villous explants using siRNA..........206

1 Optimisation of methodology .........................................................................206
2 Results.........................................................................................................................207
  2.1 Transfection of placental villous explants with siGLO transfection indicator .......207
  2.2 siRNA knockdown of TauT in placental villous explants ........................................210

Final Word Count: 40,157
List of Figures

Figure 1.1: Placental structure at term................................................................. 25
Figure 1.2: A proposed scheme for syncytiotrophoblast renewal in normal pregnancy.. 30
Figure 1.3: Spiral artery remodelling in normal pregnancy and pre-eclampsia............ 35
Figure 1.4: Biosynthesis of taurine.................................................................... 41
Figure 1.5: Taurine transport across the syncytiotrophoblast................................. 52
Figure 1.6: Syncytiotrophoblast TauT activity in pre-eclampsia........................... 54
Figure 1.7: Proposed membrane topology of the taurine transporter...................... 57
Figure 1.8: Amino acid sequence of TauT.......................................................... 57
Figure 1.9: Generation of reactive oxygen/reactive nitrogen species in the placenta... 61
Figure 2.1: Suspension of placental villous tissue............................................. 69
Figure 2.2: Schematic of method used to measure syncytiotrophoblast TauT activity in placental villous fragments........................................................................ 71
Figure 2.3: Immunoprecipitation procedure using Protein G Immunoprecipitation Kit... 81
Figure 2.4: Example images for the quantification of M30 staining....................... 88
Figure 2.5: Example images for the quantification of ki67/E-cadherin dual staining..... 89
Figure 2.6: Example images for the quantification of cytokeratin 7 staining............ 90
Figure 3.1: Placental TauT activity in normal pregnancy and pre-eclampsia (PE)..... 99
Figure 3.2: Placental TauT activity in relation to fetal growth and onset of disease...... 101
Figure 3.3: Placental TauT activity in relation to maternal BMI............................ 103
Figure 3.4: Placental TauT activity in normal pregnancy (NP) and pre-eclampsia (PE)...... 105
Figure 4.1: Western blot analysis of TauT protein expression in membrane enriched placental homogenates.................................................................. 120
Figure 4.2: TauT protein expression in syncytiotrophoblast microvillous membrane vesicles................................................................................................. 121
Figure 4.3: Relationship between placental TauT activity and TauT protein expression in normal pregnancy and pre-eclampsia............................................ 123
Figure 4.4: Immunostaining for nitrotyrosine in placental samples from normal pregnancy and pre-eclampsia................................................................. 125
Figure 4.5: Nitrotyrosine protein expression in membrane enriched placental homogenates........................................................................................................ 127
Figure 4.6: Western blot analysis of membrane enriched placental homogenates following immunoprecipitation of TauT..................................................... 129
Figure 5.1 a: \(^3\)H-taurine accumulation by placental villous fragments over 24 hours...... 144
**Figure 5.1 b:** Intracellular taurine in explants cultured with β-alanine.......................... 144

**Figure 5.2:** Effect of peroxynitrite (ONOO\(^-\)), SIN-1 and hydrogen peroxide (H\(_2\)O\(_2\)) on TauT activity in placental explants.................................................................................. 145

**Figure 5.3:** Immunostaining for nitrotyrosine in placental explants............................... 147

**Figure 5.4:** Syncytiotrophoblast nitrotyrosine staining intensity scores in control and placental explants treated with 0.1 mM ONOO\(^-\) and 1mM SIN-1......................................................... 148

**Figure 5.5:** M30 staining for apoptosis in control and SIN-1 treated placental explants.. 150

**Figure 5.6:** Dual staining for proliferating cytotrophoblasts (CTBs) in control and SIN-1 treated placental explants...................................................................................................... 151

**Figure 5.7:** Cytokeratin 7 staining in control and SIN-1 treated placental explants.......... 152

**Figure 5.8:** Quantification of cytokeratin 7 staining in control and SIN-1 treated placental explants.............................................................................................................................. 153

**Figure 5.9:** Intracellular taurine in placental explants treated with 2.5 mM β-alanine as a percent of control................................................................................................................ 154

**Figure 5.10:** M30 staining for apoptosis in control and β-alanine treated placental explants. ............................................................................................................................... 156

**Figure 5.11:** Dual staining for proliferating cytotrophoblasts (CTBs) in control and SIN-1 treated placental explants...................................................................................................... 157

**Figure 5.12:** Cytokeratin 7 staining for syncytiotrophoblast (STB) integrity in control and β-alanine treated placental explants.................................................................................... 159

**Figure 5.13:** Quantification of cytokeratin 7 staining in control and β-alanine treated placental explants....................................................................................................................... 159

**Figure 6.1:** Proposed causes and consequences of altered placental taurine uptake in pre-eclampsia.............................................................................................................................. 184

**Appendix:**

**Figure 1:** siGLO fluorescence images for placental villous explants.............................. 208

**Figure 2:** siGLO fluorescence images for placental villous explants.............................. 209

**Figure 3:** Effect of TauT specific siRNA and non-targeting (NT) siRNA on taurine uptake into placental villous explants .......................................................................................... 211
List of Tables

Table 1.1: Risk factors of pre-eclampsia ........................................................................................................ 21
Table 1.2: Body mass index classifications according to the World Health Organisation 21
Table 1.3: Amino acid transporters in the syncytiotrophoblast .................................................................. 33
Table 1.4: Biological functions of taurine ...................................................................................................... 42
Table 1.5: Regulation of TauT mRNA/protein expression, activity and trafficking in non placental tissues .................................................................................................................................................. 55

Table 2.1: BMI category definitions used in this study as defined by the World Health Organisation ........................................................................................................................................................................ 67
Table 2.2: Composition of the gels used in gel electrophoresis ................................................................... 77
Table 2.3: Composition of the reagents used for Western blotting ................................................................. 77
Table 2.4: Details of primary and secondary antibodies used for immunohistochemistry ........................... 84
Table 2.5: Details of primary and secondary antibodies used for dual staining immunohistochemistry ............... 86

Table 3.1: Demographic data for the cohort in which placental TauT activity was determined ....................... 97
Table 4.1: Demographic data for the samples used in TauT protein expression studies ................................. 118
Table 5.1: Effects of treatment of placental villous explants with SIN-1 and β-alanine on syncytiotrophoblast turnover events on day 7 compared to untreated controls .............................................. 168

Appendix:

Table 1: Summary of changes in TauT activity following treatment of placental villous explants with siRNA or non-targeting siRNA ........................................................................................................ 210
Abstract: Submitted by Chloe Rebecca Hirst in January 2015 for the degree of Doctor of Philosophy (PhD) and entitled: Placental taurine transport in pre-eclampsia.

Pre-eclampsia (PE) is a serious disease affecting approximately 5% of pregnancies per annum. The disease etiology is complex but its origin lies in abnormal placental development and function. PE is associated with inflammation, increased nitrite stress and abnormal renewal of syncytiotrophoblast (STB), the transporting epithelium of the placenta. STB is renewed by cytotrophoblasts (CTBs) that proliferate, differentiate and fuse with STB and this is balanced by apoptosis. The amino acid taurine facilitates proliferation, differentiation and apoptosis in non-placental tissues. Taurine is also cytoprotective, protecting cells from damage by inflammatory cytokines. Taurine is transported from maternal blood into STB by the amino acid transporter TauT. In isolated STB membranes, TauT activity is inhibited by agents that nitrate tyrosine residues. This thesis tested the hypothesis that STB TauT activity is down-regulated in PE due to post-translational modification of TauT through tyrosine nitration which lowers intracellular taurine and contributes to altered STB renewal.

Placentas were collected from normal pregnancy (NP) and PE (blood pressure >140/90mmHg after 20 weeks gestation in previously normotensive women plus proteinuria >300 mg/L in a 24-hour collection). STB TauT activity, measured as Na+-dependent uptake of ³H-taurine into placental villous fragments, was significantly lower in PE (n=24) compared to NP (n=44). Western blotting of membrane enriched homogenates showed that TauT protein expression (normalised to β-actin) was significantly higher in placentas from PE (n=8) compared to NP (n=9). The presence of nitrotyrosine residues (marker of nitrite stress) in placentas of women with PE and NP was assessed by immunohistochemistry (IHC). The intensity of STB nitrotyrosine staining was greater in PE placentas that had reduced TauT activity (n=8) than in NP (n=7).

To determine the effect of nitrite stress on TauT activity and STB renewal, placental villous explants from NP were cultured (7 days; n=6) and treated with SIN-1 (1mM; days 5,6) to induce nitrite stress. STB nitrotyrosine (IHC) and TauT activity (³H-taurine uptake) was determined on day 7 and STB renewal was assessed by IHC for apoptosis (M30), proliferation (dual staining for Ki67 and the CTB marker E-cadherin) and STB integrity (cytokeratin 7). SIN-1 increased STB nitrotyrosine staining intensity compared to controls, confirming induction of nitrite stress. SIN-1 reduced STB TauT activity, increased apoptosis, reduced CTB proliferation and altered STB regeneration compared to control. To determine the effect of reducing intracellular taurine on STB renewal, villous explants were cultured for 7 days with 2.5mM β-alanine to competitively inhibit taurine uptake (n=6). At day 7, intracellular taurine, measured as the steady-state accumulation of ³H-taurine, was 15% of normal. STB turnover was assessed at day 7 as described above. β-alanine significantly increased apoptosis and altered STB regeneration compared to controls. Following statistical analysis all p <0.05.

In conclusion, STB TauT activity was lower, and protein expression higher, in PE compared to NP. STB nitrotyrosine was elevated in PE and nitrite stress inhibited STB TauT activity and disrupted STB renewal in vitro. Reducing intracellular taurine also disrupted STB renewal in vitro. Overall the data support the hypothesis that post-translational modification of TauT by nitration inhibits TauT activity in PE. This reduces intracellular taurine which contributes to abnormal renewal of STB. Further work is needed (a) to confirm that TauT is nitrated in PE and that reduced STB TauT activity lowers intracellular taurine and reduces taurine delivery to the fetus and (b) to determine the mechanism/s by which taurine regulates CTB apoptosis and facilitates renewal of STB.
Declaration

A small portion of the work referred to in the thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

A small portion of the results referred to in this thesis were submitted for the degree of MRes awarded by the University of Manchester in September 2011. These results have been incorporated into some of the final datasets presented in Chapters 3 and 4 of this thesis. 11/64 (17%) of the observations in Chapter 3 and 4/30 (13%) of the observations on the effects of SIN-1 on villous explants in Chapter 4 were submitted for the MRes (collectively they contribute 7% of the results presented in the thesis).

No portion of the remaining work referred to in the thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

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### List of abbreviations

- **AGA**: Appropriately grown for gestational age
- **BM**: Basal membrane
- **BMI**: Body mass index
- **BSA**: Bovine serum albumin
- **Cl⁻**: Chloride
- **CTB**: Cytotrophoblast
- **DAB**: Diaminobenzidine
- **DEA**: Diethanolamine
- **DMEM**: Dulbecco’s modified eagle’s medium
- **ECL**: Enhanced Chemiluminescence
- **ELCS**: Elective caesarean section
- **ELISA**: Enzyme linked immunosorbent assay
- **EMCS**: Emergency caesarean section
- **eNOS**: Endothelial nitric oxide synthase
- **ERK**: Extracellular signal-regulated kinase
- **EVT**: Extravillous trophoblast
- **FBS**: Fetal bovine serum
- **FGR**: Fetal growth restriction
- **GJIC**: Gap junction intracellular communication
- **H**: Hydrogen peroxide
- **hCG**: Human chorionic gonadotrophin
- **HCl**: Hydrochloric acid
- **HELLP**: Haemolysis elevated liver enzymes low platelet count
- **HEPES**: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
- **HPLC**: High pressure liquid chromatography
- **HSE**: Health survey for England
- **IBC**: Individualised birthweight centile
- **IHC**: Immunohistochemistry
- **iNOS**: Inducible nitric oxide synthast
- **K⁺**: Potassium
- **kDa**: Kilo Daltons
- **LGA**: Large for gestational age
- **MAPK**: Mitogen-activated protein kinase
- **mmHg**: Millimetres of mercury
- **mTOR**: Mammalian target of rapamycin
- **MVM**: Microvillous membrane
- **Na⁺**: Sodium
- **NaOH**: Sodium hydroxide
- **NBF**: Neutral buffered formalin
- **NIB**: Non immune block
- **NO**: Nitric oxide
- **NP**: Normal pregnancy
- **NPC**: Neuronal progenitor cell
- **NPY**: Neuropeptide Y
- **NT**: Non targetting
- **O₂⁻**: Superoxide
- **OAT**: Organic anion transporter
- **OH**: Hydroxyl radicals
<table>
<thead>
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<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
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<tr>
<td>ONOO⁻</td>
<td>Peroxynitrite</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PE</td>
<td>Pre-eclampsia</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein kinase A</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
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<td>RNA</td>
<td>Ribonucleic acid</td>
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<td>Reactive nitrogen species</td>
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<td>small interfering ribonucleic acid</td>
</tr>
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<td>Superoxide dismutase</td>
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<td>Syncytiotrophoblast</td>
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<tr>
<td>STBM</td>
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</tr>
<tr>
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<td>Volume regulated anion channel</td>
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<td>WHO</td>
<td>World health organisation</td>
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Publications arising from this work

Abstracts


Papers

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Chapter 1: Introduction

1.1 Introduction

Pre-eclampsia (PE) is a serious disease affecting approximately 5% of pregnancies each year worldwide (Abalos et al., 2013). It is a multisystem disorder characterised by systemic inflammation and endothelial dysfunction in the mother (Sibai et al. 2005). PE has serious consequences for both mother and infant and is associated with increased maternal and fetal mortality and morbidity. The aetiology of PE is complex and remains to be fully understood; however, the origin lies in altered placental development and function. In particular, a dysregulation of cellular renewal of the syncytiotrophoblast (STB) transport epithelium of the placenta is widely recognised as characteristic of the disease (Crocker 2007). PE is more common in women who are obese compared to their ideal weight counterparts. The reasons for this are unclear but both PE and maternal obesity are associated with increased placental inflammation and oxidative/nitrative stress; factors which have the potential to affect STB cellular turnover and nutrient transporter function.

The amino acid taurine plays an important role in fetal growth and development (Aerts and Van Assche, 2002; Sturman, 1993). The fetus and placenta cannot synthesise taurine from precursor amino acids and, as it is conditionally essential for fetal tissues during pregnancy, their requirement for taurine must be met by uptake from the maternal blood. Taurine facilitates cell turnover processes in many tissues and has a cytoprotective role, limiting damage caused by oxidative/nitrative stress and inflammation (Lang et al., 2000; Oliveira et al., 2010; Wettstein & Haussinger, 2000). Work in this laboratory has shown that the uptake of taurine into STB is lower in obese women than women of ideal weight (Desforges et al., 2013a). Furthermore, preliminary data show that STB taurine uptake is also lower in PE compared to normal pregnancy. This reduced taurine uptake in PE could deplete STB taurine concentration and contribute to abnormal STB renewal and susceptibility to cellular damage. This thesis initially tested the hypothesis that STB taurine uptake is lower in PE compared to normal pregnancy. Having demonstrated that taurine uptake is reduced in PE, the causes and consequences of reduced STB taurine uptake were explored. Understanding cause and consequence is important to determine whether future treatment strategies to
increase taurine uptake have the potential to restore STB renewal and improve maternal and fetal health in PE.

1.2 Overview of pre-eclampsia

The etiopathology of PE is multifactorial and incompletely understood. However, it is recognised that the placenta is the crucial stimulus for PE as the disease has developed in molar pregnancies where only the placenta is present (Curry et al., 1975). The origin of PE lies in the maternal response to abnormal placental development and placenta derived factors. A current hypothesis for PE pathogenesis is that abnormal uterine vascular remodelling in early pregnancy results in reduced and turbulent blood flow to the placenta which establishes an environment of oxidative/nitrative stress (considered in detail in section 1.8.3) and inflammation. This has detrimental effects on the maintenance of STB, the nutrient transporting epithelium of the human placenta, which leads to (a) necrotic/inflammatory material being deported from the placenta into maternal blood which activates the maternal endothelium to trigger PE and (b) compromised placental nutrient and endocrine function that can compromise fetal growth.

1.2.1 Diagnosis, consequences and treatment

PE is diagnosed clinically by the presence of hypertension (blood pressure ≥140/90 mmHg) in previously normotensive women plus proteinuria (urinary excretion ≥0.3 g in 24 hour collection) after 20 weeks gestation (Cudihy and Lee, 2009). Multiple phenotypes of PE exist and sub-classification of PE into early or late onset (symptoms occurring before or after 34 weeks gestation respectively) is a relatively new concept (Dadelszen et al., 2009). Typically early onset PE is a more severe phenotype associated with a higher rate of fetal growth restriction (FGR) and premature delivery (Pettit and Brown, 2012). PE is associated with increased maternal and fetal morbidity and mortality. The maternal consequences of PE include eclampsia, stroke, haemolysis elevated liver enzymes low platelet count (HELLP) syndrome, acute renal failure, liver failure, pulmonary oedema and, if the disease progresses, death (Sibai et al., 2005). PE can also have severe complications for the fetus. The disease accounts for 15% of premature births in the UK and is a major risk factor for FGR which is the failure of the fetus to reach its genetically predetermined growth potential that would have been achieved in a healthy pregnancy (Lain, 2002; Scifres and Nelson, 2009). Other consequences for the infant include hypoxia-neurologic insult and
perinatal death. PE also impacts on the long term health of both mother and baby, increasing the risk of cardiovascular disease in later life (Barker et al., 1993, 1989; Bellamy et al., 2007; Smith et al., 2009). For the mother there is also an increased risk of a recurrence of PE in future pregnancies (Pettit and Brown, 2012).

Despite extensive research, no effective biomarker has been found to reliably identify those women at risk of developing PE. Discovery of biomarkers with high sensitivity, specificity and low false positive rates will enable improved antenatal and perinatal treatments (Huppertz, 2008a). If the risk of developing PE is detected early enough, this could open a long window before the onset of symptoms to implement prevention strategies. However, should biomarkers be identified, advances will be hampered by the lack of effective treatments available. Anticonvulsants (magnesium sulphate) and blood pressure medications are given to alleviate symptoms and prevent progression to eclampsia; however, the only effective treatment is early delivery of the placenta and the baby. This is not ideal, as premature delivery of the infant is associated with increased fetal morbidity and mortality. Many potential biomarkers have been identified, often through metabolomic and proteomic approaches, including: VEGF receptor 1 (sFLT-1), PIGF, PP13, PAPP-A, leptin-receptor, inhibin-A and activin-A (Chappell et al., 2013; Kenny et al., 2014; Lai et al., 2013; Meiri et al., 2014; Myers et al., 2013; Ozdamar et al., 2014). As there is strong evidence of increased oxidative stress in PE (section 1.8.3) antioxidant treatments such as vitamin C and E were assessed as potential treatments. Although antioxidants showed promise in early clinical trials, they failed to show any useful clinical benefit in a large multicentre trial (Poston et al., 2006).

1.2.2 Risk factors for pre-eclampsia

There are several factors that increase the risk of a woman developing PE (Table 1.1) and of these maternal obesity is a major contributor. The risk of PE increases progressively with BMI; women with a BMI>30 in early pregnancy are 3 times more likely, and with a BMI>40 are 4 times more likely, to develop PE than women of ideal weight (Bianco et al., 1998; Bodnar et al., 2005; Mbah et al., 2010). This is worrying as the rate of obesity, in particular morbid obesity, is increasing worldwide. Most commonly, the calculation of body mass index (kg/m²: BMI) is used to classify weight. The classifications of BMI according to the World Health Organisation (WHO) are shown in Table 1.2.
### Table 1.1: Risk factors of pre-eclampsia. Adapted from: Sibai et al. (2005)

<table>
<thead>
<tr>
<th>Risk Factors of Pre-eclampsia</th>
</tr>
</thead>
<tbody>
<tr>
<td>Obesity (BMI&gt;30)</td>
</tr>
<tr>
<td>Family history</td>
</tr>
<tr>
<td>Previous PE</td>
</tr>
<tr>
<td>Extremes of maternal age</td>
</tr>
<tr>
<td>Renal disease</td>
</tr>
<tr>
<td>Nulliparity</td>
</tr>
<tr>
<td>Primipaternity</td>
</tr>
<tr>
<td>Multiple gestation</td>
</tr>
<tr>
<td>Maternal infection</td>
</tr>
<tr>
<td>Assisted reproduction</td>
</tr>
<tr>
<td>Pre-existing hypertensive and metabolic disorders</td>
</tr>
</tbody>
</table>

Table 1.2: Body mass index classifications according to the World Health Organisation

<table>
<thead>
<tr>
<th>Classification</th>
<th>BMI range (kg/m²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lean</td>
<td>≤18.5</td>
</tr>
<tr>
<td>Normal weight</td>
<td>18.5-24.9</td>
</tr>
<tr>
<td>Overweight</td>
<td>25-29.9</td>
</tr>
<tr>
<td>Obese class I</td>
<td>30-34.9</td>
</tr>
<tr>
<td>Obese class II</td>
<td>35-39.9</td>
</tr>
<tr>
<td>Obese class III (Morbidly obese)</td>
<td>≥40</td>
</tr>
</tbody>
</table>
Obesity has been classified as a worldwide pandemic affecting 400 million adults and the prevalence is increasing rapidly. According to WHO's most recent data, 23% of women in the UK are obese; however, this figure is expected to rise to 50% by 2050. The Health Survey for England (HSE) has estimated that if the current trend in obesity continues, the cost to the NHS by 2050 will be £4.2 billion. This worrying trend is also affecting women of reproductive age; the HSE reported an increase in obesity in women of reproductive age from 12% in 1993 to 18.5% in 2006. In the north west of England in 2006, 46% of women were overweight and 18% were obese at the start of pregnancy (Heslehurst et al., 2010). Obesity in the pregnant population has been predicted to be as much as 25.3% on an international scale (Heslehurst et al., 2010). In addition to the rising incidence of obesity it is suggested that the degree of obesity in those who are already obese is also increasing (Knight et al., 2010). The UK has seen an increase in morbid obesity in women aged 25-34 from 1.0% to 2.2% between 1993 and 2007 (Knight et al., 2010). As obesity, and in particular morbid obesity, is increasing in prevalence it is expected that the incidence of PE will increase in parallel with this.

Independent of increasing the risk of PE, there are serious complications associated with obesity in pregnancy for both mother and fetus. Detrimental effects include increased likelihood of large for gestational age (LGA) births, stillbirth with FGR, premature delivery, miscarriage, congenital abnormalities and neonatal death (Andreasen et al., 2004; Guelinckx et al., 2008; Wallace et al., 2012). As there is a wealth of evidence that disorders of fetal growth are associated with cardiovascular and metabolic disease in later life (Barker et al., 1993, 1989), obesity in pregnancy severely impacts on the health of the next generation.

In summary, PE is a serious condition of pregnancy, arising from placental disease. It is well recognised that raised maternal BMI increases the risk of PE but the reasons why obese mothers are more likely to develop PE than their ideal weight counterparts are not understood. However, as PE originates from a disease of the placenta, it is probable that the increased risk of PE in maternal obesity is mediated by effects on the placenta. The next section considers placental development in normal pregnancy.
1.3 The placenta in normal pregnancy

1.3.1 Placental development

The development of the placenta is a carefully orchestrated process which involves the adaptation of the maternal circulation by embryonic derived cells to allow sufficient perfusion of the feto-placental unit with maternal blood. This ensures the efficient transfer of nutrients across the placenta to satisfy the increasing demands of the growing fetus.

From day 12, after implantation, a population of cytotrophoblast (CTB) cells proliferate and differentiate into STB and extravillous trophoblasts (EVTs) which assume an invasive phenotype. The EVTs form columns which migrate and invade into the endometrium and top third of the myometrium. EVTs are crucial not only for anchorage of the placenta but also for the adaption of the maternal circulation to establish sufficient perfusion of the feto-placental unit with maternal blood (Benirschke et al., 2006). EVTs invade into uterine spiral arteries and transform them from narrow coiled vessels (as seen in non-pregnant women) into large, high flow, low resistance vessels. This remodelling process involves the replacement of smooth muscle cells with EVTs which leads to a loss of elasticity and maternal vasomotor control. This is a mechanism to prioritise the needs of the growing fetus and guarantees blood supply to the fetal-placental unit regardless of maternal attempts to redistribute blood flow e.g. during exercise (Gude et al., 2004). Spiral artery remodelling in early pregnancy is absolutely essential for a healthy pregnancy and failure to adequately remodel these vessels is associated with several pregnancy complications including FGR, spontaneous miscarriage, stillbirth and PE (Kam, 1999; Lyall, 2005).

Non migratory CTBs proliferate, differentiate and fuse to form an overlying STB layer. The early villous stage of placental development begins 13 days post conception. Primary villi are formed by protrusions of STB into the intervillous space (Benirschke et al., 2006; Huppertz, 2008b) (Figure 1.1). Fetal mesenchyme then penetrates into the primary villi, giving them a mesenchymal core, to form secondary villi. The vascularisation of the secondary villi with fetal capillaries transforms them into tertiary villi by the third week of gestation (Huppertz, 2008b).
1.3.2 Placental structure at term

The mature placenta is a large disk-shaped organ that is connected to the fetus by the umbilical cord. The placenta consists of a maternal and fetal side and is composed of three principle tissues (1) the trophoblast, (2) the connective tissue with chorionic membrane and blood vessels and (3) the amnion (Benirschke, 1998). There is wide variation between placentas including differences in shape, size, thickness and location of cord insertion. The fetal side consists of the chorionic plate which is covered by the amnion. The chorionic plate contains blood vessels from the umbilical cord which branch across its surface (Figure 1.1 a). Further branching of the chorionic vessels as they descend into the placenta forms the main functional unit, the villous trees. There are three main types of placental villi; stem, intermediate and terminal villi (Figure 1.1 b). The extent of this branching gives a very large surface area for efficient transfer of nutrients, of which the majority is thought to occur via the terminal villi. The villous trees are surrounded by STB which is a highly specialised multinucleate epithelial cell (Figure 1.1 c). The villous STB is in direct contact with maternal blood which perfuses the intervillous space (Figure 1.1 a). The blood is supplied to this space by the maternal spiral arteries and drains through the endometrial veins (Gude et al., 2004). It is important to note that the maternal and fetal circulations remain separate for the entirety of pregnancy (Pijnenborg et al., 2006). The maternal side of the placenta is known as the basal plate. This side of the placenta is divided in 10-40 regions known as lobules (Huppertz, 2008b). The fetus is enclosed in a membranous sac consisting of an inner amnion and outer chorion.
Figure 1.1: Placental structure at term

(a) Cross section of the placenta. Vessels in the umbilical cord branch across the chorionic plate and branch further as they descend into the placenta forming villous trees. Villous trees are bathed in maternal blood, supplied by the endometrial spiral arteries, which fills the intervillous space. Adapted from: Moore et al. (2003). (b) A mature villous tree indicating the three main types of villi; stem, intermediate and terminal villi. Adapted from: Lecuit et al. (2004). (c) Cross section of a terminal villous. Fetal capillaries at the villous core are surrounded by the outer trophoblast layer comprised of cytotrophoblasts and the syncytiotrophoblast. Adapted from: Malassine et al. (2008).
1.3.3 Syncytiotrophoblast renewal

STB is a highly specialised nutrient transporting epithelium that forms a continuous multinucleated layer surrounding the villous tree. STB has an apical, maternal facing microvillous membrane (MVM) in contact with maternal blood and a fetal facing basal membrane (BM) in close proximity to the fetal capillaries. The STB is terminally differentiated and must therefore be maintained by fusion of underlying CTBs. CTBs are mononucleated progenitor cells which maintain the STB by undergoing proliferation and differentiation before finally fusing into the STB (Mayhew, 2014) (Figure 1.2). To maintain the CTB population, when CTBs proliferate, one daughter cell remains as a progenitor cell whilst the other goes on to differentiate and fuse into the STB (Mayhew, 2014). As well as having a maintenance role it is also thought that fusion of the CTB into the STB is necessary to deliver molecules that cannot be synthesised by this layer but are necessary to maintain function (Castellucci and Kaufmann, 2006).

STB is renewed continuously throughout pregnancy by the process of cellular turnover. The proliferation, differentiation and fusion of the CTBs into the STB must be balanced by apoptosis in order to maintain tissue homeostasis (Heazell and Crocker, 2008). The mechanisms underlying the process of CTB proliferation and differentiation are not well understood and the role of apoptosis in STB turnover is subject to debate.

The involvement of several growth factors for CTB proliferation has been demonstrated in CTB cells and intact tissue including EGF, TGF-α and IGF-I and II (Forbes et al., 2008; Li and Zhuang, 1997).

The importance of several proteins has been implicated in promoting CTB differentiation and fusion including cytokines, growth factors, protein kinases, transcription factors and proteases (Huppertz and Gauster, 2011). Gap junctions are clusters of transmembrane channels composed of connexin hexamers (Cronier et al., 2003). Protein and mRNA expression of connexin 43 has been demonstrated in trophoblast (Cronier et al., 2002). Treatment of CTB with heptanol, a non-specific junctional un-coupler which blocks all connexin channels, demonstrated the importance of connexins for trophoblast gap junction intracellular communication (GJIC) (Cronier et al., 1994). Inhibition of connexin 43 in isolated primary CTBs suppressed their fusion in vitro (Frendo et al., 2003a) and GJIC is a prerequisite for CTB differentiation and fusion (Cronier et al., 2003; Malassiné and Cronier, 2005). Other proteins including, ADAM 12, phosphatidylycerine, GCM-1, cadherins and
Syncytins 1 and 2 are also important for CTB differentiation and fusion (Aplin et al., 2009; Frendo et al., 2003b; Getsios and MacCalman, 2003; Huppertz et al., 2006a). Syncytins are retroviral envelope glycoproteins encoded by the HERV-W (syncytin 1) and HERV-FRD (syncytin 2) genes. The syncytins are fusogenic glycoproteins which are highly expressed in placenta (Blaise et al., 2005; Lee et al., 2001; Mi et al., 2000). Stimulation of CTB differentiation is associated with an increase in syncytin mRNA and protein expression and inhibition of syncytin reduces fusion of primary CTBs in vitro (Frendo et al., 2003b; Langbein et al., 2008).

Mounting evidence indicates that apoptosis is involved in the development, remodelling and aging of the placenta. In addition there is evidence that members of the apoptotic pathway, caspase 8 and caspase 14, play a role in the fusion of CTBs into the STB (Black et al., 2004; Gauster et al., 2010; White et al., 2007). However, the evidence for the role of caspases in STB turnover is inconsistent and contradictory. There are two pathways of apoptosis; the extrinsic or receptor mediated pathway and the intrinsic or mitochondrial pathway. Using placental villous tissue sections several studies have demonstrated that CTB and STB express anti-apoptotic proteins such as Bcl-2, Bax and Mcl-1 (Huppertz et al., 1998; Ratts et al., 2000; Soni et al., 2010), proteins of the extrinsic apoptosis pathway such as fas, fas-L and caspase 8 (De Falco et al., 2004; Huppertz et al., 1998; Runic et al., 1996; Uckan et al., 1997) and proteins of the intrinsic pathway such as caspase 9 and Apaf-1 (De Falco et al., 2004). The importance of apoptosis for the fusion of CTBs into the STB is supported by evidence that blocking members of the apoptotic process (e.g. caspase 8) inhibits CTB fusion (Black et al., 2004). However, the literature on the role of caspase 8 is inconsistent. Two reviews of the involvement of caspase 8 in CTB differentiation concluded that activated-caspase 8 may be involved in CTB differentiation and fusion into the STB (Gauster and Huppertz, 2010; Rote et al., 2010). However, Guilbert and colleagues re-examined the role of caspase 8 activation in CTB differentiation by using three inhibitors of caspase 8 and found no evidence that differentiation of isolated CTBs into multinucleated STB in culture is blocked by inhibition of caspase 8 (Guilbert et al., 2010).

Several issues have been highlighted surrounding the involvement of apoptosis in STB turnover. Firstly, as Burton and Jones (2009) point out, if the initiation of the apoptotic cascade is required for CTB fusion, this process must then be suspended for a period of 2-3 weeks during their time in the STB, to prevent widespread apoptosis in this layer (Burton
and Jones, 2009). Exactly how this would occur is unknown, although it has been suggested that as CTBs are continuously recruited to the STB they donate anti-apoptotic proteins such as Bcl-2 and Mcl-1 which act to prolong the suspension of apoptosis for several weeks (De Falco et al., 2005). There has also been controversy more recently over the phenotype of the trophoblast which is undergoing apoptosis. The reason for these conflicting results lies in part in the difficulty in identifying and discerning the CTBs from the STB nuclei. Longtine et al. (2012) recently demonstrated that CTBs are frequently found interdigitated into the STB which could therefore be mistaken for STB nuclei unless a marker of CTBs (such as E-cadherin) is used. In addition, the study by Longtine et al. (2012) provided compelling data that apoptosis is actually confined to CTBs, and does not occur in STB. Furthermore, it was suggested that the absence of apoptosis in the STB is an important mechanism to prevent apoptosis from spreading uncontrollably throughout the STB. Finally, it has been noted that the apoptotic theory of STB turnover is not supported by morphological observations e.g. membrane blebbing of nuclei has never been observed despite this being a characteristic of end stage apoptosis (Burton and Jones, 2009; Huppertz et al., 2006b).

The final element of trophoblast turnover in normal pregnancy is the clustering of syncytial nuclei into syncytial nuclear aggregates (syncytial knots) (Figure 1.2). It has been proposed that these knots are then shed into the maternal circulation. Particles from STB have been identified in the uterine venous blood (Johansen et al., 1999). The deported material from STB then circulates to the maternal lungs where macrophages phagocytose apoptotic material (Askelund and Chamley, 2011; Pantham et al., 2011). Apoptosis has also been suggested to have a role in the removal of aged nuclei from the STB (Huppertz, 2008b). It was suggested that CTB proliferation and fusion into the overlying STB culminates in the aggregation of aged and apoptotic nuclei into syncytial knots which are then shed into the maternal circulation; a hypothesis which proved popular for many years (Huppertz, 2008b). However, recent evidence has challenged the apoptotic nature of syncytial knots as well as the mechanism by which STB material is deported into the maternal blood. A recent study has demonstrated, using thorough immunohistochemistry (IHC) and morphological examination, that although syncytial knots are a feature of normal pregnancy they show little evidence of apoptosis (Coleman et al., 2013). Sectioning tissue for assessment of syncytial knots by IHC produces sectioning artefacts and false knots (intervillous bridges). Sectioning artefacts were found to comprise approximately 80% of apparent syncytial nuclear aggregates in the study by Coleman et al. (2013). It has been suggested that serial
sectioning and IHC markers of transcriptional activity and oxidative damage should be employed to distinguish true knots and false knots (Coleman et al., 2013; Mayhew, 2014).

Although the mechanisms of STB renewal in normal pregnancy remain controversial, there is substantial evidence to support abnormalities in the processes of STB renewal in pregnancies complicated by FGR, PE and maternal obesity (considered further in section 1.5.1); notably an increase in apoptosis and the shedding of apoptotic material (Allaire et al., 2000; Burton and Jones, 2009; Higgins et al., 2013; Huppertz et al., 2006b; Ishihara et al., 2002) and disrupted proliferation/differentiation (Arnholdt et al., 1991; Brown et al., 2005; Higgins et al., 2013; Langbein et al., 2008).
Cytotrophoblasts (CTB) maintain the syncytiotrophoblast (STB) by proliferating and differentiating before fusing into the overlying STB. Incorporation of CTB nuclei is balanced by apoptosis. In this scheme, apoptotic nuclei are shown to gather into protrusions termed syncytial knots which are shed into the maternal blood (taken from: (Huppertz, 2008b)). Recently this model has been challenged and although nuclei aggregate into clusters which can protrude from the STB, they may not be apoptotic and the mechanism/s by which material is deported from STB remain poorly understood (see text section 1.3.3; (Burton and Jones, 2009; Coleman et al., 2013; Mayhew, 2014)).
1.3.4 Syncytiotrophoblast function

The essential functions of the placenta in supporting the growth of the fetus throughout gestation are performed by the STB and include nutrient and gas transfer, immunoprotection and endocrine production/secretion (Burton and Jones, 2009).

As the STB is the site of nutrient and gas exchange between mother and fetus, any disruption to this compromises nutrient supply to the fetus. There are potentially three routes for nutrient exchange across the STB; paracellular diffusion, transporter-protein mediated transfer and endocytosis/exocytosis (Sibley et al., 1997). Glucose is transported across the placenta by facilitated diffusion through specific glucose transporters (GLUTs) (Lager and Powell, 2012). Primary active transport, utilising hydrolysis of ATP, mediates the transport of nutrients such as calcium and secondary active transport, utilising ion gradients, is responsible for the transport of many nutrients including amino acids, phosphorus and lactate (Lager and Powell, 2012). Several specific amino acid transporters have been identified and well characterised on the STB MVM and BM (Table 1.3). The transport of nutrients across the BM to the fetus is less well characterised than the transport of solutes across the MVM. Both amino acid exchangers and accumulative transporters are present on the BM. However, as amino acid exchangers can only alter amino acid composition and not total concentration and accumulative transports facilitate transport in the direction of the Na⁺ gradient (i.e into the STB), these cannot mediate the efflux of amino acids to the fetus (Cleal and Lewis, 2008). Cleal and colleagues (2011) examined the potential role of facilitated transporters in the efflux of amino acids across the BM. Using Western blotting the presence of the facilitated transporters TAT1, LAT3 and LAT4 on the BM was demonstrated. In addition, using the isolated placental perfusion model non-exchange transport systems were demonstrated for L-alanine, L-tyrosine, L-phenylalanine, L-isoleucine and L-valine; all substrates for TAT1, LAT3 and LAT4 (Cleal et al., 2011).

The placenta plays a protective role in two ways: firstly by preventing the transfer of toxic substances to the fetus and secondly by ‘shielding’ the fetus from the maternal immune system (Burton et al., 2006; Gude et al., 2004). A number of active transport proteins and enzymes are expressed by the placenta which protects the fetus by preventing the transfer of bacteria and viruses as well as xenobiotics, drugs and other toxic substances. The expression of a multitude of cytochrome P450 enzymes which are responsible for the
detoxification of drugs and xenobiotics have been identified in the human placenta (Prouillac and Lecoeur, 2010). The placenta also contains a number of enzymes involved in steroid hormone metabolism, importantly 11β-hydroxysteroid dehydrogenase which is responsible for the conversion of cortisol to inactive cortisone thus protecting the fetus against maternal glucocorticoids (Benediktsson et al., 1997; Bernal et al., 1980). The placenta is also involved in protecting the fetus from rejection by the maternal immune system, although exactly how this occurs is still unknown as the placenta as well as the fetus is semi-allogeneic.

STB produces and secretes a variety of hormones which sustain pregnancy and support fetal growth, these include human chorionic gonadotrophin (hCG), progesterone, oestrogen, placental lactogen, pregnancy specific B1-glycoprotein, growth hormones, collagenases and thrombomodulin (Burton et al., 2006). The maintenance of a healthy placenta throughout pregnancy is essential to its function. Abnormalities of STB renewal are evident in PE and have severe consequences for both fetal and maternal health including (a) initiating PE in vulnerable mothers and (b) compromising nutrient transport to the fetus.
### Table 1.3: Amino acid transporters in the syncytiotrophoblast

<table>
<thead>
<tr>
<th>Gene Name (protein)</th>
<th>System</th>
<th>Specificity</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>SLC38A1 (SNAT1)</td>
<td>A</td>
<td>Ala, Ser, Cys, Gln, Asn, His, (Met), MeAIB</td>
<td>Highly polarised to MVM, also on BM</td>
</tr>
<tr>
<td>SLC38A2 (SNAT2)</td>
<td>Na⁺ dependent cotransporter</td>
<td>Gly, Pro, Ala, Ser, Cys, Gln, Asn, His, MeAIB</td>
<td></td>
</tr>
<tr>
<td>SLC38A4 (SNAT4)</td>
<td>Na⁺ dependent cotransporter</td>
<td>Gly, Ala, Ser, Cys, Asn, (Met), (MeAIB)</td>
<td></td>
</tr>
<tr>
<td>SLC1A4 (ASCT1)</td>
<td>ASC</td>
<td>Ala, Ser, Cys</td>
<td>BM</td>
</tr>
<tr>
<td>SLC1A5 (ASCT2)</td>
<td>Na⁺ dependent exchanger</td>
<td>Ala, Ser, Cys, Thr, Gln</td>
<td></td>
</tr>
<tr>
<td>SLC6A6 (TauT)</td>
<td>β/TauT Na⁺ dependent cotransporter</td>
<td>β-alanine, taurine</td>
<td>Highly polarised to MVM, also on BM</td>
</tr>
<tr>
<td>SLC38A5 (SNAT5)</td>
<td>N Na⁺ dependent, Gln and H⁺ gradient dependent</td>
<td>His, Gln, Asn</td>
<td>MVM</td>
</tr>
<tr>
<td>SLC7A5 (LAT1)</td>
<td>Na⁺ independent exchanger</td>
<td>Leu, His, Iso, Val, Met, Tyr, Phe, Trp, BCH, L-DOPA</td>
<td>MVM, BM</td>
</tr>
<tr>
<td>SLC7A8 (LAT2)</td>
<td>Na⁺ independent exchanger</td>
<td>Ala, Ser, Cys, Thr, Asn, Gln, His, Met, Leu, Iso, Val, Phe, Tyr, Trp</td>
<td></td>
</tr>
<tr>
<td>SLC43A1 (LAT3)</td>
<td>Facilitated Diffusion</td>
<td>Leu, Phe, Iso, Val, Met, BCH</td>
<td>? – Low/no mRNA present</td>
</tr>
<tr>
<td>SLC43A2 (LAT4)</td>
<td>Facilitated Diffusion</td>
<td>Phe, Leu, Iso, Met, Leu⁺</td>
<td>? – mRNA present</td>
</tr>
<tr>
<td>SLC7A1 (CAT1)</td>
<td>y⁺ Uniporter</td>
<td>Lys, Arg, His</td>
<td>MVM, BM</td>
</tr>
<tr>
<td>SLC7A2 (CAT2B)</td>
<td>y⁺ Uniporter</td>
<td>Lys, Arg, His</td>
<td></td>
</tr>
<tr>
<td>SLC7A4 (CAT4)</td>
<td>y⁺ Uniporter</td>
<td>Lys, Arg</td>
<td></td>
</tr>
<tr>
<td>SLC7A7 (y⁺LAT1)</td>
<td>y⁺L Na⁺ dependent for neutral amino acids</td>
<td>Lys, Arg, Gln, His, Met, Leu⁺</td>
<td>MVM, BM</td>
</tr>
<tr>
<td>SLC7A6 (y⁺LAT2)</td>
<td>y⁺L Na⁺ dependent for neutral amino acids</td>
<td>Lys, Arg, Gln, His, Met, Leu⁺, Ala, Cys</td>
<td></td>
</tr>
<tr>
<td>SLC1A1 (EAAT3)</td>
<td>X⁻AG Na⁺ dependent cotransporter</td>
<td>Glu, Asp, Cys</td>
<td>MVM, BM</td>
</tr>
<tr>
<td>SLC1A2 (EAAT2)</td>
<td>Na⁺ dependent cotransporter</td>
<td>Glu, Asp</td>
<td></td>
</tr>
<tr>
<td>SLC1A3 (EAAT1)</td>
<td>Na⁺ dependent cotransporter</td>
<td>Glu, Asp</td>
<td></td>
</tr>
<tr>
<td>SLC16A10 (TAT1)</td>
<td>TAT1 Facilitated diffusion</td>
<td>Phe, Tyr, Trp, (Ala, Leu), L-DOPA</td>
<td>BM</td>
</tr>
</tbody>
</table>

Characteristics of the amino acid transporters which have been identified in the STB including solute specificity and location. Adapted from: Jansson (2001) and Cleal & Lewis (2008).

BCH = 2-aminobicyclo-(2,2,1)-heptane-2-carboxylic acid, Ala=L-alanine, Arg =L-arginine, Asn=L-asparagine, Asp=L-aspartate, Cys=L-cysteine, Glu=L-glutamate, Gln=L-glutamine, Gly=L-glycine, His=L-histidine, Iso=L-isoleucine, Leu=L-leucine, Lys=L-lysine, Met=L-methionine, Phe=L-phenylalanine, Pro=L-proline, Ser=L-serine, Thr=L-threonine, Trp=L-tryptophan, Tyr=L-tyrosine, Val=L-valine.
1.4 The placenta in pre-eclampsia

1.4.1 Abnormal spiral artery remodelling

A key feature of PE is incomplete uterine spiral artery remodelling in early pregnancy. As described in section 1.3.1, in normal pregnancy EVTs invade into the endometrium and top third of the myometrium, replacing spiral artery smooth muscle cells to remodel the vessels, creating dilated, low resistance channels. In PE there is shallow trophoblast invasion which is limited to the decidual regions (Figure 1.3) (Pijnenborg et al., 2006). The arteries remain narrow high resistance vessels which most likely leads to decreased placental perfusion; Doppler ultrasound measurements of the uterine artery confirm abnormal flow-velocity waveforms in PE indicating raised downstream vascular resistance (Aardema et al., 2001). Evidence to support a link between aberrant spiral artery remodelling and reduced placental perfusion comes from studies that have related Doppler measurements of uterine artery blood to histological analysis of spiral arteries (Pijnenborg et al., 2006). Mechanisms proposed for the reduced spiral artery remodelling in PE include reduced expression of adhesion molecules in the maternal endothelium (e.g. PCAM, VCAM, αβ3-integrins), impaired natural killer cell function and exaggerated maternal defence against trophoblast invasion (increased activation of maternal macrophages) (Goldman-Wohl and Yagel, 2002; Kaufmann et al., 2003; Zhou et al., 1993).
Figure 1.3: Spiral artery remodelling in normal pregnancy and pre-eclampsia

In normal pregnancy spiral arteries in the decidua and top third of the myometrium are remodelled by invading trophoblasts, producing dilated low resistance vessels. In pre-eclampsia this remodelling is limited to the decidual region which leads to reduced placental perfusion. Taken from: Bell (2004).
1.5 Syncytiotrophoblast turnover in pre-eclampsia

1.5.1 Abnormal syncytiotrophoblast turnover associated with pre-eclampsia

Abnormal renewal of the STB is a key feature of PE and involves the dysregulation of the normal processes of cellular renewal (section 1.3.3).

Despite the controversy surrounding the mechanism of STB turnover in normal pregnancy it is clear that apoptosis is elevated in the pregnancy diseases of FGR and PE (Burton and Jones, 2009; Huppertz et al., 2006b). Alterations in pro and anti-apoptotic markers in placenta have been demonstrated in a number of studies. Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) identifies DNA fragmentation and is used as a marker of apoptosis. Trophoblast TUNEL staining is increased in PE compared to normal pregnancy (Allaire et al., 2000; Ishihara et al., 2002). The Fas receptor is a member of the cell death receptor family and binding with its ligand, FasL, induces apoptosis. A decrease in FasL (Allaire et al., 2000; Tomas et al., 2011) and an increase in Fas receptor protein expression (Allaire et al., 2000) have been demonstrated in placenta in PE. An increase in pro-apoptotic proteins p53, Bax and smac and a decrease in anti-apoptotic proteins Bcl-2 and Mdm2 has also been demonstrated in PE (Cobellis et al., 2007; Heazell et al., 2008a, 2005; Ishihara et al., 2002). Other markers have been used to show increased placental apoptosis in PE including increased M30 staining, which identifies a cleavage product of cytokeratin 18, increased caspase 9 activity, and increased DNA fragmentation (Prusac et al., 2011; Shaker and Sadik, 2013; Tomas et al., 2011). A recent study by Longtine et al. (2012) demonstrated increased apoptosis isolated to CTBs by identifying increased expression of cleavage products of cytokeratin 18 and PARP1 in combination with E-cadherin staining by high resolution confocal microscopy.

Using isolated CTBs from normal term placenta and those complicated by PE, Crocker and colleagues (2003) have demonstrated an increased susceptibility of CTBs from PE to cytokines and hypoxia-induced apoptosis (Crocker et al., 2003). Consistent with this, placental villous explants taken from pregnancies complicated by PE demonstrate increased apoptosis in response to hypoxia and tumour necrosis factor-α (TNF-α) (Crocker et al., 2004a). These data suggest the placenta in PE has an increased susceptibility to apoptosis induced by hypoxia/inflammatory cytokines. Studies have also demonstrated that an exaggerated apoptotic response can be induced in placental villous explants from
normal pregnancy in response to hypoxia, inflammatory cytokines and reactive oxygen species (ROS), all of which are features present in vivo in PE (Crocker et al., 2004a; Heazell et al., 2008b; Moll et al., 2007).

Less work has been carried out investigating the proliferation, differentiation and fusion of CTBs in PE. In the limited number of studies available, proliferation has been shown to be both increased and unchanged in PE. BrdU and Ki67 immunostaining of villous tissue identified an increase in CTB proliferation in PE (Arnholdt et al., 1991). Additionally, a recent study by Unek and colleagues (2014) demonstrated an increase in proliferation markers Ki67 and proliferating cell nuclear antigen (PCNA) in villous tissue from PE compared to normal pregnancy (Unek et al., 2014). The study also identified increased staining intensity of cell cycle inhibitors p27 and p57. Conversely, Prusac et al. (2011) found no difference in proliferation between placentas from PE and normal pregnancy using Ki67 (Prusac et al. 2011). Therefore further work is necessary to elucidate if alterations to the proliferation and cell cycle arrest mechanisms contribute to the dysregulation of STB turnover in PE. Brown et al. (2005) used E-cadherin as a marker of CTB differentiation, as the expression of this cell-cell adhesion protein is lost in CTBs as they differentiate. In placental tissue samples from PE, a raised expression of E-cadherin was confirmed by IHC and Western blot analysis. This could mean that CTB differentiation is compromised in PE, or it could indicate increased proliferation; however, both have relevance to the pathology of PE (Brown et al., 2005).

There is also evidence to suggest that fusion of CTBs into the STB is altered in PE compared with normal pregnancy. Syncytin mRNA expression has been shown to be reduced in the placenta in PE compared to normal pregnancy and the syncytin protein improperly localised to the apical membrane instead of the basal membrane (Knerr et al., 2002; Lee et al., 2001). Furthermore, using quantitative reverse transcriptase polymerase chain reaction and Western blot analysis, Vargas and colleagues (2011) demonstrated a reduction in syncytin mRNA and protein expression in the placenta in PE and a correlation between reduced syncytin expression and severity of PE (Vargas et al., 2011).

In summary, evidence suggests that in PE there is an imbalance between proliferation and apoptosis and possibly differentiation/fusion which will compromise STB maintenance and function.
1.5.2 Causes of dysregulated syncytiotrophoblast renewal

Inflammatory cytokines which may be locally produced as consequence of ischaemia-reperfusion in PE have been shown to disrupt turnover events in *in vitro* models (Crocker et al., 2004b, 2001). In addition PE tissue is more susceptible to these cytokines than normal pregnancy (Crocker et al., 2003). Several papers have demonstrated that apoptosis can be induced in trophoblast cells and villous tissue by exposure to ROS. Moll et al. (2007) showed that apoptosis is significantly increased in placental explants after exposure to H$_2$O$_2$. Similarly Heazell et al. (2009) have demonstrated that exposure of BeWo cells, a trophoblast–derived choriocarcinoma cell line, to H$_2$O$_2$ increases apoptosis, decreases cell viability, decreases hCG secretion and decreases cell fusion. Furthermore, Kudo et al. (2003) assessed the effect of hypoxia (2% O$_2$) on the rate of cell fusion in BeWo cells and found fusion to be suppressed in hypoxic conditions (Kudo et al., 2003). Heazell et al. (2007) also demonstrated that H$_2$O$_2$ increases the formation of syncytial knots. Collectively these studies provide evidence that cell turnover is disrupted in response to ROS.

1.5.3 Consequences of dysregulated syncytiotrophoblast turnover

1.5.3.1 Increased deportation of syncytiotrophoblast material into maternal blood

It has been demonstrated that deportation of STB material is elevated in PE compared to normal pregnancy (Askelund and Chamley, 2011). In addition, damage occurring to the STB in PE through disrupted STB turnover causes necrosis to become the primary cell death mechanism and exposes the CTB (Huppertz and Kingdom, 2004). It is further proposed that release of this necrotic material may be responsible for causing maternal endothelial dysfunction characteristic of PE.

The apoptotic nature of syncytial knots is currently under debate. It has also been noted that a key feature of apoptosis is that it does not stimulate an inflammatory response as material is packaged into sealed syncytial vesicles (Burton and Jones, 2009). However, increased release of necrotic material from the STB, not in membrane sealed structures, would be pro-inflammatory. A recent study demonstrated using IHC that there are more syncytial knots in placentas from PE compared to normal pregnancy (Calvert et al., 2013). It is suggested that the turbulent maternal blood flow in the intervillous space that is suggested to occur in PE could dislodge aggregates protruding from the STB more
frequently (Burton and Jones, 2009). STB material is found in the maternal circulation in normal pregnancy and several papers provide evidence for significantly higher levels of circulating STB microparticles (STBM) in PE (Aly et al., 2004; Germain et al., 2007; Knight et al., 1998). It has also been found that circulating STBMs appear in higher concentrations in early onset PE than late onset PE (Y. Chen et al., 2012; Goswami et al., 2006). It has been suggested that STB material is cleared by phagocytosis in the lungs which protects endothelial cells against activation (Q. Chen et al., 2012). The presence of STB material in the maternal lungs has been demonstrated in several studies (reviewed by: Askelund and Chamley, 2011). STBMs isolated from PE have been shown to be capable of stimulating the production of several inflammatory cytokines including IL-18 and IFN-γ (Germain et al., 2007). Furthermore it has been demonstrated that phagocytosis of necrotic material induces activation of endothelial cells and necrotic material has been shown to lead to hypertension in rats (Chen et al., 2006; Lau et al., 2013a). Therefore the disruption to STB turnover and increased deportation of necrotic material in PE may be responsible for inducing endothelial dysfunction in PE.

In addition to avoiding the deportation of pro-inflammatory material, STB integrity is of the upmost importance for the efficient transfer of nutrients between mother and fetus. Fetal growth is directly related to STB volume and in PE altered STB cellular renewal from proliferation through to apoptosis results in altered STB volume and thickness (Crocker, 2007). Therefore altered renewal of STB leading to compromised nutrient transfer could underlie FGR in PE.

1.5.3.2 Syncytiotrophoblast function in pre-eclampsia

There have been few studies of nutrient transport by the STB in PE. Shibata et al (2008) demonstrated no difference in placental system A activity in PE compared to normal pregnancy. However, a preliminary study in this laboratory demonstrated that the activity of the taurine transporter protein (TauT) in STB was significantly lower in PE compared to normal pregnancy (section 1.7.2, Figure 1.6) (Hirst et al., 2012). Taurine is an amino acid with unique properties that include promoting organ growth and differentiation, and it is required for normal development of the placenta and fetus. Therefore, depletion of taurine in the STB could have adverse consequences for both STB renewal and fetal growth in PE. The functions of taurine are discussed in detail in the following sections.
1.6 Taurine

1.6.1 Properties and synthesis of taurine

Taurine, 2-aminoethanolsulphonic acid, is one of the most abundant amino acids in the human body (Huxtable, 1992; Lourenço and Camilo, 2002). Taurine is highly conserved throughout the animal kingdom and is present at a high concentration in many cell types allowing it to perform a wide variety of functions which collectively benefit cell and tissue wellbeing (Section 1.6.2). Taurine differs from other amino acids in that it is a β and not an α-amino acid and it contains a sulfonate group instead of a carboxylate group (Lambert et al., 2014). The presence of a carboxylate group means that taurine has no net charge and is therefore a zwitterion. This zwitterionic nature infers the characteristics of water solubility and low lipophilicity (Huxtable, 1992). Low lipophilicity means that diffusion of taurine through lipophilic membranes is slow. Taurine is ubiquitous throughout the human body with the highest concentrations in bile (200 µmol/L), breast milk (337 µmol/L), retina (30-40 µmol/g) and platelets (16-24 µmol/g) (Lourenço and Camilo, 2002). High intracellular concentrations of taurine can be achieved due to the action of the taurine transporter, the lipophobic properties of taurine and the fact that taurine is not metabolised or incorporated into protein and therefore remains free in the intracellular environment (Han et al., 2006; Huxtable, 1992).

The main dietary sources of taurine are meat and seafood; however adults can synthesise taurine from cysteine and methionine via the cysteine sulfinate pathway (Figure 1.4).
1.6.2 Functions of taurine

Taurine has a wide diversity of functions in a variety of tissues including the brain, cardiovascular system, liver, kidneys, musculature and reproductive system (Table 1.4). Although these functions attributed to taurine are well established, the signalling mechanisms by which taurine mediates these effects have not been extensively studied. The varied functions of taurine confer many beneficial effects which are exemplified by its use therapeutically e.g. in cardiovascular disease, hypercholesterolemia, epilepsy, Alzheimer’s, alcoholism and cystic fibrosis without any side effects (Birdsall, 1998). Magnesium sulphate is routinely administered in an attempt to delay the onset of eclampsia. However, magnesium taurate has been suggested as a better alternative to magnesium sulphate due to the wider therapeutic benefits including antivasospastic, antihypertensive, platelet-stabilizing, anticonvulsant and hypoxia-protective properties (McCarty, 1996).
<table>
<thead>
<tr>
<th>System</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cardiovascular</td>
<td>Antiarrythmic</td>
</tr>
<tr>
<td></td>
<td>Hypotensive</td>
</tr>
<tr>
<td></td>
<td>Chronotropic</td>
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<tr>
<td></td>
<td>Modulation of calcium</td>
</tr>
<tr>
<td>Brain</td>
<td>Anticonvulsant</td>
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<tr>
<td></td>
<td>Modulation of neuronal excitability</td>
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<tr>
<td></td>
<td>Maintenance of cerebellar function</td>
</tr>
<tr>
<td></td>
<td>Thermoregulation</td>
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<tr>
<td>Central nervous system</td>
<td>Neuroprotection</td>
</tr>
<tr>
<td></td>
<td>Modulation of neurotransmitter release</td>
</tr>
<tr>
<td>Retina</td>
<td>Promotes development and maintains structure of photoreceptors and outer segments</td>
</tr>
<tr>
<td>Liver</td>
<td>Bile salt synthesis</td>
</tr>
<tr>
<td></td>
<td>Bile acid conjugation</td>
</tr>
<tr>
<td>Reproductive System</td>
<td>Sperm motility</td>
</tr>
<tr>
<td>Musculature</td>
<td>Muscle membrane stabilizer</td>
</tr>
<tr>
<td></td>
<td>Increase exercise capacity</td>
</tr>
<tr>
<td>General</td>
<td>Osmoregulation</td>
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<tr>
<td></td>
<td>Antioxidation</td>
</tr>
<tr>
<td></td>
<td>Modulation of apoptosis</td>
</tr>
<tr>
<td></td>
<td>Regulation of phosphorylation</td>
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<td></td>
<td>Cell viability, proliferation, differentiation, migration</td>
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<td></td>
<td>Calcium homeostasis</td>
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<td></td>
<td>Protects against inflammatory cytokines</td>
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<td></td>
<td>Protects gap junctions against oxidative stress</td>
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<td></td>
<td>Protects against ischaemia-reperfusion injury</td>
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<tr>
<td></td>
<td>Promotes mitochondrial function</td>
</tr>
<tr>
<td></td>
<td>Detoxification of xenobiotics</td>
</tr>
</tbody>
</table>

This table details some of the biological actions of taurine in several systems (Huxtable, 1992; Lambert et al., 2014; Lourenço and Camilo, 2002; Ripps and Shen, 2012; Schaffer et al., 2014)
There are three main functions attributed to taurine that might be important in maintaining STB in health and disease: a) its ability to regulate cell volume b) its role in facilitating appropriate proliferation, differentiation, fusion and apoptosis and c) its role in protecting cells against ischaemia-reperfusion injury, oxidative stress and inflammatory cytokines. These are further considered in the following sections.

1.6.2.1 Osmoregulation

The osmoregulatory role of taurine is one of the longest recognised functions (Huxtable, 1992). It is vital that cells adapt to osmotic stresses as osmotic imbalances that are left uncorrected will lead to changes in cell volume. Cells face a constant challenge to maintain their volume as physiological and pathophysiological conditions such as nutrient transport, epithelial absorption/secretion, hormonal stimulation, induction of apoptosis, hypoxia and ischaemia alter cell volume (Lambert et al., 2008). Maintaining the correct cell volume is important; an increase can lead to cell rupture and a decrease can alter concentrations of cell constituents which will impact on biochemical processes and intracellular signalling cascades thus affecting cell viability (Huxtable, 1992; Lambert et al., 2008). Maintenance of cellular hydration state is also essential for tissue renewal as it must be regulated during processes such as proliferation, differentiation and apoptosis (Lang et al., 1998). It is therefore necessary that cells have mechanisms in place to alter the movement of water and restore cell volume and function (Lambert et al., 2008).

To avoid volume perturbations cells must maintain osmotic equilibrium across their membrane (Lang et al., 1998). One mechanism for cell volume regulation involves altering the transport of solutes across the membrane. Due to the physiochemical properties of taurine it meets almost perfectly the characteristics for the ideal osmoregulator leading to it becoming known as a compatible osmolyte (Han et al., 2006; Huxtable, 1992). Following osmotic swelling taurine is lost from the cell via a volume sensitive taurine efflux pathway and the uptake of taurine via TauT is decreased (Lambert, 2004). To regulate their hydration state most cells activate volume-sensitive efflux pathways for osmotically active substances such as ions and organic osmolytes in response to cell swelling (Lambert, 2004). As taurine is not metabolised, the activity of both TauT and the unknown taurine efflux pathway are responsible for determining intracellular concentrations of taurine. The osmoregulatory role of taurine has been demonstrated in several non placental tissues,
including the heart, brain and renal cells (Zhang et al., 2008) and in the STB (Remsbury Thesis, University of Manchester; Vallejos & Riquelme 2007).

Therefore it is necessary for cells to maintain high intracellular taurine in order to facilitate maintenance of cellular hydration state. In turn, maintenance of hydration state is essential for tissue renewal processes such as cell proliferation, differentiation and apoptosis (Lang et al., 2000). The interaction between volume regulatory mechanisms and the cell machinery leading to proliferation and apoptosis are not well understood (Lang et al., 2000). However, it remains a possibility that a reduction in intracellular taurine concentration may contribute to altered STB renewal involving altered proliferation and apoptosis in PE.

1.6.2.2 Taurine as a modulator of apoptosis

One of the mechanisms for taurine modulation of apoptosis is through its role as an osmoregulator, discussed above. Cell shrinkage is an essential early event in apoptosis required to activate the enzymes that initiate the apoptotic cascade (Maeno et al., 2000). Nishimura et al. (2010) exposed a rat STB cell line (TR-TBT 18d-1 cells) to hypertonic stress to induce apoptosis, in the presence and absence of taurine. The addition of taurine to the culture media blocked hypertonicity induced apoptosis (Nishimura et al., 2010). However, whether taurine regulates apoptosis in human STB through effects on cell volume have not been investigated.

Aside from regulating cell volume, taurine has a role in attenuating apoptosis and/or protecting cells from apoptotic stimuli in a variety of tissues. For example, taurine protects against hyperglycaemia induced apoptosis (Ulrich-Merzenich et al., 2007; Verzola et al., 2002; Wu et al., 1999) and prevents islet cell apoptosis in fetuses of rat dams fed on a protein restricted diet (Boujendar et al., 2002). The mechanisms for how taurine regulates apoptosis remain to be fully understood; however, the evidence suggests that taurine could act at several points in the apoptotic pathway.

Using rat neonatal cardiomyocytes, Takatani et al. (2004) have shown that ischaemia-induced apoptosis is attenuated by taurine. This study presented evidence that taurine may bring about its anti-apoptotic effects through the inhibition of APAF-1/caspase 9 apoptosome formation which is necessary for the apoptotic cascade to continue (Takatani et al., 2004b). Furthermore, the same group has demonstrated that the Akt-caspase 9 pathway is also inhibited by taurine through the prevention of caspase 9 cleavage (Takatani
et al., 2004a). Maher and colleagues have shown that IL-2 and CD3 induced T cell apoptosis is inhibited by pre-treatment with taurine, through down-regulation of the Fas-L protein (Maher et al., 2005). Taurine has a key role as an antioxidant (see section 1.6.2.4) and in human umbilical vein endothelial cells taurine prevented apoptosis induced by ROS (Wu et al., 1999).

Studies on primary cultures of CTB from human placenta have shown that taurine protects cells from apoptosis induced by the inflammatory cytokine TNF-α. CTB cells with siRNA-mediated knockdown of TauT demonstrated a 12 fold increase in apoptosis in response to controls (Desforges et al., 2013b). This could be of significance for PE because TNF-α is elevated in maternal plasma in this condition (Lau et al., 2013b; Wang and Walsh, 1996).

1.6.2.3 The role of taurine in cell proliferation, differentiation and fusion

It has been demonstrated in several tissues that taurine can promote cell proliferation. The mechanisms for the stimulatory effect of taurine are incompletely understood and may differ between cell types. In both embryonic and adult mouse neuronal progenitor cells (NPC), the addition of taurine to culture media significantly increased proliferation (Hernández-Benítez et al., 2012, 2010). In adult mouse NPCs an increase in BrdU positive cells and a lack of effect on cell viability led the authors to conclude that the increase in NPC number was due to elevated proliferation and not enhanced cell survival (Hernández-Benítez et al., 2012). Taurine supplementation also increased proliferation of NPCs from the mouse dentate gyrus (Shivaraj et al., 2012) and from human fetal brain (Hernández-Benítez et al., 2013). In experiments on human embryonic kidney 293 cells, knockdown of TauT, the taurine transporter, by RNAi significantly reduced cell proliferation (Han and Chesney, 2013).

A number of mechanisms have been proposed for the pro-proliferative effects of taurine. Hernandez and colleagues noted that it appears that taurine does not act as a mitogen itself but has a stimulatory effect once a cell has initiated proliferation (Hernández-Benítez et al., 2012). It has also been noted that rather than directly affecting the DNA replication process, taurine acts by increasing the number of healthy cells and provides better conditions for the cell to enter the proliferative cycle (Ramos-Mandujano et al., 2014). The same study showed that taurine increased the proportion of cells in the S phase (DNA replication stage) of the proliferation cycle, and decreased the proportion in the G0/G1 phase (resting phase) without affecting the length of the cell cycle. Consistent with this, knockdown of TauT in embryonic kidney 293 cells demonstrated reduced proliferation due
to cell cycle arrest in the G2 phase (Han and Chesney, 2013). This study also showed that several genes involved in cell cycle regulation such as CDK6 and CDC7 were significantly reduced following knockdown of TauT. It could be that taurine improves conditions to encourage transition between phases of the cell cycle (Ramos-Mandujano et al., 2014). Other possibilities include an effect of taurine on modulating calcium homeostasis, a general effect on membrane stability or the antioxidant role of taurine preventing redox imbalance and maintaining favourable conditions for proliferation (Hernández-Benítez et al., 2012; Ramos-Mandujano et al., 2014). The role of taurine as an osmoregulator has also been implicated in its stimulatory effect on proliferation as cell swelling is involved in proliferation. Several signalling pathways have also been implicated including the extracellular signal-regulated kinase (ERK), mitogen-activated protein kinase (MAPK), Wnt and sonic hedgehog pathways (Hernández-Benítez et al., 2012; Jeon et al., 2007; Ramos-Mandujano et al., 2014).

There is limited evidence regarding the role of taurine in differentiation, however in the rat liver taurine has been shown to preserve GJIC, which is important for CTB differentiation (Fukuda et al., 2000). Taurine has been demonstrated to be important for differentiation and fusion of skeletal muscle and placenta. Skeletal muscle and STB are fairly unique in that fusion to make syncytia is part of their differentiation process. Taurine supplementation has been shown to promote differentiation and fusion of mouse myoblasts in culture (Miyazaki et al., 2013). In addition, reduced intracellular taurine following knockdown of TauT has been shown to compromise differentiation and fusion in primary CTBs (Desforges et al., 2013b).

1.6.2.4 Taurine protects against ischaemia-reperfusion injury and oxidative stress

Taurine is cytoprotective in a number of ways; it protects against ischaemia-reperfusion injury, inflammatory cytokines and oxidative stress through its role as an antioxidant.

The mechanism behind the ability of taurine to act as an antioxidant remained elusive for many years. It was demonstrated over 20 years ago that taurine is unable to directly scavenge ROS (Aruoma et al., 1988). Taurine therefore must act indirectly to protect against ROS-induced damage. This could be achieved in part by the ability of taurine to increase or restore levels of several antioxidant enzymes (Nonaka et al., 2001; Vohra and Hui, 2001; Yildirim et al., 2007). Emerging evidence suggests the ability of taurine to prevent ROS formation may be due to its role in protecting mitochondrial function.
Damaged mitochondria and defects in mitochondrial protein synthesis and electron transport chain activity can lead to a severe increase in mitochondrial oxidative stress generation (Jong et al., 2012, 2010). It has been demonstrated that taurine is required for the efficient translation of mitochondrial proteins and respiratory complex formation which are essential to maintain normal mitochondrial function (Jong et al., 2012, 2010). An important role for taurine as a mitochondrial matrix buffer stabilising oxidative metabolism has also been proposed (Hansen et al., 2006). These studies suggest that by stabilising the environment and promoting mitochondrial function, taurine can act indirectly as an antioxidant by preventing the release of ROS. Interestingly, mitochondrial dysfunction has been reported in PE (Mandò et al., 2014).

Using a variety of techniques to induce oxidative stress in several different tissues, studies have demonstrated the protective effects of taurine. Hagar et al. (2004) examined the effects of taurine supplementation on Cyclosporin A-induced oxidative stress and hepatotoxicity in rats. The study found that taurine protected against oxidative stress and hepatotoxicity through a reduction in lipid peroxidation, and an increase in antioxidant enzyme activity (glutathione peroxidase) (Hagar, 2004). Similarly, Oudit et al. (2004), using iron overload-induced oxidative stress in mouse myocardial cells, demonstrated that taurine reduced oxidative stress through a reduction in lipid peroxidation (Oudit et al., 2004). Similar results have been shown in rat cardiomyocytes and rat hepatocytes (Fukuda et al., 2000; Ghosh et al., 2009). Fukuda et al. (2008) also demonstrated that taurine protects hepatocyte GJIC, necessary for maintaining liver function, from oxidative stress induced by H$_2$O$_2$.

ROS generation is a major phase of ischaemia reperfusion injury and an important determinant of the outcome following such an injury. As the rate of ROS generation is dependent on oxygen availability, during the ischaemic insult the ROS generation is low, however still sufficient to cause damage. During reperfusion there is a massive influx of oxygen and consequently a significant increase in ROS production (Schaffer et al., 2014). Taurine is protective against ischaemia-reperfusion injury as it regulates several events that determine the outcome of ischaemia-reperfusion, including inflammation, maintenance of ion homeostasis, osmoregulation and antioxidant capacity (Schaffer et al., 2014). Several studies have shown that taurine attenuates the effects of ischaemia-reperfusion injury in rat hearts by increasing antioxidant availability, decreasing lipid peroxide accumulation and
reducing apoptosis/necrosis (Oriyanhan et al., 2005; Shiny et al., 2005; Takahashi et al., 2003). Hanna and colleagues (2004) also demonstrated that supplementation of drinking water with 0.1 mM taurine for 6 months prior to injury protected rat hearts against ROS generation during an ischaemia-reperfusion injury (Hanna et al., 2004). Interestingly, a later study compared the effects of the addition 10 mM taurine prior to an ischaemic insult to the presence of 10 mM taurine for the first 10 minutes of reperfusion (i.e. when ROS production is at its highest) in isolated rat hearts. In both circumstances taurine had a protective effect; however the most potent effect was demonstrated with taurine present during reperfusion (Ueno et al., 2007). Furthermore it is not just treatment with taurine prior to ischaemia-reperfusion injury that is shown to have beneficial impact. A study which induced an ischaemia-reperfusion injury by occluding the middle cerebral artery in rats found that taurine supplementation post treatment offered a dose-dependent neuroprotective effect by decreasing neuronal loss and reducing apoptosis, a finding that could have a clinical implication for stroke patients (Wang et al., 2007). Similar cytoprotective effects of taurine have been demonstrated in the liver, intestine and skeletal muscle in several animal models through a reduction in lipid peroxidation, apoptosis, necrosis and TNF-α levels (Kincius et al., 2007; McLaughlin et al., 2000; Tong et al., 2006; Wettstein and Haussinger, 2000; Zhang et al., 2008). In humans, taurine has been shown to have a clinical protective benefit against ischaemia-reperfusion injury during coronary bypass surgery. Taurine supplementation three hours prior to surgery significantly reduced lipoperoxidation, mitochondrial cell damage and myocardial necrosis during reperfusion compared to a placebo group (Milei et al., 1992).
1.7 Taurine in pregnancy

1.7.1 Taurine is required for normal fetal growth and development

Taurine is conditionally essential for the growing fetus. In contrast to adults, fetal and placental tissues cannot synthesise taurine from cysteine and methionine as they lack the necessary enzymes (Gaull et al., 1972). Therefore the fetal tissues rely entirely on the supply of taurine from the mother and the transport across the placenta. The highest concentrations of taurine are found in the fetus in the developing brain when the concentrations of other amino acids are relatively low, highlighting the importance of taurine for brain development. As development progresses the concentration of taurine in the brain falls producing concentrations in adults about a third of those found in neonates (Huxtable, 1992).

The importance of taurine for fetal development has been demonstrated in several animal studies. Cats have been used extensively as an animal model to study the effects of taurine deficiency since they possess a very limited capacity to synthesise taurine and rely on dietary intake. In cats, a taurine deficient diet leads to growth restriction and spontaneous abortions. Those kittens which do survive exhibit a variety of neurological abnormalities including delayed cerebellar cell division and migration and abnormal cortical development (Sturman, 1993). The kittens also display impaired neurologic function later in development. Several groups have used TauT knockout mice to prevent the uptake of taurine into cells and explore the importance of taurine for development and demonstrated that taurine deficiency results in a wide range of abnormalities including: fetal growth restriction, retinal degradation, decreased renal function, cardiac and skeletal muscle abnormalities (Heller-Stilb et al., 2002; Ito et al., 2010; Warskulat et al., 2007). In humans, the concentration of taurine in placenta was found to be significantly lower in low birth weight infants compared to normally grown infants (Ghisolfi et al., 1989). It has also been demonstrated that cord venous plasma taurine concentration is lower in FGR compared to normally grown fetuses suggesting the involvement of taurine in this pregnancy pathology (Cetin et al., 1990; Economides et al., 1989). Furthermore a study conducted by Mortensen et al. (2010) demonstrated that maternal low protein diet-induced FGR in mice could be rescued by the supplementation of taurine in the drinking water of the dams (Mortensen et al., 2010). These studies demonstrate the importance of
taurine for fetal growth and brain, kidney, retinal and muscle development. However, there is a less information on the importance of taurine for placental development.

The importance of taurine for neonatal development is underscored by its addition to infant formulas. The concentration of taurine is particularly high in human milk but in contrast cow’s milk (the major protein source of most of the worlds infant formula) contains only 10% of the taurine in human milk (Aerts and Van Assche, 2002; Chesney et al., 1998). Since the newborn has a very limited capacity to synthesise taurine, intake via maternal milk is essential to satisfy demand. Due to the absence of taurine in infant formula, early studies compared the plasma and urine concentrations in human milk fed and formula fed infants. Formula fed infants had higher free amino acid concentrations in both plasma and urine compared to human milk fed infants with taurine being the notable exception. The plasma and urine concentration of taurine in formula fed infants fell throughout the duration of the studies (Gaull et al., 1977). Falling urine taurine values are indicative of an attempt to conserve taurine by the kidney (Chesney et al., 1998). Several animal and human studies started in the 1970’s, investigated the consequences of taurine deficiency or supplementation on development. Studies inducing taurine deficiency in cats and monkeys demonstrated significant retinal abnormalities in taurine deficient animals compared to taurine supplemented animals (Hayes et al., 1975; Sturman et al., 1984). In human studies, children receiving >95% of their calorie intake for extended periods of time from parenteral solutions, deficient in taurine, had reduced plasma taurine concentrations and retinal abnormalities which were corrected by the addition of taurine (Ament et al., 1986). The findings of these studies and the conditionally essential nature of taurine in newborn nutrition led to the addition of taurine to infant formulas from 1981 (Chesney et al., 1998; Gaull, 1989).

1.7.2 Syncytiotrophoblast taurine transport in normal and compromised pregnancy

Taurine is actively transported into tissues by the amino acid transporter TauT, previously known as system-β due to its high affinity for β amino acids (Karl and Fisher, 1990; Lambert, 2004). TauT is a sodium (Na⁺) and chloride (Cl⁻) dependent transporter which is encoded by the gene SLC6A6. The transport of taurine into cells occurs by secondary active transport which involves utilising the movement of Na⁺ down its electrochemical gradient
to provide the energy for taurine uptake (Han et al., 2006). Low intracellular Na⁺, and thus a Na⁺ concentration gradient, is achieved by the action of Na⁺/K⁺ ATP-ase (Figure 1.5). TauT transports Na⁺, Cl⁻ and taurine into cells in the ratio 2:1:1 (Ramamoorthy et al., 1994b). Taurine can also be transported across the brush border membrane of human intestines by the Na⁺/Cl⁻ independent, H⁺ coupled transporter PAT1 (SLC36A1) (Anderson et al., 2009). Transport of taurine by an organic anion transporter (OAT) (SLC22A13) has also been shown in human embryonic kidney 293 cells (Schulz et al., 2014). Taurine uptake into cells is balanced by the passive release via a yet unidentified transport protein. Several putative volume-sensitive anion transporters/channels for taurine export have been proposed including ICln, ClC-3, Cl⁻/HCO₃⁻ exchanger the “Maxi” Cl⁻ channel and volume regulated anion channels (VRAC) (Lambert, 2004; Lang et al., 1998; Riquelme, 2009; Vallejos and Riquelme, 2007).

MVM studies of STB confirmed the presence of a Na⁺ and Cl⁻ dependent taurine transporter with a stoichiometry for Na⁺, Cl⁻, taurine of 2:1:1 over two decades ago (Miyamoto et al., 1988; Ramamoorthy et al., 1994b). Later studies comparing TauT protein activity on STB MVM and BM showed that TauT is strongly polarised to the MVM (Figure 1.8) with activity on the BM only 6% of that on the MVM (Norberg et al. 1998; Jansson 2001). This activity data has been consolidated by a study demonstrating that the protein expression of TauT is primarily localised to the MVM (Roos et al., 2004). At the BM, TauT would take taurine up from the fetus into the STB according to the electrochemical gradient for Na⁺. The activity of TauT allows the STB to accumulate taurine producing concentrations in the STB approximately 150 times greater than in the maternal blood (Philipps et al., 1978). Taurine efflux across the BM is not well characterised and occurs via an as yet unidentified transporter. Taurine is thought to be transported across the BM to the fetus via volume regulated anion channels (VRAC) (Vallejos and Riquelme, 2007). By treating STB MVM vesicles with tyrosine specific reagents, Kulanthaivel et al. (1988) demonstrated that tyrosine residues are essential for the optimal activity of TauT.
Figure 1.5: Taurine transport across the syncytiotrophoblast

Taurine is co-transported into the syncytiotrophoblast with Na\(^+\) and Cl\(^-\) by the Na\(^+\)-dependent amino acid transporter TauT, present on the microvillous membrane (MVM) and on the basal membrane (BM) with a lower activity. Taurine efflux across the BM to the fetus is less well characterised but probably occurs via volume regulated anion channels (VRAC). The action of a Na\(^+\)/K\(^+\) ATP-ase maintains the Na\(^+\) concentration gradient which provides energy for taurine uptake. The concentration of taurine in maternal blood is estimated at 60 μM. The action of TauT allows the syncytiotrophoblast to accumulate taurine to a concentration of around 10mM; the concentration in fetal blood is estimated at 135 μM (Philipps et al., 1978).
It is recognised that taurine is the most abundant free amino acid in the STB (Philipps et al., 1978). Despite the fact that the STB accumulates taurine so effectively the significance of maintaining such a high taurine concentration for STB wellbeing has yet to be investigated.

Placental TauT activity has been examined in three pregnancy complications, FGR, maternal obesity and in a preliminary study in PE. These conditions are associated with disordered fetal growth as well as abnormal STB renewal, and in all cases activity is reduced compared to normal pregnancy. TauT activity, but not protein expression, is lower in STB MVM from pregnancies complicated by FGR compared to appropriately grown infants (Norberg et al., 1998; Roos et al., 2004). This probably reduces taurine delivery to the fetus as plasma taurine concentration is lower in FGR compared to normally grown fetuses; these data support the importance of taurine for appropriate fetal growth (Cetin et al., 1990; Economides et al., 1989). STB TauT activity has also been examined in relation to maternal BMI in women having babies that were an appropriate weight for gestational age. TauT activity was significantly lower in obese (BMI>30kg/m²) compared to ideal weight women and negatively related to maternal BMI (Desforges et al., 2013a). In recent preliminary studies, STB TauT activity has also been determined in women experiencing PE compared to normal pregnancy. Figure 1.6 shows preliminary data that TauT activity is significantly lower in PE. However, in this small study four babies born to women with PE were FGR, and as STB TauT activity is lower in placentas of FGR babies (Norberg et al., 1998) it remains to be confirmed in a larger study that PE is associated with reduced placental TauT activity.
Bearing in mind the functions of taurine in regulating proliferation, differentiation, fusion and apoptosis, and in offering cytoprotection (reviewed in sections 1.6.2.2 - 1.6.2.4) it is plausible that a reduction in intracellular taurine consequent on reduced STB TauT activity in pregnancy complications could compromise STB renewal and function. To test this proposal, experiments were performed on primary cultures of CTB’s isolated from normal term placenta and TauT mRNA and protein expression was knocked down with TauT specific siRNA. Treatment with TauT siRNA (a) reduced TauT mRNA expression by 71%, (b) reduced TauT protein expression, evidenced by reduced immunofluorescence of TauT, (c) reduced TauT activity by 64% and (d) reduced intracellular taurine to 31% of that in control cells (Desforges et al., 2013b). This was associated with a significantly lower fusion of CTB’s to form multinucleated cells, indicating the importance of maintaining intracellular taurine for facilitating CTB differentiation and fusion, processes that are essential for STB renewal in vivo. In addition, following knockdown of TauT, CTB apoptosis in response to the inflammatory cytokine TNF-α was increased 12 fold. These experiments demonstrate a cytoprotective role for taurine in the placental trophoblast (Desforges et al., 2013b).
Therefore it is proposed that intracellular taurine depletion in vivo, as a consequence of reduced TauT activity, could contribute to the abnormal STB renewal that is a key feature of PE. The mechanism of the reduction in STB TauT activity has not yet been investigated and is important to determine factors that regulate TauT to develop potential strategies to restore STB intracellular taurine in the future.

### 1.7.3 Regulation of TauT

Regulation of TauT mRNA expression, protein expression, activity and trafficking as well as post translational regulation of transporter activity, has been reported in various tissue/cell types (Table 1.5).

**Table 1.5: Regulation of TauT mRNA/protein expression, activity and trafficking in non placental tissues**

<table>
<thead>
<tr>
<th>Regulator</th>
<th>Cell Type</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Substrate availability</strong></td>
<td>Pig kidney proximal tubule cell line (LLC-PK1), Madin Darby canine kidney cells (MDCK)</td>
<td>(Chesney et al., 1990; Han et al., 1998; Jones et al., 1990)</td>
</tr>
<tr>
<td><strong>Osmolarity/tonicity</strong></td>
<td>MDCK, brain capillary endothelial cell line (TR-BBB13), mouse fibroblast cells (NIH3T3)</td>
<td>(Hansen et al., 2012; Kang et al., 2002; Uchida et al., 1991)</td>
</tr>
<tr>
<td><strong>Protein kinases (PKC, PKA)</strong></td>
<td>Xenopus oocytes, LLC-PK1, MDCK</td>
<td>(Han et al., 1999; Jones et al., 1991; Loo et al., 1996; Mollerup and Lambert, 1996; Voss et al., 2004)</td>
</tr>
<tr>
<td><strong>Casein kinase 2</strong></td>
<td>NIH3T3</td>
<td>(Jacobsen et al., 2008)</td>
</tr>
<tr>
<td><strong>TNF-α</strong></td>
<td>NIH3T3</td>
<td>(Voss et al., 2004)</td>
</tr>
<tr>
<td><strong>Reactive oxygen species</strong></td>
<td>NIH3T3</td>
<td>(Hansen et al., 2012)</td>
</tr>
<tr>
<td><strong>Ca²⁺/calmodulin</strong></td>
<td>LLC-PK1, human retinal pigment epithelial cell line (HRPE)</td>
<td>(Han et al., 2000; Ramamoorthy et al., 1994a)</td>
</tr>
<tr>
<td><strong>cisplatin</strong></td>
<td>LLC-PK1</td>
<td>(Han &amp; Chesney 2005)</td>
</tr>
<tr>
<td><strong>Calcitriol and retinoic acid</strong></td>
<td>LLC-PK1</td>
<td>(Han and Chesney, 2005; Han et al., 2002)</td>
</tr>
<tr>
<td><strong>P53</strong></td>
<td>Human embryonic kidney 293 cells, rat renal proximal tubule cell line (NRK-52E)</td>
<td>(Han and Chesney, 2005; Han et al., 2002)</td>
</tr>
<tr>
<td><strong>Transcription factors e.g. WT1</strong></td>
<td>293 cells</td>
<td>(Han &amp; Chesney 2003)</td>
</tr>
</tbody>
</table>

Factors regulating TauT and the cell types in which they were identified.
TauT can be post-translationally modified by phosphorylation, glycosylation or nitration. Hydropathy plots of TauT suggest that there are twelve hydrophobic transmembrane domains with four potential N-glycosylation sites, several potential phosphorylation sites for protein kinase C (PKC), protein kinase A (PKA) and casein kinase II (CK2) (Figure 1.7) (Lambert and Hansen, 2011; Lambert, 2004; Uchida et al., 1992) and 34 tyrosine residues which are potential nitration sites (Figure 1.8). The serine 322 residue was identified as the critical site for PKC regulation of TauT (Figure 1.7/1.8) (Han et al., 1999). Phosphorylation of TauT by PKC inhibits TauT activity (Tappaz, 2004).
Figure 1.7: Proposed membrane topology of the taurine transporter

Cloning of TauT from MDCK cells suggests the protein is 621 amino acids long and has twelve transmembrane domains. Putative N-linked glycosylation sites (Y) and phosphorylation sites (p) are indicated. Ser-322 has been identified as the critical site of phosphorylation of TauT by PKC (indicated by the green circle). Adapted from: Uchida et al. (1992)

Figure 1.8: Amino acid sequence of TauT

The amino acid sequence of TauT indicating Ser-322 (S), the critical site of phosphorylation by PKC, the 34 tyrosine residues which are putative nitration sites (Y) and four potential N-linked glycosylation sites (N) (Han et al., 1998; Jhiang et al., 1993).
There have been a number of studies that have addressed the mechanisms that regulate TauT in trophoblast. In common with several cell types (Jones et al., 1990), TauT activity is regulated by substrate availability. This adaptive regulation, whereby TauT activity is down-regulated when extracellular taurine is increased, has been demonstrated in the JAr choriocarcinoma cell line (Jayanthi et al., 1995). Again using JAr cells, Kulanthaivel et al. (1991) showed that activators of PKC inhibited TauT activity. Later work by Roos et al. (2004) confirmed that activation of PKC reduced TauT activity in STB. Furthermore, neuropeptide Y (NPY), which activates PKC in STB and is elevated in maternal plasma in PE, inhibited TauT activity in placental villous explants (Desforges et al. 2013). Inhibition of the Ser/Thr protein kinase, mammalian target of rapamycin (mTOR), has been shown to reduce TauT activity in cultured primary CTBs (Roos et al., 2009). It has also been demonstrated in fetal blood cells that TauT can be glycosylated (Iruloh et al., 2007) but this has not been explored in placenta.

Post translational modification of TauT by nitration is another potential regulator of TauT that has been explored in placenta. Using nitrating agents which specifically target tyrosine residues, Kulanthaivel and colleagues (1989) demonstrated in STB MVM that tyrosine groups on TauT can be nitrated, and that nitration leads to a reduction in TauT activity. Treatment of STB MVM, and villous fragments, with the drug 3-morpholinosydnonimine (SIN-1) to induce nitrative stress, significantly reduced TauT activity (Khullar et al., 2004). Similarly, a reduction in taurine uptake in a rat STB cell line (TR-TBT cells) treated with SIN-1 has been demonstrated (Lee and Kang, 2010). There is preliminary evidence that the proportion of TauT that is nitrated is higher in PE compared to normal pregnancy (Quoted in a review by: (Webster et al., 2008)). As PE and maternal obesity are associated with elevated placental nitrate stress (described in section 1.8.3), the possibility arises that STB TauT activity is reduced in these pregnancy conditions as a result of TauT tyrosine nitration.

1.8 Placental oxidative and nitratative stress in pre-eclampsia

1.8.1 Important reactive oxygen/nitrogen species in the placenta

It has been suggested that defective spiral artery remodelling in PE results in an intermittent, turbulent blood supply to the placenta which will lead to periods of hypoxia followed by re-oxygenation, the basis of ischaemia-reperfusion type injury (Myatt, 2010; Raijmakers et al., 2004). Ischaemia-reperfusion injury is recognised as an important consequence of reduced perfusion in many organs (Hung et al., 2001). During ischaemia
cells are deprived of oxygen, however during reperfusion there is massive influx of oxygen and as a result ROS production rapidly increases (Schaffer et al., 2014). This increase in ROS leads to the production and release of inflammatory mediators. Ischaemia reperfusion injury is also associated with increased shedding of necrotic material from the STB as a consequence of the turbulent flow (section 1.5.3).

Oxidative and nitrative stress occurs in normal pregnancy but is exaggerated in pregnancy pathologies such as FGR, PE (Myatt et al., 1996) and gestational diabetes (Lyall et al., 1998). Oxidative and nitrative stress arise when the production of ROS and reactive nitrogen species (RNS) exceed the antioxidant defences. The main ROS/RNS produced by the placenta (Figure 1.9) are superoxide ($O_2^-$), nitric oxide (NO), peroxynitrite (ONOO$^-$) and hydrogen peroxide ($H_2O_2$). $O_2^-$ is formed from the reduction of molecular oxygen by NADPH oxidases, xanthine oxidase and the mitochondrial electron transfer chain (Poston and Raijmakers, 2004). NO is synthesised by endothelial nitric oxide synthase (eNOS) and under inflammatory conditions, inducible nitric oxide synthase (iNOS; reviewed by: (Pacher et al., 2007) and (Webster et al., 2008)). The antioxidant defences in the placenta include superoxide dismutase (SOD), catalase, glutathione peroxidise, glutathione and ascorbate which scavenge $O_2^-$. NO is removed through its rapid diffusion through tissues into red blood cells where it is converted to nitrate by oxyhaemoglobin (Joshi et al., 2002). These effective means of minimising $O_2^-$ and NO accumulation means that neither $O_2^-$ or NO are particularly toxic in vivo (Pacher et al., 2007). It is important that the balance between $O_2^-$ and NO production is tightly regulated as this determines their physiological effects (Myatt, 2010). The rate of reaction between $O_2^-$ and NO is much quicker than $O_2^-$ and SOD. It is estimated that the rate of reaction between $O_2^-$ and NO is $\sim 10 \times 10^9 \text{M}^{-1} \text{s}^{-1}$ whereas $O_2^-$ reacts with SOD at a rate of $\sim 1-2 \times 10^3 \text{M}^{-1} \text{s}^{-1}$ (Radi, 2004). Therefore when $O_2^-$ and NO are produced within a few cell distances of each other, NO outcompetes SOD for $O_2^-$ and ONOO$^-$ is spontaneously formed (Radi, 2004). NO can diffuse readily through membranes up to a distance of 100 µm in its half life of 0.5 sec however $O_2^-$ has a much shorter half life (<1 ms) and is restricted to a diffusion distance of 0.4 µm (Denicola et al., 1996; Myatt, 2010). Due to the rapid and ready diffusion of NO through membranes, NO and $O_2^-$ do not have to be produced within the same cell to produce ONOO$^-$ in a diffusion limited reaction.

ONOO$^-$ is a potent pro-oxidant and the major species responsible for nitrative stress in the placenta (Webster et al., 2008). ONOO$^-$ nitrates tyrosine residues (incorporation of a nitro–
NO$_2$ group) in proteins forming nitrotyrosine. ONOO$^-$ is very unstable and difficult to detect directly therefore evidence of its formation and action comes indirectly from the formation of nitrotyrosine which is used as a fingerprint of ONOO$^-$ formation and a marker of nitrative stress (Radi, 2004). Nitration by ONOO$^-$ is highly selective as it is limited to specific tyrosine residues in specific proteins (Pacher et al., 2007). ONOO$^-$ has a short half life of ~100 ms; however, this is long enough for ONOO$^-$ to diffuse across a distance of 5-20 μm (1-2 cell diameters). ONOO$^-$ can also cross cell membranes via anion channels (Pacher et al., 2007).

Other factors regulating nitration by ONOO$^-$ include the abundance of the protein and its tyrosine content (Souza et al., 1999) and the environment (hydrophobic environments favour ONOO$^-$ diffusion) (Bartesaghi et al., 2007, 2006). ONOO$^-$ also has limited reactivity with electron rich groups which makes it a relatively selective nitrating agent.
Important ROS/RNS in the placenta are superoxide (O$_2^-$) and nitric oxide (NO). O$_2^-$ is produced intracellularly by the conversion of molecular oxygen by the enzymes xanthine oxidase, NADPH oxidase and by mitochondrial electron transfer. NO is produced by nitric oxide synthase (eNOS and iNOS). O$_2^-$ and NO spontaneously combine to produce peroxynitrite (ONOO$^-$), a potent pro-oxidant that induces tissue damage by nitrating tyrosine residues in proteins. Superoxide dismutase (SOD) rapidly converts O$_2^-$ into hydrogen peroxide (H$_2$O$_2$) which is reduced to water, oxygen and hydroxyl radicals (OH) which induce tissue damage through oxidation.

Figure 1.9: Generation of reactive oxygen/reactive nitrogen species in the placenta
1.8.2 Consequences of elevated reactive oxygen/nitrogen species in the placenta

ROS and RNS can be dangerous if their production is left unchecked as they interact with proteins and lipids to alter function. Lipid peroxidation occurs when ROS attack polyunsaturated fatty acids in membranes and lipoproteins. The primary products of lipid peroxidation, lipid hydroperoxides, are involved in the regulation of enzymes and genes in normal placental physiology (Hubel, 1999). However, excessive lipid peroxidation is implicated in the pathogenesis of PE (Atamer et al., 2005; Hubel et al., 1989; Poranena et al., 1996). ONOO\(^-\) can be very damaging in tissues for several reasons. Firstly it depletes NO which is an important regulator of placental vascular resistance (reviewed by (Webster et al., 2008)). Secondly ONOO\(^-\) can cause both oxidation and nitration of DNA, lipids and protein residues including cysteine, histidine, methionine and tyrosine (Webster et al., 2008). Several nitrated proteins have been identified in the placenta including p38 MAPK, acetyl CoA acyltransferase, p53, P2X(4) purinergic receptors and TauT (Myatt and Cui, 2004; Webster et al., 2008, 2006). Nitration of many proteins occurs in normal physiology suggesting this post-translational modification is a regulatory mechanism of protein function. Nitration can have no effect or can lead to a gain of function, but most commonly causes loss of function (Myatt and Cui, 2004). Other functional consequences of nitration include faster protein degradation (Webster et al. 2008) and cytotoxicity as nitration inhibits mitochondrial electron transport leading to inhibition of cellular respiration (Myatt and Cui 2004).

1.8.3 Evidence for increased placental oxidative/nitrative stress in pre-eclampsia

There is growing evidence for increased placental nitrative stress in PE. Myatt et al. (1996) have demonstrated significantly higher nitrotyrosine staining in the villous vascular endothelium, vascular smooth muscle and villous stroma in placentas from PE compared to normal. This suggests that there is chronically increased formation of ONOO\(^-\) and subsequent nitrative stress in PE (Myatt et al., 1996). Increased nitration of p38 MAPK and the purinergic receptor P2X(4) in the placenta in PE has also been reported (Roberts et al., 2007; Webster et al., 2006). Interestingly, it has been shown that nitration of SOD by ONOO\(^-\) leads to the inactivation of SOD which could account for the decreased SOD activity in PE (MacMillan-Crow et al., 1998). In addition immunostaining has demonstrated an increase in eNOS expression, an enzyme responsible for NO production, present in the STB and villous vascular endothelial cells in placentas from PE (Myatt et al., 1997). NO is
converted to nitrate by oxyhaemoglobin, therefore measuring nitrate concentrations can be used as an estimate of NO synthesis. It has been demonstrated that umbilical venous serum concentrations of nitrate are significantly higher in PE (Lyall et al., 1995). It has also been demonstrated that nitrotyrosine formation is increased in the placentas of obese women compared to their ideal weight counterparts (Roberts et al., 2009).

Regarding oxidative stress, Sikkema et al. (2001) demonstrated using electron paramagnetic spin trap resonance, that $\text{O}_2^-$ levels are significantly increased in placentas from PE women (Sikkema et al., 2001). Similarly, Wang and Walsh (2001) showed using spectrophotometric assays that $\text{O}_2^-$ generation is increased in isolated trophoblast cells from pregnancies complicated by PE (Wang and Walsh, 2001). The levels and activity of xanthine oxidase, an enzyme involved in the production of $\text{O}_2^-$, have been shown to be increased in invasive trophoblast isolated from placental bed biopsies in PE (Many et al., 2000). A consequence of increased ROS is lipid peroxidation. A significant increase in lipid peroxidation has been reported in placental tissue and plasma from women with PE (Gratacós et al., 2009; Gupta et al., 2009; Llurba et al., 2004; Patil et al., 2009; Serdar et al., 2002).

In addition to the studies above showing that ROS/RNS are increased in PE, there is also evidence that antioxidant defences are compromised. Proteomic analysis of placentas from pregnancies complicated by PE has provided evidence that proteins with antioxidant activity including SOD, peroxiredoxin 2, 3, and 6, glutathione S-transferase, thioredoxin reductase and glutathione peroxidase are reduced in PE (Gharesi-Fard et al., 2010; Johnstone et al., 2011; Vanderlelie et al., 2005). Therefore, evidence that xanthine oxidase and eNOS are increased in PE and antioxidant defences are decreased, suggests that an environment favouring the production of $\text{O}_2^-$ and NO exists in PE. This environment is detrimental as it favours the formation of the powerful nitrating agent, ONOO$^-$, which has been shown to impair nutrient uptake in placental villous fragments and STB MVM (Khullar et al., 2004). Treatment with SIN-1, which generates NO and $\text{O}_2^-$ significantly reduced the transport of arginine, leucine, MeAIB and taurine (Khullar et al., 2004) in STB MVM vesicles probably through nitration of transport proteins following the formation of ONOO$^-$. 

Insufficient remodelling of the uterine spiral arteries in early pregnancy in PE may result in an ischaemia reperfusion type injury in the placenta, producing periods of hypoxia followed
by reoxygenation. It has been demonstrated that hypoxia alone is not sufficient to cause placental oxidative stress or account for the placental changes in PE (Hung et al., 2001), however there is evidence for ischaemia reperfusion injury as the basis of ROS/RNS generation in PE. In cardiac tissue, ischaemia-reperfusion injury has been shown to increase nitrotyrosine formation (Myatt and Cui, 2004). Hung et al. (2001) exposed placental tissue to periods of hypoxia followed by reoxygenation to mimic the conditions of ischaemia-reperfusion in vivo and assessed oxidative status during these periods. When hypoxic tissues were re-oxygenated rapid generation of ROS was seen in the villous endothelium, STB and stromal cells. Alongside this, increased formation of nitrotyrosine was demonstrated, indicating the formation of ONOO\(^{-}\). Preincubation of tissue with free radical scavengers desferrioxamine and α-phenyl-N-tert-butylnitrone reduced the levels of ROS produced during reoxygenation (Hung et al., 2001). It has been shown that under normal conditions the production of NO is greater than the production of O\(_2\)\(^{-}\), however during periods of reperfusion, O\(_2\)\(^{-}\) formation is favoured. As a result, O\(_2\)\(^{-}\) reacts with NO more readily than SOD producing ONOO\(^{-}\), providing further support for the ischaemia-reperfusion injury hypothesis (Beckman and Koppenol, 1996; Hung et al., 2001).

In summary, there is strong evidence that the generation of O\(_2\)\(^{-}\) and NO and their product ONOO\(^{-}\) is significantly increased in the placenta in PE compared to normal pregnancy. Furthermore, there is increasing evidence that nitrotyrosine formation is increased indicative of chronically elevated nitrative stress in PE. Alongside this, a decrease in antioxidant defences including, SOD, glutathione peroxidise, catalase and vitamin C and E has been demonstrated.

### 1.9 Summary

PE is a leading cause of maternal and fetal mortality and morbidity. The disease is associated with dysregulated renewal of STB, the nutrient transporting placental epithelium, by altered proliferation, differentiation, fusion and apoptosis. The mechanisms underlying STB dysregulation are unknown but PE is associated with elevated oxidative/nitrative stress and inflammation, possibly due to inadequate remodelling of the uterine spiral arteries in early pregnancy causing irregular maternal blood flow to the placenta. PE is more common in women who are obese and, as the prevalence of obesity is
rising, an increase in the incidence of PE is anticipated in the future. The reasons why maternal obesity predisposes to developing PE have not been elucidated; however both conditions share common features including increased placental oxidative/nitrative stress, heightened inflammation and dysregulation of STB renewal. In maternal obesity there is a reduction in the uptake of the amino acid taurine into the STB due to a fall in the activity of the taurine transporter, TauT. Preliminary studies have also demonstrated a reduction in STB TauT activity in PE, although this remains to be confirmed.

Taurine is the most abundant amino acid in STB and is likely to be important in pregnancy for three reasons; (a) the fetus requires taurine for normal growth and development (b) taurine regulates cell proliferation, differentiation, fusion and apoptosis; events involved in renewal of STB (c) taurine protects cells against ischaemia-reperfusion, free-radicals and inflammatory cytokines; factors which can disrupt STB renewal and are elevated in the placenta in PE and maternal obesity.

Depletion of STB taurine following a reduction in TauT activity could increase the susceptibility to altered STB renewal in both PE and maternal obesity. Furthermore, reduced TauT activity could result in inadequate taurine delivery to the developing fetus therefore compromising fetal growth and development and increasing the risk of FGR. The reasons for reduced STB TauT activity in obesity and PE remain to be determined. The activity of TauT in STB membranes in vitro is inhibited by nitration of tyrosine groups on the transporter protein and it is plausible that elevated placental nitrative stress associated with obesity and PE down-regulates TauT activity through nitration.

Understanding STB taurine transport by TauT and its regulation in normal pregnancy and pregnancy pathologies may identify interventions to restore taurine delivery to STB and the fetus and improve pregnancy outcome in PE and maternal obesity.
1.10 Hypothesis and aims

The overall hypothesis for this research project is that STB TauT activity is reduced in PE through nitration which contributes to altered STB renewal.

Aims and approaches:

The main aims of this study were to determine whether:

1. Placental TauT activity is lower in women with PE compared to normal pregnancy.

2. Reduced placental TauT activity in PE is due to (a) reduced TauT protein expression and/or (b) increased nitration of TauT compared to normal pregnancy.

3. Chronic exposure of placental villous fragments in vitro to RNS will down regulate TauT activity and disrupt STB cell turnover.

4. Maintaining STB taurine is required for appropriate STB renewal.
Chapter 2: Materials and methods

2.1 Source of reagents

All chemicals were from Sigma Aldrich Ltd, Dorset, UK unless otherwise stated.

2.2 Study groups and placental collection

Placentas were collected from women delivering at St Mary’s Hospital Manchester following their written informed consent as approved by the local research ethics committee. Placentas were collected within 30 minutes of vaginal delivery or caesarean section from normal pregnancy or pregnancy complicated by PE. Normal pregnancy was defined as the delivery of a singleton infant at term (37-40 weeks) following an uncomplicated pregnancy. PE was defined by onset of hypertension (blood pressure >140/90 mmHg) after 20 weeks gestation in previously normotensive women plus significant proteinuria (excretion of >0.3 g of protein every 24 hours or a protein:creatinine ratio of at least 0.3). Exclusion criteria for PE included pre-existing hypertension, gestational diabetes, renal disease and delivery of a LGA baby (defined as the delivery of a baby with an individualised birthweight ratio (IBR) > 90). Both early and late onset PE (defined as symptoms before and after 34 weeks gestation respectively), with or without fetal growth restriction (FGR), were included in the study. FGR was defined as the delivery of an infant with an IBR between 0-5. Maternal body mass index (BMI: kg/m²), measured at first ante-natal visit (~12 weeks), was recorded allowing separation of women into the following BMI categories (Table 2.1):

<table>
<thead>
<tr>
<th>Weight Category</th>
<th>BMI range (kg/m²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ideal weight</td>
<td>18.5-24.9</td>
</tr>
<tr>
<td>Overweight</td>
<td>25-29.9</td>
</tr>
<tr>
<td>Obese</td>
<td>30 and above</td>
</tr>
</tbody>
</table>

Table 2.1: BMI category definitions used in this study as defined by the World Health Organisation
2.3 Measurement of TauT activity

2.3.1 Placental dissection

Placental villous tissue fragments were sampled from normal pregnancies and pregnancies complicated by PE to examine STB TauT activity. Four cubes (1cm$^3$) of placental villous tissue were dissected from sites across the placenta including centre, middle and edge and placed in a 1:1 Dulbecco’s Modified Eagles Medium (DMEM; Gibco, Life Technologies UK): Tyrode’s buffer (135 mM NaCl, 5.0 mM KCl, 1.8 mM CaCl$_2$, 1.0 mM MgCl$_2$, 10 mM HEPES, 5.6 mM D-glucose pH 7.4) solution containing 100 μM taurine to approximate the taurine concentration in maternal plasma (Philipps et al., 1978).

2.3.2 $^3$H-taurine uptake assay

The cubes of villous tissue were further dissected into small fragments which were tied to hooks with cotton and suspended in a 1:1 DMEM: Tyrode’s buffer with 100 μM taurine at room temperature until all tissue required for the experiment was similarly prepared (Figure 2.1).
The hooks were subsequently placed in DMEM:Tyrode’s buffer in a water bath at 37°C for 20 minutes to equilibrate to 37°C (Figure 2.2). $^3$H-taurine uptake by villous fragments was measured in the presence and absence of Na$^+$ as transport of taurine via TauT is Na$^+$-dependent and any uptake occurring in the absence of Na$^+$ occurs via non-specific routes. Subtracting $^3$H-taurine uptake in the absence of Na$^+$ from that in the presence of Na$^+$ gives the Na$^+$-dependent taurine uptake which corresponds to uptake that is specifically mediated by TauT. Following equilibration, fragments were washed for 2 minutes in 4 ml of prewash solution, Tyrode’s buffer +/- Na$^+$ (Na$^+$ free Tyrode’s buffer: 135 mM NaCl replaced by 135 mM choline chloride) as appropriate, before being transferred to 4 ml of Tyrode’s buffer (+/- Na$^+$) containing $^3$H-taurine (Hartmann Analytic, Germany) (0.5 µCi/ml = 25 nM; incubation solutions, no unlabelled taurine present). The fragments were suspended in the incubation solutions for 30, 60, 90 and 120 minutes at 37°C. Following incubation the fragments received two 15 second washes in ice cold Tyrode’s buffer +/- Na$^+$ (post wash solutions) to stop any further uptake and remove any extracellular isotope. The fragments

Figure 2.1: Suspension of placental villous tissue

Diagram showing placental villous fragments suspended in DMEM:Tyrode’s buffer for measurement of TauT activity. Villous fragments were tied to hooks with cotton, three of which were suspended from each rod allowing measurements to be made in triplicate.
were then suspended in water for 18 hours at room temperature to allow cell lysis and the release of $^3$H-taurine taken up by the tissue.

Following lysis the tissue fragments were removed from the water and placed in 3 ml 0.3 M sodium hydroxide (NaOH) overnight at 37°C to denature the tissue. 16 ml of Scintisafe scintillation fluid (Fisher Scientific, UK) was added to each of the water lysates which were then counted in a β-scintillation counter (Tri-carb 2100TR liquid scintillation analyzer, Packard). The $^3$H-taurine radioactivity of the Tyrode’s buffer incubation solution was determined by counting 100 μl of the incubation solution + 3.9 ml water + 16 ml scintillation fluid in duplicate as standards. The samples and the standards were counted for 10 minutes along with background counts (4 ml water + 16 ml scintillation fluid).
Figure 2.2: Schematic of method used to measure syncytiotrophoblast TauT activity in placental villous fragments

The experiment was carried out in a water bath maintained at 37°C. After fragments were tied onto the hooks they were suspended in DMEM:Tyrode’s + 100 µM taurine for 20 minutes to equilibrate. Fragments were then placed in either control or Na⁺-free Tyrode’s buffer for a 2 minute pre-wash to remove extracellular Na⁺ for assessing ³H-taurine uptake in the absence of Na⁺. Following this the fragments were suspended in control or Na⁺-free Tyrode’s buffer containing radiolabelled taurine for 30, 60, 90 or 120 minutes. After incubation fragments were washed twice for 15 seconds in ice-cold Tyrode’s buffer before being placed in water to lyse for 18 hours at room temperature. Fragments were finally denatured in 0.3 M NaOH for 24 hours at 37°C.
2.3.3 Protein assay

Following incubation in 0.3 M NaOH overnight, denatured fragments were stored at 4°C prior to assay for protein content. A BioRad protein assay was used to determine the protein content of the villous fragments. A standard curve was generated using known concentrations of bovine serum albumin (BSA: 0-250 µg/ml). 20 µl of the standards and samples were pipetted in duplicate into wells of a 96 well plate. 180 µl of neutralising solution (1:1.25 mix of 0.3 M NaOH and 0.3 M HCl) was pipetted into each well followed by 50 µl of the BioRad dye reagent (BioRad labs, Hemel Hempstead, UK). Each well was mixed thoroughly and the absorbance was measured at 595 nm using a Versamax microplate reader (Molecular Devices, California, USA) and Softmax Pro Software (Molecular Devices). Protein content of the samples was then calculated by extrapolation of values from the standard curve using Graphpad Prism 5.0 software (San Diego, Graphpad software, 2010).

2.3.4 Calculation of $^3$H-taurine uptake

The $^3$H-taurine uptake into the villous fragments was expressed as fmol/mg protein. Firstly the average background count was subtracted from all samples and standards. To calculate counts per fmol the mean counts in the duplicate control and Na$^+$-free standards was calculated and multiplied by 10 to give counts per ml. This number was then divided by 25 as the concentration of $^3$H-taurine in the incubation solution was 25 nM which gave counts/pmol. Finally the counts/pmol was divided by 1000 to give counts/fmol. Sample counts were divided by this value to give the uptake by each fragment in fmol. This value was then divided by the mg protein for each sample, as determined by the protein assay, to give fmol/mg protein uptake of $^3$H-taurine. Since each time point was measured in triplicate for Na$^+$-free and control conditions, the mean fmol uptake/mg protein was calculated for each time point. The Na$^+$-dependent uptake of $^3$H-taurine/mg protein was determined by subtracting the mean fmol uptake/mg protein in the absence of Na$^+$ from the mean fmol uptake/mg protein in the presence of Na$^+$ at each time point.
2.4 Assessment of TauT protein expression and tyrosine nitration in placenta from normal pregnancy and pre-eclampsia

2.4.1 Placental dissection

TauT protein expression was examined in membrane enriched homogenates of placental villous tissue from normal pregnancy and PE (for which TauT activity had previously been determined in this study) and in isolated placental microvillous membranes (MVM) from normal pregnancy and PE (not matched for TauT activity) using Western blot analysis. For membrane enriched homogenates, six cubes of placental villous tissue (1 cm$^3$) were dissected and frozen at -80°C within 30 minutes of delivery of the placenta until further processing (section 2.4.2). 100-200 g of villous tissue was processed for preparation of MVM (section 2.4.3).

Tyrosine nitration was also examined in placental villous membrane enriched homogenates from normal pregnancy and PE (for which TauT activity had previously been determined) using Western blot analysis. In addition, immunohistochemistry (IHC) for nitrotyrosine was used to assess STB nitration in formalin fixed, wax embedded placental villous tissue from normal pregnancy and PE (for which TauT activity had previously been determined). The IHC procedure is described in section 2.7.

2.4.2 Preparation of membrane enriched placental villous homogenates

Three frozen fragments of villous tissue per placenta were defrosted and pooled in 10 ml ice cold lysis buffer (Buffer A: 300 mM Mannitol, 10 mM HEPES, pH 7.6) + 0.1% protease inhibitor cocktail and 1% phosphatase inhibitor cocktail 2 and 3) before being homogenised on ice using a polytron homogeniser. The samples were then centrifuged at 2500 g for 5 minutes at 4°C using a Sorvall Discovery™ 100SE Ultracentrifuge (Hitachi) with a T-1250 Fixed Angle titanium rotor (Thermo Scientific). The sample supernatant was collected and spun at 100,000 g for 30 minutes at 4°C using the ultracentrifuge. The supernatant was removed and discarded and the pellet re-suspended in 300 μl of lysis buffer. The protein content of the samples was determined using a BioRad protein assay (as described in
section 2.3.3) and the samples then aliquoted (10 x 30 μl), and stored frozen at -80°C prior to use for Western blotting.

2.4.3 Preparation of microvillous membranes

The entire MVM preparation was performed at 4°C. The chorionic plate was removed and approximately 100-200 g of placental villous tissue weighed. The tissue was homogenised in a Waring blender at high speed for 20 seconds in 2.5 x volume of Browns buffer (300 mM Mannitol, 10 mM Tris-Hepes, 1 mM MgSO\(_4\), pH 7.4). 5 ml of the resulting crude placental homogenate was removed for subsequent protein and alkaline phosphatase assays (sections 2.4.4 and 2.4.5 respectively). Magnesium chloride was added to the homogenate to a final concentration of 10 mM and the solution stirred for 10 minutes. The solution was centrifuged at 2300 g for 15 minutes in a Sorvall Discovery 100SE centrifuge precooled to 4°C. The supernatant was then centrifuged at 23500 g for 40 minutes and the pellet discarded. The subsequent pellet was then re-suspended in 2.5 x volume of Browns buffer using a hand held homogeniser. Magnesium chloride was added to the homogenised solution to a final concentration of 10 mM and stirred for 10 minutes. The solution was then centrifuged at 2300 g for 15 minutes. The supernatant was removed, taking care not to dislodge the pellet, and spun at 23500 g for 40 minutes and the pellet discarded. The resulting pellet (representing the MVM) was re-suspended in 4 x volume of intravesicular buffer (5 mM Tris, 5 mM HEPES, 290 mM sucrose, 0.1% protease inhibitor cocktail, 1% phosphatase inhibitor cocktails 2 and 3, pH 7.4) using a hand held homogeniser. The MVM preparation was then stored at -80°C.

2.4.4 Protein assay of microvillous membranes

A Lowry protein assay (microplate method) was used to determine the protein content of the crude placental homogenate and the final MVM preparation. A standard curve was generated using known concentrations of BSA (range 0-2 mg/ml).
2.4.4.1 Sample preparation

The crude placental homogenate was diluted 1:40 in dH₂O and the MVM vesicles diluted 1:50 in dH₂O. 100 μl of each standard and samples were pipetted into a 96 well microplate in triplicate. 100 μl of 2 M NaOH was added to each well and mixed by pipetting up and down and left at room temperature for 15 minutes to dissolve the protein.

2.4.4.2 Assay procedure

20 μl of the solubilised standards and samples were pipetted into a new 96 well microplate and 20 μl of dH₂O added to each well. 200 μl of Reagent C (50 ml 2% Na₂CO₃, 500 μl 1% CuSO₄, 500 μl Na/K tartrate) was added to each well and mixed and left for 10 minutes at room temperature. 20 μl of Folin D was added to each well and mixed and left for 30 minutes at room temperature. The plate was then read at 750 nm using a Versamax microplate reader (Molecular Devices, California, USA) and Softmax Pro Software (Molecular Devices). Protein content of the samples was then calculated by extrapolation of values from the standard curve using Graphpad Prism 5.0 software (San Diego, Graphpad software, 2010).

2.4.5 Alkaline phosphatase enrichment assay

An alkaline phosphatase enrichment assay was used to determine the MVM enrichment factor by comparing the alkaline phosphatase levels in the crude placental homogenate and final MVM preparation.

2.4.5.1 Sample preparation

The crude placental homogenate was diluted 1:40 in dH₂O and the MVM preparation diluted 1:300 in dH₂O.

2.4.5.2 Assay procedure

5 μl of the diluted crude placental homogenate and MVM samples were pipetted in triplicate into a 96 well plate. 250 μl of diethanolamine buffer (DEA; 1 M diethanolamine, 0.5 mM MgCl₂, pH 9.8) was added to each well. 25 μl of Comix (p-nitrophenylphosphatase in DEA) was added to each well and mixed by pipetting up and down. The absorbance was read at 410 nm immediately after the addition of Comix (t=10 seconds) and then 2 minutes later (t=2 minutes 10 seconds). The difference between the two values gives the activity of
alkaline phosphatase over 2 minutes which was then normalised to protein concentration to calculate the enrichment of alkaline phosphatase in the MVM samples.

2.4.6 Preparation of reagents for Western blotting

The composition of the gels used in gel electrophoresis and the reagents used for Western blotting are described in Table 2.2 and 2.3.
### Table 2.2: Composition of the gels used in gel electrophoresis.

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Protogel</strong></td>
<td>Ultrapure Protogel (37.5:1 Acrylamide:bis-acrylamide) (National Diagnostics (UK) Ltd.)</td>
</tr>
</tbody>
</table>
| **Solution B** (Resolving gel buffer) | 1.8M TRIZMA® base (Tris(hydroxymethyl)aminomethane)  
17.3mM sodium dodecylsulphate (SDS)  
(pH to 8.8 using HCl)               |
| **Solution C** (Stacking gel buffer) | 500mM TRIZMA® base  
13.8mM SDS  
(pH to 6.8 using HCl)               |
| **7% resolving gel (2 gels)** | 6.5M ammonium persulphate (APS)  
20% solution B  
23% protogel  
0.1% Tetramethylethylenediamine (TEMED) |
| **3% stacking gel (2 gels)**   | 4.4M APS  
25% Solution C  
10% protogel  
0.1% TEMED |

### Table 2.3: Composition of the reagents used for Western blotting

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Composition in dH2O</th>
</tr>
</thead>
</table>
| **2 x Laemmli sample loading buffer** | 100mM Tris-HCl, pH 6.8  
4% SDS  
20% glycerol  
0.04% Bromophenol Blue  
2% β-mercaptoethanol |
| **Running Buffer** | 24.7mM TRIZMA® base  
3.5mM SDS  
191.8mM glycine |
| **Transfer Buffer** | 25mM TRIZMA® base  
191.8mM glycine  
20% methanol |
| **Tris-buffered saline (TBS)** | 150mM NaCl  
19.8mM TRIZMA® base (pH 7.0 with HCl) |
| **Strip Buffer** | 0.1M glycine  
(pH 2.5 with HCl) |
2.4.7 Gel electrophoresis

A BioRad mini-Protean III system was used for polyacrylamide gel electrophoresis. A 7% resolving gel was prepared as described in Table 2.2, overlaid with ethanol and allowed to set before a 3% stacking gel (Table 2.2) was poured on top and a well-defining comb inserted immediately. Once the stacking gel had set the comb was removed, the wells were rinsed with running buffer (Table 2.3) and the gel mounted into the electrophoresis unit. 30 µg sample was mixed with 2 x Laemmli sample loading buffer (Table 2.3) in a 1:1 ratio and boiled at 95°C for 5 minutes in preparation for electrophoresis. These samples were then loaded into the wells of the stacking gel along with a Kaleidoscope™ pre-stained molecular weight marker (BioRad Laboratories Ltd., UK) and the electrophoresis tank filled with running buffer. Electrophoresis was carried out at 120 V for 70 minutes using a BioRad PowerPac 200 electrophoresis power supply (BioRad Laboratories Ltd., UK).

2.4.8 Gel transfer

In preparation for gel transfer, filter paper and nitrocellulose membranes (Amersham, GE Healthcare Life Sciences, UK) (cut to the size of the gel) were soaked in transfer buffer (Table 2.3) for 20 minutes. Once electrophoresis had finished the gels were removed and rinsed in transfer buffer. The transfer cassette was then assembled as follows: 1 x sponge, 1 x filter paper, 1 x nitrocellulose membrane, resolving gel, 1 x filter paper, 1 x sponge. Any air bubbles were removed by rolling over with a small test tube. The transfer cassette was then placed in the transfer tank with an ice reservoir and filled with transfer buffer. Transfer was carried out at 200 mA for 70 minutes. Upon completion nitrocellulose membranes were removed from the transfer cassette and placed in TBS overnight at 4°C prior to probing with antibodies.

2.4.9 Antibody probing

Nitrocellulose membranes were blocked by incubating in Blotto (3% milk powder in TBS) for 1 hour at room temperature. The membranes were then washed in Blotto (3 x 5 minutes). A rabbit polyclonal anti-TauT antibody (final concentration 2.5 µg/ml in Blotto) or a rabbit polyclonal anti-nitrotyrosine antibody (final concentration 1 µg/ml in Blotto) was then applied to the nitrocellulose membrane for 2 hours at room temperature. Primary antibodies were supplied by Merck Millipore, UK. Following incubation with primary antibodies the membranes were washed 3 x 5 minutes in TBS-0.5% Tween (TBS-T). The appropriate horseradish peroxidise conjugated secondary antibody (1:1000 dilution in TBS-
T) was then applied to all membranes for 1 hour at room temperature. Membranes were subsequently washed 3 x 5 minutes in TBS-T prior to development.

2.4.10 Enhanced chemiluminescence (ECL) development

Membranes were incubated with ECL Western blotting substrate (Amersham, GE Healthcare Life Sciences, UK) for 1 minute before being placed into developing cassettes with film (Amersham Hyperfilm ECL; Amersham, GE Healthcare Life Sciences, UK) for a defined period of time before developing and fixing the film using Kodak processing chemicals for autoradiography films: GBX developer and GBX fixer.

2.4.11 Re-probing of nitrocellulose membranes

Nitrocellulose membranes were stripped for 1 hour at room temperature using strip buffer (Table 2.3). Membranes were washed for 3 x 5 minutes in TBS-T before blocking in Blotto and re-probing for the housekeeping protein, β-actin, using a mouse monoclonal antibody (clone AC-74, final concentration 0.04 μg/ml in Blotto), for 1 hour at room temperature. The membranes were then probed with the appropriate secondary antibody and Western blotting procedure continued as described in section 2.4.9 and 2.4.10.

2.4.12 Densitometry

Developed film was scanned and the mean signal intensity of the immunoreactive species determined using Image J image processing software version 1.44 (National Institutes of Health). To account for any variability in sample loading, TauT and nitrotyrosine signal intensity for each sample was normalised to the corresponding β-actin signal intensity.
2.5 Assessment of TauT nitration by immunoprecipitation

2.5.1 Sample preparation
Nitration of TauT was examined in placental membrane enriched homogenates (prepared as described in section 2.4.2) from normal pregnancy and PE using Western blot analysis of nitrotyrosine following immunoprecipitation of TauT.

2.5.2 TauT immunoprecipitation
A Protein G Immunoprecipitation Kit (catalogue # IP50) was used to purify TauT protein from the placental membrane enriched homogenates following the manufacturer’s instructions. An optional pre-clearance step was included to reduce background. In brief, 50 µl washed protein G agarose beads were incubated with 200 µg placental membrane enriched homogenate sample for 3 hours at 4°C. The beads were then pelleted by centrifugation at 12,000 g for 30 seconds and the supernatant (pre-cleared cell lysate) collected.

The pre-cleared cell lysate was added to a spin column (provided in the kit) along with 2 µl rabbit polyclonal anti-TauT antibody (1 µg/ml, Millipore). Figure 2.3 summarises the immunoprecipitation procedure as instructed by the manufacturer of the kit.

2.5.3 Western blot analysis of immunoprecipitated samples
Electrophoresis of immunoprecipitated sample and Western blot analysis of nitrotyrosine using a monoclonal mouse anti-nitrotyrosine antibody (Millipore, final concentration 10 µg/ml) was carried out as described in sections 2.4.7 – 2.4.10.
Pre-cleared cell lysate sample was incubated for 1 hour at 4°C with the anti-TauT antibody which binds the protein of interest (1). 50 µl washed protein G agarose beads were added to the cell lysate which physically isolates the protein of interest from the other proteins in the sample (2). To remove non-specific binding, the sample was extensively washed by resuspending in IP buffer (provided in the kit) and spinning in a microcentrifuge at 12,000 g for 30 seconds (3). Laemmli buffer was added to the sample before heating at 95°C for 5 minutes to separate the beads from the protein-antibody complex. The sample was then centrifuged again and the elutant collected for Western blot analysis (4).
Placental Explant Studies

2.6 Placental explant culture preparation

Placental villous explant culture is a well established method used to study chronic regulation of placental function and the mechanisms of STB renewal (Audette et al., 2014; Crocker et al., 2004a; Heazell et al., 2009; Simán et al., 2001). It has been demonstrated using electron microscopy that the STB degenerates and is shed over the first few days of culture but that by day 4 there is evidence of STB regeneration. Furthermore, the newly formed STB remains viable for at least 11 days in culture (Simán et al., 2001).

2.6.1 Placental dissection

To assess the effect of (a) chronic exposure to nitrative stress induced by RNS on STB TauT activity and the process of STB renewal and (b) the effect of reducing intracellular taurine on STB renewal, placentas were collected from women experiencing normal pregnancy (including both vaginal delivery and caesarean section) who had a BMI no greater than 30 kg/m². Six cubes of villous tissue were dissected from random sites and placed in warmed sterile phosphate buffered saline (PBS). Under sterile conditions, the cubes were further dissected into small fragments (2-3 mm³) and washed three times in PBS before receiving a final wash in explant culture media (CMRL-1066, 26 mM sodium hydrogen carbonate, 100 μg/ml streptomycin sulphate, 100 IU/ml penicillin G, 1 μg/ml insulin, 0.1 μg/ml retinol acetate, 0.1 μg/ml hydrocortisone, 100 μg/ml L-glutamine, 5% Fetal Calf Serum pH 7.2).

2.6.2 Experimental procedure

Placental villous fragments were cultured in 12 well plates on individual Costar Netwell supports (15 mm diameter, 90μM mesh, Corning Lifesciences) in 1.5 ml of culture media with 3 fragments (explants) per well. Explants were maintained in a humidified incubation chamber (95% air and 5% CO₂) at 37°C for 7 days. To induce (a) nitrative stress, explants were treated with SIN-1 or ONOO⁻ (detailed methods in Chapter 5 section 5.2.1) or (b) a reduction in TauT activity and intracellular taurine, explants were treated with β-alanine which competitively inhibits TauT (detailed methods in Chapter 5 section 5.2.2).
On day 7, 6 explants (from 2 Netwells) were fixed in 4% neutral buffered formalin (NBF) overnight and then washed and stored in PBS at 4°C prior to wax embedding for the purposes of immunohistochemistry (IHC) analysis of M30 to assess apoptosis, ki67 and E-cadherin dual staining to identify proliferating CTBs and cytokeratin 7 as a marker of STB regeneration. Remaining explants were taken to measure STB TauT activity at 90 minutes (as described in 2.3.2 - 2.3.4) and intracellular taurine accumulation (Chapter 5 section 5.2.2.2).

2.7 Assessment of nitrotyrosine formation and syncytiotrophoblast renewal by immunohistochemistry

2.7.1 Tissue dissection and processing

IHC for nitrotyrosine was used to assess syncytiotrophoblast nitration in freshly fixed placental villous tissue from normal pregnancy and PE (section 2.4.1) and in explants treated with ONOO-/SIN-1. In addition, control, SIN-1 and β-alanine treated explants were immunostained for caspase-cleaved cytokeratin 18 (using M30: a marker of apoptosis), cytokeratin 7 (an epithelial marker allowing assessment of syncytiotrophoblast regeneration) and dual stained for Ki67 (a marker of proliferation) and E-cadherin (a marker of cytotrophoblast cells) to assess the effect of nitrative stress/low intracellular taurine concentration on STB renewal by trophoblast cell turnover.

Collection of samples from normal pregnancy and PE took place within 30 minutes of delivery of the placenta. Three cubes of placental villous tissue (approx 1 cm³) were dissected at random and washed briefly in PBS before placing into fixative (4% NBF) overnight at 4°C. SIN-1 and β-alanine treated explants were fixed on day 7 of culture using the same method. After fixation in NBF, samples were washed 3 x in PBS and stored in PBS at 4°C prior to routine processing and paraffin wax embedding.

Embedded tissue was cut into 5 μm sections using a microtome (Leica RM2245, Wetzler, Germany) and mounted onto poly-L-lysine coated slides which were dried overnight at 37°C.
2.7.2 Immunohistochemistry for nitrotyrosine, cytokeratin 7 and M30

Sections were warmed for 10 minutes at 60°C and then dewaxed in Histoclear (National Diagnostics, Georgia, USA) by three 10 minute immersions. Sections were rehydrated by two 3 minute immersions in 100% ethanol and two 3 minute immersions in 70% ethanol before being placed in tap water. For antigen retrieval, slides were placed into 400 ml of 0.01 M sodium citrate buffer (pH 6.0), boiled twice for 5 minutes in a microwave and then left to cool for 20 minutes in the hot buffer. Endogenous peroxidase was quenched by applying 3% hydrogen peroxide (diluted in H$_2$O) to the sections for 10 minutes at room temperature. Slides were then washed twice in TBS (5 mM TRIZMA® base, 3 M NaCl, pH 7.6) for 5 minutes and excess liquid removed by dabbing around the sections with tissue. Non specific antibody binding sites were blocked by incubating the sections in non-immune block (NIB; 10% normal swine serum, 2% human serum in TBS-Tween (0.1%)) (for nitrotyrosine) or in 5% BSA in TBS (for M30 and cytokeratin 7) for 30 minutes at room temperature in a humidified chamber. Excess NIB/BSA was tapped off and sections dabbed with tissue before applying 50 μl of primary antibody (see Table 2.4).

Table 2.4: Details of primary and secondary antibodies used for immunohistochemistry

<table>
<thead>
<tr>
<th>Primary Antibody</th>
<th>Clone</th>
<th>Dilution</th>
<th>Supplier</th>
<th>Secondary Antibody</th>
<th>Dilution</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-nitrotyrosine (Rabbit polyclonal, stock concentration 1 mg/ml)</td>
<td>-</td>
<td>1:400 in NIB</td>
<td>Milipore California</td>
<td>Biotinylated swine anti-rabbit</td>
<td>1:400 in TBS</td>
<td>Dako, Denmark</td>
</tr>
<tr>
<td>Cytokeratin 7 (Mouse monoclonal, stock concentration 196 mg/L)</td>
<td>OV-TL 12/30</td>
<td>1:500 in BSA</td>
<td>Dako, Denmark</td>
<td>Biotinylated goat anti-mouse</td>
<td>1:200 in TBS</td>
<td>Dako, Denmark</td>
</tr>
<tr>
<td>M30 (Mouse monoclonal, stock concentration – information not provided)</td>
<td>M30</td>
<td>1:100 in BSA</td>
<td>Roche, Germany</td>
<td>Biotinylated goat anti-mouse</td>
<td>1:200 in TBS</td>
<td>Dako, Denmark</td>
</tr>
</tbody>
</table>
For negative controls, the primary antibody was omitted or substituted with an equal concentration of rabbit or mouse non-immune IgG as appropriate. A positive control for nitrotyrosine was also included by adding 50 μl of 10 mM ONOO− (stock solution described in Chapter 5 section 5.2.1) directly to the tissue section on the slide for 10 minutes prior to the antigen retrieval step. Sections were incubated with primary antibody or non-immune IgG at 4°C overnight.

Slides were washed in TBS for 5 minutes, in TBS-Tween (0.6%) for 2 x 5 minutes and again in TBS for 5 minutes and excess liquid removed. 50 μl of secondary antibody (see Table 2.4) was added to each section and incubated for 30 minutes at room temperature in a humidified chamber. The secondary antibody was washed off using TBS for 1 x 5 minutes, followed by TBS-Tween (0.6%) for 2 x 5 minutes, and TBS 1 x 5 minutes and excess liquid dabbed off. 50 μl of avidin peroxidase (5 μg/ml, diluted in TBS) was applied to each section and slides were incubated for a further 30 minutes at room temperature. Sections were again washed in TBS 1 x 5 minutes, TBS-Tween 2 x 5 minutes, and finally in TBS 1 x 5 minutes and excess liquid removed.

### 2.7.3 Colour development and mounting

50 μl of chromogen diaminobenzidine (DAB) was added to each section for 1 minute (M30 and Ki67) or 2 minutes (nitrotyrosine). Slides were washed with distilled water and then counterstained in filtered Harris’s hemotoxylin for 1 minute. Sections were placed in running tap water before being dipped in acid-alcohol for 2-3 seconds to remove excess haemotoxylin and washed again in running tap water. Slides were incubated for 5 minutes in warm running tap water to “blue” haemotoxylin staining. Slides were dehydrated by immersion twice in 70% ethanol and 95% ethanol for 5 minutes, and three times in 100% ethanol for 5 minutes. Slides were then immersed in Histoclear for 1 x 10 minutes, 2 x 30 minutes before being mounted with DPX mounting medium.

### 2.7.4 Dual staining for Ki67 and E-cadherin

To assess CTB proliferation in control, SIN-1 and β-alanine treated explants, tissue sections were dual stained for Ki67 (as a marker of proliferation) and E-cadherin (to identify CTBs). IHC for Ki67 was performed according to section 2.7.4 using NIB for the blocking step and 50 μl Ki67 primary antibody (Table 2.5).
50 µl DAB was added to each section for 1 minute and the slides then washed in TBS 3 x 5 minutes. Sections were blocked again in NIB for 30 minutes at room temperature in a humidified chamber. Excess NIB was tapped off and removed with tissue and 50 µl E-cadherin primary antibody added to each section (Table 2.5) and incubated at 4°C overnight.

<table>
<thead>
<tr>
<th>Primary Antibody</th>
<th>Clone</th>
<th>Dilution</th>
<th>Supplier</th>
<th>Secondary Antibody</th>
<th>Dilution</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ki67 (Monoclonal mouse stock concentration 46 mg/L)</td>
<td>MIB-1</td>
<td>1:500 in NIB</td>
<td>Dako, Denmark</td>
<td>Biotinylated goat anti-mouse</td>
<td>1:200 in TBS</td>
<td>Dako, Denmark</td>
</tr>
<tr>
<td>E-cadherin (Monoclonal mouse, stock concentration 250µg/ml)</td>
<td>NCH-38</td>
<td>1:1500 in NIB</td>
<td>Dako, Denmark</td>
<td>Alkaline phosphatase conjugate goat anti-mouse</td>
<td>1:50 in TBS</td>
<td>Sigma Aldrich, UK</td>
</tr>
</tbody>
</table>

Excess primary antibody was tapped off and sections washed in TBS 3 x 5 minutes. 50 µl alkaline phosphatase conjugated goat anti-mouse secondary antibody was then added to each section for 30 minutes at room temperature. Excess secondary antibody was tapped off and sections washed in TBS 3 x 5 minutes.

2.7.5 Liquid permanent red colour development

1 drop of liquid permanent red (LPR) chromagen was added to 3 mls LPR substrate buffer (Dako, Denmark) and 50 µl of this solution added to each section for 10 minutes at room temperature. The slides were washed in dH2O and lightly counterstained with Harris’s haematoxylin for 5 seconds. Sections were placed in running tap water before being dipped in acid-alcohol for 2 seconds to remove excess haematoxylin and washed again in running tap water. Slides were incubated for 5 minutes in warm running tap water to “blue”
haemotoxylin staining. Slides were dehydrated by immersion once in 70% ethanol, 95% and 100% ethanol for 3 minutes. Slides were then immersed in Histoclear for 1 x 10 minutes, 2 x 30 minutes before being mounted with DPX mounting medium.

### 2.7.6 Analysis of staining

Following immunostaining of placental tissue sections, images of the stained sections were taken using an Olympus BX41 microscope (Olympus, Tokyo, Japan) with a QICAM fast 1394 camera. Images were obtained using Image ProPlus software version 7.0 (Media Cybernetics, Buckinghamshire, UK). For nitrotyrosine staining of placentas from normal pregnancy and PE, 8 images per placenta were captured. For explant cultures 6 fragments were fixed and embedded per treatment and 6 images per treatment were captured (i.e. 1 image per fragment).

Nitrotyrosine staining was assessed by two observers blind to the experimental group using a scoring system of 0-4 based on intensity of nitrotyrosine staining in STB. All villi in the field of view were scored (a minimum of 5 villi per field of view). All images were scored by both observers.

M30 staining was assessed using Image ProPlus software to calculate the area of M30 positive staining as a percent of the total villous area (Figure 2.4).

Ki67/E-cadherin staining was assessed by counting the number of Ki67 + E-cadherin positive nuclei and expressing this as a percent of the total number of E-cadherin positive nuclei in the field of view (Figure 2.5).

Cytokeratin 7 staining was assessed by an observer blind to the experimental group to determine (a) the number of villi with intact STB, and the number of villi with shed STB that had (b) or had not (c) regenerated a new cellular layer (Figure 2.6). Based on this, regeneration of STB was assessed in four ways: (1) the number of villi with an intact STB (category a) expressed as a percent of total villi (2) villi with shed STB (b and c) as a percent of total villi (3) the number of villi with a complete STB layer i.e intact villi plus shed STB with complete cellular regeneration (a and b) expressed as a percent of total number of villi (4) percent of shed villi (b plus c) with cellular regeneration (b).
Figure 2.4: Example images for the quantification of M30 staining

The total villous area was tagged in red (a) and the sum of this area was calculated by image pro plus software. The total M30 (brown) staining (b) was tagged in yellow (c) and the sum of this area calculated. The area of M30 staining was then expressed as a percent of total villous area. Images taken at x 250 magnification.
Figure 2.5: Example images for the quantification of ki67/E-cadherin dual staining

High magnification images (a-c) and an example of a typical image used for quantification purposes (d); taken at x 400 magnification on day 7 of explant culture. Blue arrows indicate proliferating cytotrophoblast cells (brown nuclei surrounded by red staining). Red arrows indicate examples of non proliferating cytotrophoblast cells (blue nuclei with surrounding red staining). Proliferating cytotrophoblast cells were expressed as a percent of total cytotrophoblast cell population. The black arrow (d) indicates a nucleus close to the trophoblast but with no surrounding E-cadherin staining (i.e. a stromal nucleus). This illustrates the necessity for E-cadherin dual staining to accurately identify cytotrophoblast nuclei.
Figure 2.6: Example images for the quantification of cytokeratin 7 staining
(a) villi with intact (in) syncytiotrophoblast (b) villi with shed syncytiotrophoblast (s) and complete cellular regeneration (black arrows) (c) villi with shed syncytiotrophoblast (s) with no cellular regeneration (red arrows). Original images taken at x 250 magnification on day 7 of explant culture.
2.8 Expression of results and statistics

Details of the expression of results and statistical analysis are provided in individual methods sections for each results Chapter (see Chapters 3, 4 and 5).

The time course of placental taurine uptake (30-120 minutes) and TauT activity was analysed by least squares linear regression. Over this time frame uptake was linear and differences between slope and intercept were compared using least squares linear regression. Additionally taurine uptake at 120 minutes was used to compare TauT activity between patient groups as described below.

For studies on fresh tissue numbers were based on previous similar data on amino acid uptake and protein expression of amino acid transporter proteins and other proteins (by Western blotting or IHC) (Desforges et al., 2013; khullar et al., 2004; Roos et al., 2004; Shibata et al., 2008). On this basis an n of 9 was considered appropriate in patient groups to compare normal pregnancy and pre-eclampsia in ideal weight and obese women. For in vitro studies numbers were based on similar publications using the placental villous explant model (Audette et al., 2014; Desforges et al., 2013) and an n of 6 was considered appropriate to show statistical significant differences in response to treatment of placental villous fragments. Where the numbers were equal to 6, or the data were not normally distributed, non parametric statistics were employed (Mann Whitney U test, Wilcoxon matched pairs signed rank as appropriate) to compare between groups.
Chapter 3: Placental TauT activity in normal pregnancy and pre-eclampsia

3.1 Introduction

PE complicates up to 5% of pregnancies and is a leading cause of maternal and perinatal morbidity and mortality accounting for the death of 3-5 women and 600 babies a year in the UK (Abalos et al., 2013; CESDI 5th Annual report 1998). Those babies that do survive are more likely to have suffered from FGR and life-threatening diseases in adulthood (Barker et al., 1989; Bellamy et al., 2007; Kajantie et al., 2009; Vatten, 2003). The aetiology of PE is complex but activation of the endothelium underpins maternal disease and factors initiating endothelial dysfunction derive from placenta because symptoms resolve after delivery. There are no means to prevent or treat PE and the only effective management is delivery of the placenta.

STB is a specialised multinucleate cell in the human placenta that functions as a solute transporting epithelium and endocrine/paracrine organ, maintaining nutrient delivery to the fetus and producing hormones that sustain pregnancy. STB is renewed throughout pregnancy by cellular turnover (Huppertz, 2008b). In PE, STB renewal is dysregulated with altered proliferation, apoptosis and syncytial knots and reduced STB volume (Arnholdt et al., 1991; Calvert et al., 2013; Daayana et al., 2004; Longtine et al., 2012; Sharp et al., 2010). Abnormal turnover compromises STB integrity and renewal, culminating in reduced nutrient delivery to the fetus, which can lead to FGR and the release of cytokines and necrotic material into maternal blood which initiates a maternal inflammatory response and endothelial cell activation in PE.

Obesity is a major risk factor for PE and the risk of PE increases progressively with increasing BMI (Bodnar et al., 2005; Catov et al., 2007; Mbah et al., 2010). Obesity has been classified as a worldwide pandemic affecting 400 million adults and the prevalence is increasing rapidly. As the incidence of maternal obesity is rising it is expected that the incidence of PE will increase in parallel. In common with PE, dysregulation of STB turnover is evident in maternal obesity (Higgins et al. 2013) and both conditions are associated with increased placental oxidative/nitrative stress (Myatt et al., 1996; Roberts et al., 2009). In addition, studies from this laboratory have shown that the uptake of taurine into the STB is
significantly lower in obese women compared to women of ideal weight (Desforges et al., 2013a). Preliminary studies suggest that taurine uptake into the STB is also reduced in PE compared to normal pregnancy, indicating another potential abnormality common to obesity and PE.

Taurine is a β amino acid which is transported into the STB by its transporter, TauT (see Chapter 1, Section 1.6.1). The activity of TauT allows the STB to accumulate taurine to achieve levels approximately 150 x greater than those in maternal blood (Philipps et al., 1978). Taurine is conditionally essential for both fetus and placenta, as fetal tissues cannot synthesise taurine (Gaull et al., 1972), therefore they rely entirely on the supply of taurine from the maternal circulation. The importance of taurine for fetal growth evident in FGR where placental taurine uptake and fetal plasma taurine concentration is lower than normally grown babies (Cetin et al., 1990; Economides et al., 1989; Norberg et al., 1998). Maintaining intracellular taurine has been shown in many tissues to be important for facilitating cell turnover processes such as proliferation and apoptosis (Han and Chesney, 2013; Hernández-Benítez et al., 2012; Takatani et al., 2004b). Inhibition of TauT by siRNA-mediated knockdown in disrupts differentiation and fusion in primary CTBs (Desforges et al., 2013b). In addition taurine is cytoprotective limiting damage caused by ischaemia reperfusion injury, inflammation and oxidative/nitrative stress; all features of the placenta in PE (Oriyvanhan et al., 2005; Tong et al., 2006; Yang et al., 2013; Zhang et al., 2008).

Bearing in mind the functions of taurine, it is possible that taurine depletion in STB, through reduced TauT activity, may contribute to altered STB renewal in PE. However, it has yet to be confirmed whether TauT activity is reduced in PE compared to normal pregnancy and how maternal BMI impacts on this.

**Hypothesis and aims**

The experiments performed in this Chapter tested the hypothesis that STB TauT activity is lower in pregnancies complicated by PE compared to normal pregnancy.

The main aims were to:

1) Determine whether STB TauT activity in villous tissue from placentas of women having PE was reduced compared to women experiencing normal pregnancy by measuring the Na⁺-dependent uptake of ³H-taurine in freshly isolated placental villous fragments.
2) Determine whether the negative relationship between STB TauT activity and maternal BMI previously reported in normal pregnancy exists in women with PE.

3.2 Methods

3.2.1 Measuring TauT activity in placental villous fragments

Placental TauT activity was measured in fresh placental villous fragments by the Na\(^+\)-dependent uptake of \(^3\)H-taurine over 30-120 minutes and expressed in fmol/mg protein (for detailed methods see Chapter 2 section 2.3). Villous fragments were prepared from placentas of women having normal pregnancy (n=44) and women experiencing PE (n=24) over a range of maternal BMI (19.8-48 kg/m\(^2\)).

Demographic data collected from women participating in this study included age, ethnicity, parity and gravidity. Information on mode of delivery, infant sex and weight were collected at the time of delivery. Smoking status of women was determined from patient notes where self-reported smoking status is recorded following a routine antenatal questionnaire. The individualised birth weight ratio (IBR) was calculated using GROW (Gestation Related Optimal Weight) customised centile software (Gestation Network). This software takes into account the maternal characteristics of height, weight, ethnicity, parity and the infant characteristics; sex, birth weight and gestational age. FGR was defined as the delivery of an infant with an IBR of ≤ 5. Exclusion criteria for PE participants included gestational diabetes, pre-existing hypertension, renal disease and delivery of an LGA baby (IBR > 90).

3.2.2 Presentation of results and statistical analysis

Data were represented as median and interquartile range unless otherwise stated.

Least squares linear regression was used to analyse the relationship between Na\(^+\)-dependent \(^3\)H-taurine uptake (TauT activity) and time in villous fragments from normal pregnancy and PE. As the relationship between TauT activity and time was linear over 30 - 120 minutes, the 120 minute time point was chosen to represent TauT activity in all subsequent analyses. Least squares linear regression was used to analyse the relationship between maternal BMI and TauT activity in normal pregnancy (n=44) and between
maternal BMI and TauT activity in PE (n=24). TauT activity in normal pregnancy (n=35) and PE (n=23) over a matched BMI range (19.8-39 kg/m²) was expressed as a box and whisker plot and analysed using a Mann Whitney U test.

To assess the influence of BMI on TauT activity in PE, TauT activity was compared between ideal weight women having normal pregnancy (n=9) and PE (n=8), ideal weight women (n=8) and obese women (BMI 30-39 kg/m²; n=8) experiencing PE, and obese women (BMI 30-39 kg/m²; n=16) having normal pregnancy and PE (n=8). Differences between these groups were assessed using a Mann Whitney U test.

To assess the effect of FGR on TauT activity in PE, activity was compared in placentas of women with PE having a FGR baby (n=9) and women having a baby appropriately grown for gestational age (AGA; n=15). Placental TauT activity from women with PE was used to assess differences between early (n=13) and late (n=11) onset disease (onset of symptoms before or after 34 weeks gestation respectively). Data were expressed as scatter dot plots and medians compared using a Mann Whitney U test.

All data analyses were performed using GraphPad Prism version 5.0 (GraphPad Inc, CA, USA). Statistical significance was set at p<0.05.
3.3 Results

3.3.1 Demographics

Table 3.1 shows the demographic information for the women whose placentas were included in this study to determine TauT activity. Activity was examined in 68 placentas, 44 from women having normal pregnancy (9 ideal weight, 9 overweight and 26 obese) and 24 from women having PE (8 ideal weight, 8 overweight and 8 obese). Maternal age was significantly lower in women of ideal weight having PE compared to normal pregnancy. The majority of placentas in normal pregnancy were delivered by elective caesarean section. In PE there was a higher incidence of emergency caesarean section and vaginal deliveries with/without instrumental aid. Gestation in the overweight women with PE was significantly lower than in overweight women having normal pregnancy. Overall gestation tended to be lower in PE compared to normal pregnancy. Fetal birth weight and IBR were significantly lower in the overweight women with PE compared to overweight women with normal pregnancy and obese women with PE. In the pregnancies complicated by PE, 4 infants from the ideal weight group (50%), 3 from the overweight group (37%) and 2 from the obese group (25%) were FGR (IBR below the 5th centile).
Table 3.1: Demographic data for the cohort in which placental TauT activity was determined

<table>
<thead>
<tr>
<th></th>
<th>Normal pregnancy</th>
<th>Normal pregnancy</th>
<th>Normal pregnancy</th>
<th>Pre-eclampsia ideal</th>
<th>Pre-eclampsia ideal</th>
<th>Pre-eclampsia ideal</th>
<th>Pre-eclampsia ideal</th>
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<td></td>
<td>ideal weight</td>
<td>overweight</td>
<td>obese</td>
<td>weight</td>
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<td>obese</td>
<td>Overweight</td>
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<tr>
<td></td>
<td>n=9</td>
<td>n=9</td>
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<td>BMI (kg/m²)</td>
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<td>27 (25-29)</td>
<td>37 (30-48)</td>
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<td>35 (19-39)</td>
<td>32 (25-40)</td>
<td>23 (19-27)</td>
<td>24 (20-37)</td>
<td>24 (18-34)</td>
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<td>7 (77%) Caucasian</td>
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<tr>
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<td>1 (11%) Asian</td>
<td>1 (11%) Israeli</td>
<td>1 (4%) Pakistani</td>
<td>1 (12.5%) Pakistani</td>
<td>1 (12%) Syrian</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
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<td>1 (11%) Asian</td>
<td>2 (4%) African</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td>ELCS</td>
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<td>9 (100%)</td>
<td>24 (92%)</td>
<td>5 (62.5%)</td>
<td>1 (12.5%)</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EMCS</td>
<td></td>
<td></td>
<td>1 (4%)</td>
<td>2 (25%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td>VD</td>
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<td></td>
<td>1 (4%)</td>
<td>1 (12.5%) (INST)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gestation (Days)</td>
<td>273 (271-288)</td>
<td>273 (262-280)</td>
<td>273 (266-276)</td>
<td>249 (213-279)</td>
<td>231 (204-254)</td>
<td>259 (194-293)</td>
<td>0.0016</td>
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</tr>
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<td>3 (1-5)</td>
<td>2 (2-5)</td>
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<td>2 (1-4)</td>
<td>1 (1-6)</td>
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<td>1 (0-4)</td>
<td>2 (0-4)</td>
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<td>1 (0-3)</td>
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<td>2 (25%)</td>
<td>No</td>
<td>No</td>
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<td>Fetal Sex</td>
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</tr>
<tr>
<td>Male</td>
<td>3 (33%)</td>
<td>5 (55%)</td>
<td>11 (42%)</td>
<td>3 (37.5%)</td>
<td>5 (62.5%)</td>
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<td></td>
</tr>
<tr>
<td>Female</td>
<td>6 (67%)</td>
<td>4 (45%)</td>
<td>15 (58%)</td>
<td>5 (55.5%)</td>
<td>3 (37.5%)</td>
<td>3 (37.5%)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Fetal Weight</td>
<td>3480 (2730-3940)</td>
<td>3450 (2800-3920)</td>
<td>3628 (2760-4140)</td>
<td>1765 (1260-3380)</td>
<td>1530 (638-2880)</td>
<td>3652 (1300-4320)</td>
<td>0.0001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IBR</td>
<td>65 (10-89)</td>
<td>46 (40-88)</td>
<td>63 (19-93)</td>
<td>6 (0-59)</td>
<td>6 (0-29)</td>
<td>29 (0-76)</td>
<td>0.0012</td>
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</tr>
<tr>
<td>Number of FGR</td>
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<td></td>
<td></td>
<td>4 FGR</td>
<td>3 FGR</td>
<td>2 FGR</td>
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<td>Onset of PE</td>
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</tr>
<tr>
<td>Early onset</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>4 (50%)</td>
<td>6 (75%)</td>
<td>3 (37.5%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Late onset</td>
<td></td>
<td></td>
<td></td>
<td>4 (50%)</td>
<td>2 (25%)</td>
<td>5 (62.5%)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Data are median and range. BMI = body mass index, ELCS = elective caesarean section, EMCS = emergency caesarean section, VD = vaginal delivery, INST = vaginal delivery with instrumental aid, IBR = individualised birth weight ratio, FGR = fetal growth restriction, defined as an IBR below the 5th centile. Early and late onset pre-eclampsia defined as the onset of symptoms before or after 34 weeks gestation respectively. Data are compared between categories using a Kruskall Wallis test with a Dunn’s multiple comparison test. Statistical significance was set at p<0.05. Letters indicate columns between which statistical significance was identified.
3.3.2 Placental TauT activity in normal pregnancy and pre-eclampsia

Uptake of $^3$H-taurine into placental villous fragments was investigated over a time course of 30, 60, 90 and 120 minutes. Figure 3.1 shows the uptake of $^3$H-taurine in the presence and absence of Na$^+$ in placentas from women having normal pregnancy (Figure 3.1a) and PE (Figure 3.1b). The uptake of $^3$H in the presence of Na$^+$ was linear up to 120 minutes in both normal pregnancy and PE. Uptake of $^3$H-taurine in the absence of Na$^+$ was consistently low, did not increase with time and was not different between normal pregnancy and PE. As TauT is a Na$^+$-dependent transporter, the accumulation of $^3$H-taurine in the absence of Na$^+$ is attributed to non-specific diffusion and uptake in the presence of Na$^+$ reflects specific carrier mediated taurine transport into the STB by TauT expressed in the MVM. To calculate the Na$^+$-dependent component of $^3$H-taurine uptake in both normal pregnancy and PE the uptake of $^3$H-taurine in the absence of Na$^+$ was subtracted from the uptake in the presence of Na$^+$ (Figure 3.1). The Na$^+$-dependent uptake of taurine in normal pregnancy and PE was linear between 30 - 120 minutes and the regression line of activity with time extrapolates the x,y axis close to the origin, indicating that TauT activity is at initial rate up to 120 minutes. The Na$^+$-dependent uptake of taurine was significantly lower in PE compared to normal pregnancy (Figure 3.1c).
Figure 3.1: Placental TauT activity in normal pregnancy and pre-eclampsia (PE)

The uptake of $^3$H-taurine in the presence (blue squares) and absence (red circles) of Na$^+$ in normal pregnancy (a) and PE (b). The Na$^+$-dependent component of $^3$H-taurine uptake (black triangles) was calculated by subtracting the uptake of $^3$H-taurine in the absence of Na$^+$ from the uptake in the presence of Na$^+$. TauT activity was linear up to 120 minutes in normal pregnancy and PE (*p<0.0001; Least squares linear regression). (c) Taut activity in PE was significantly lower than in normal pregnancy (*p<0.03: least squares linear regression). Data are expressed as median and interquartile range (NP n=44, PE n=24).
3.3.3 Placental TauT activity in pre-eclampsia in relation to fetal weight and onset of disease

It has previously been reported that TauT activity is lower in STB MVM vesicles of placentas from women having a growth restricted compared to a normally grown infant (Norberg et al., 1998). To assess whether the reduction in TauT activity in PE was due to an effect of FGR in the current study, women with PE were divided into those having AGA babies and those having FGR babies (Figure 3.2 a). There was no difference in placental TauT activity (measured at 120 minutes corresponding to initial rate) in women with PE having AGA and FGR infants.

To determine whether the reduction in TauT activity in PE was due to an effect of onset of disease, the PE group was divided into early and late (before and after 34 weeks gestation respectively) disease onset. Figure 3.2 b shows that there was no difference in placental TauT activity between early and late onset PE.
Figure 3.2: Placental TauT activity in relation to fetal growth and onset of disease

Placental TauT activity was similar in women having pre-eclampsia with AGA (n=15) and FGR (n=9) babies (a) and between women with early (n=13) and late (n=11) onset pre-eclampsia (PE) (b). Line represents the median.
3.3.4 Placental TauT activity in normal pregnancy and pre-eclampsia in relation to maternal BMI

A previous study from this laboratory showed that placental TauT activity was significantly lower in obese compared to ideal weight women having normal pregnancy and negatively related to maternal BMI (Desforges et al., 2013a). To investigate the effect of maternal BMI in the current study, TauT activity at 120 minutes was plotted against maternal BMI (Figure 3.3). Figure 3.3 a shows that there was a significant negative relationship between TauT activity and maternal BMI (blue triangles) in women having normal pregnancy. Figure 3.3 a also compares TauT activity (at 120 minutes) in normal pregnancy with PE. Most of the values for TauT activity in PE fell below the regression line fitted to the data in normal pregnancy, indicating lower activity in PE (consistent with the data in Figure 3.1 c). However, in contrast to normal pregnancy, there was no significant relationship between TauT activity and maternal BMI (19.8-41 kg/m²) in PE (Figure 3.3 a pink squares). Placental TauT activity was compared between PE and normal pregnancy across a matched BMI range (19.5-39 kg/m²) to investigate the influence of maternal BMI on TauT activity in PE. Figure 3.3 b shows that there was significantly lower TauT activity in PE compared to normal pregnancy in women with matched BMI.
Figure 3.3: Placental TauT activity in relation to maternal BMI

a) Placental TauT activity in normal pregnancy (blue triangles, n=44) and PE (pink squares, n=24). There was a significant negative relationship between TauT activity and increasing maternal BMI in normal pregnancy (NP) (least squares linear regression, p<0.001, R²=0.25).

b) Placental TauT activity across a matched BMI range (19.5-39 kg/m²) was significantly lower in PE (n=23) compared to NP (n=35, Mann Whitney U test, *p<0.03). Line represents the median.
The possibility that obesity could account for the reduced placental TauT activity in PE was assessed in three ways: firstly, placental TauT activity in women of ideal weight having normal pregnancy or PE were compared (Figure 3.4 a). TauT activity was significantly lower in ideal weight women with PE compared to normal pregnancy (BMI 19.8-24.9 kg/m²).

Secondly, TauT activity was compared in women having PE that were ideal weight (BMI 19.8-24.9 kg/m²) or obese (BMI 30-38 kg/m²). There was no difference in TauT activity between ideal weight and obese women with PE (Figure 3.4 b). Lastly, placental TauT activity was compared in obese women having normal pregnancy or PE (BMI 30-39 kg/m²). TauT activity was significantly lower in obese women with PE compared to obese women having normal pregnancy (Figure 3.4 c).
Figure 3.4: Placental TauT activity in normal pregnancy (NP) and pre-eclampsia (PE)

(a) Placental TauT activity in ideal weight women (BMI 19.5-24.9 kg/m$^2$) was significantly lower in PE (n=8) compared to NP (n=9, Mann Whitney U test *p<0.03). (b) Placental TauT activity was similar in ideal weight women with PE (n=8) and obese women (BMI 30-38 kg/m$^2$) with PE (n=8). (c) Placental TauT activity in obese women (BMI 30-39 kg/m$^2$) was significantly lower in PE (n=8) compared to NP (n=16) (Mann Whitney U test, *p<0.03).
3.4 Discussion

This study demonstrated that placental TauT activity was reduced in PE compared to normal pregnancy. This effect could not be attributed to FGR and was unrelated to onset of disease. This data is consistent with a previous report that TauT activity is lower in women with raised BMI than in ideal weight women having an otherwise normal pregnancy (Desforges et al., 2013a). However, a raised maternal BMI did not account for the reduction of TauT activity in PE.

3.4.1 Methodological considerations

The villous fragment preparation has been used in several studies to estimate amino acid transport over the MVM into placental STB in intact tissue (Audette et al., 2014; Farley et al., 2010; Greenwood and Sibley, 2006; Shibata et al., 2008). Amino acid transporter activity is also studied by measuring uptake of radiolabelled substrates into STB MVM vesicles. A study by Brand et al. (2010) compared the uptake of L-serine by system A and system L into MVM vesicles and placental villous fragments from the same placenta. Although there were differences in the transporter contribution between the two methods uptake was comparable in both. It was concluded by the authors that both models are appropriate for studying amino acid transporters in the placenta. The advantage of the villous fragment model is that tissue integrity/architecture, intracellular signalling mechanisms and driving forces are maintained (Brand et al., 2010).

There are methodological challenges when using villous fragments to determine transporter activity in the STB MVM by uptake of radiolabelled solutes. It is important to distinguish between the component of tissue accumulation due to the activity of specific transporter proteins in the MVM from the components which are due to nonspecific diffusion through plasma membranes and the cut ends of the tissue. In the current experiments, the fact that TauT is a Na\(^+\)-dependent transporter was exploited to identify specific and nonspecific tissue accumulation. Villous fragments were incubated in the presence and absence of Na\(^+\); uptake in the absence of Na\(^+\) represented accumulation via nonspecific routes and was subtracted from uptake in the presence of Na\(^+\) to determine the Na\(^+\)-dependent, or carrier mediated transport, of taurine. Despite there being several cell types in villous tissue that could contribute to uptake of taurine, the Na\(^+\)-dependent component is taken to reflect unidirectional transport across STB MVM. Extrapolation of
the least squares linear regression line of TauT activity over 30-120 minutes intercepted the x,y axis close to the origin. This indicates that transport approximates to initial rate which is important as it supports the assumption that uptake reflects transport across the MVM into a single tissue compartment, the STB. In addition, the MVM is the first plasma membrane to be encountered and the STB, which covers all villous tissue, contributes the largest volume and surface area (Benirschke, 1998). Therefore, in the current study, the reduction in TauT activity demonstrated in placental villous fragments of women with PE compared to normal pregnancy implicates altered transport activity at the STB MVM.

During tissue processing for taurine uptake studies, the tissue was maintained in Tyrode’s:DMEM solution with 100 µM taurine, which is the estimated concentration of taurine in maternal plasma (Philipps et al., 1978). This was to ensure that the placental fragments were maintained in physiological conditions for as long as possible as it has been established that TauT activity and mRNA expression is up-regulated in response to low taurine availability in several cell types (Chesney et al., 1990; Jones et al., 1990) including choriocarcinoma cells (Jayanthi et al., 1995).

Taurine is not metabolised or incorporated into proteins (Lambert et al., 2014) and therefore the measurement of taurine uptake into the STB will not be affected by a loss of radiolabelled taurine through metabolism. Incubation solutions contained trace amounts of $^3$H-taurine (0.5 µCi/ml; 25 nM) to ensure that the substrate concentration did not exceed the $K_m$ of the transporter which has been determined to be 6 µM in STB MVM (Ramamoorthy et al., 1994b). Another important methodological consideration is to standardise the size of the villous fragments between experiments as transporter activity (expressed per mg protein) is negatively correlated to fragment size (Greenwood and Sibley, 2006). In this study fragment size, as estimated by protein content, was maintained between 0.5-1.0 mg and did not differ between study groups.

It has been demonstrated that, in addition to transport via TauT, taurine can also be transported by PAT 1 in human intestinal brush border membranes and by GABA in rat hepatocytes (Anderson et al., 2009; Ikeda et al., 2012). Taurine uptake by an OAT (SLC22A13) has also been reported in embryonic kidney 293 cells (Schulz et al., 2014). However, the same study also demonstrated SLC22A13 is not expressed in the placenta. OAT1 and OAT4 have been detected in the human placenta (Myllynen et al., 2009); however it is unknown whether they contribute to taurine uptake. Although these transporters are present in the placenta, they are Na$^+$-independent transporters and
therefore will not have contributed to the Na\(^+\)-dependent uptake of taurine by TauT measured in this study. It is also noteworthy that the Na\(^+\)-independent uptake of taurine was not different between villous fragments of normal pregnancy and PE.

In summary, villous fragments are an appropriate preparation in which to determine differences in placental TauT activity in normal and complicated pregnancies.

### 3.4.2 Placental TauT activity is significantly lower in pre-eclampsia compared to normal pregnancy

The time course of Na\(^+\)-dependent \(^3\)H-taurine uptake (30-120 minutes) into villous fragments showed that STB TauT activity was significantly lower in PE compared to normal pregnancy. Reduced TauT activity has been demonstrated in STB MVM vesicles from pregnancies complicated by FGR compared to normal pregnancy (Norberg et al., 1998). The PE group in this study included placentas from both FGR (n=9) and AGA (n=15) infants. However, using the villous fragment model, there was no difference in placental TauT activity in PE with FGR compared to PE with AGA infants. Therefore, these preliminary studies suggest that the reduction in TauT activity demonstrated in PE is not related to birthweight. The PE group in the current study also included placentas from both early onset (n=13) and late onset (n=11) PE. Gestation at delivery was significantly lower in the women with early onset PE compared to those with late onset PE (Mann Whitney, p<0.0001). However, there was no difference in TauT activity in early onset PE compared to late onset PE. Therefore, these preliminary studies suggest that neither onset of disease nor early gestation at delivery account for the reduction in TauT activity in PE. However, this study was not powered to make definite conclusions on the influence of FGR, gestation and onset of disease on TauT activity in PE.

Other possible factors that might have influenced placental TauT activity include maternal age and mode of delivery. Women of ideal weight having PE were significantly younger than their ideal weight counterparts having normal pregnancy. As a whole, women having normal pregnancy tended to be older and have a greater age range than women with PE. Recent work from this laboratory has shown that placental TauT activity is higher in advanced maternal age (>40 years) compared to women aged 20-30 (Lean et al., 2014). In the current study there were 3 women having normal pregnancy (2 ideal weight and one obese) that were older than 40. However, when the women over 40 were removed from the data sets, statistical significance was unaffected indicating that there was no influence
of advanced maternal age in this study. Another potential influence on STB TauT activity was the mode of delivery which varied between study groups. However, previous studies in this laboratory have shown that STB TauT activity is unaffected by mode of delivery (Ditchfield, personal communication). Therefore, the reduction in TauT activity in PE compared to normal pregnancy demonstrated in the current study can be attributed to PE per se.

3.4.3 Placental TauT activity in pre-eclampsia: effect of maternal BMI

Placentas were collected from normal pregnancy and PE across a range of BMI between 19.8 and 48 kg/m$^2$. Previous work in this laboratory has shown that placental TauT activity is significantly lower in obese women compared to women of normal weight at term (Desforges et al., 2013a). With this in mind, the influence of maternal BMI on the reduction in TauT activity in PE was investigated. TauT activity was measured in women with raised maternal BMI having otherwise normal pregnancy to firstly confirm previous studies showing that TauT activity is lower in placentas of obese compared to ideal weight women and secondly to act as temporal controls for the studies on placentas from pregnancies complicated by PE. In common with previous findings, placental TauT activity was significantly negatively related to maternal BMI in normal pregnancy. However, no significant relationship was observed between STB TauT activity and maternal BMI in PE, possibly because of the low number of women with PE having a BMI $>40$ kg/m$^2$. Measurements of TauT activity in placentas of women with PE over the same BMI range as normal pregnancy would confirm whether the negative relationship in normal pregnancy also prevails in PE.

A number of comparisons were made which collectively suggest that TauT activity is lower in STB from pregnancies complicated by PE independently of obesity. Firstly, in women of ideal weight, TauT activity was significantly lower in PE compared to normal pregnancy. Secondly, there was no difference in TauT activity in placentas of ideal weight and obese women experiencing PE. Thirdly, placental TauT activity in obese women was significantly lower in PE compared normal pregnancy. Overall the results show that both obesity and PE can independently reduce placental TauT activity. Furthermore, the data suggest that PE and obesity are not additive in their down-regulation of TauT activity. However, multiple comparisons have been performed on a relatively small sample size and the study was not powered to make definite conclusions from these comparisons. Although obesity and PE
can independently reduce STB TauT activity, it is possible that the same factor/s inhibit the transporter in both pregnancy complications.

### 3.4.4 Implications of reduced TauT activity in pre-eclampsia

The reduction in STB TauT activity in PE could lead to a depletion of taurine in the STB, assuming that there is no compensatory change in taurine efflux. However, it is not known whether STB taurine is lower in PE compared to NP. If a reduction in taurine uptake into STB in PE does lower intracellular taurine, it would have implications for the fetus and placenta including a) reduced transfer of taurine to the fetus, b) disrupted STB turnover and c) reduced cytoprotection. Reduced placental TauT activity has been demonstrated in both obese women having normal pregnancy and women experiencing PE. Maternal obesity is a major risk factor for developing PE and in this context it might be significant that TauT activity is also reduced in placentas of obese women in the first trimester of pregnancy (Desforges et al., 2013a). A reduction in taurine uptake in the first trimester could increase susceptibility to developing PE through dysregulated STB renewal and/or reduced cytoprotection from inflammatory cytokines that are elevated in obesity. It is possible that the effect of PE to lower TauT activity is selective for TauT as the activity of the system A amino acid transporter, which is also Na⁺-dependent, is unaffected by PE (Shibata et al., 2008). However, neither the cause of reduced TauT activity, nor the consequence for STB renewal, have been investigated in PE or maternal obesity.

### 3.4.5 Summary

In summary, the current study demonstrated that a) placental TauT activity is significantly lower in PE compared to normal pregnancy, b) as previously reported, placental TauT activity is negatively related to maternal BMI and c) the effect of PE is apparent in women of ideal weight and is not exacerbated by obesity. Bearing in mind the importance of taurine transport across the MVM from the maternal blood for fetal and placental growth and development it is important to establish what down-regulates TauT activity in PE. There are several mechanisms by which TauT activity may be reduced in PE including reduced TauT protein expression/localisation to the plasma membrane and/or post translational down-regulation of activity in existing transport proteins. Since the data have shown that there is no greater reduction in TauT activity in obese women with PE compared to ideal weight women with PE, it is possible that the same inhibitory factor
reduces TauT activity in both conditions. In the next chapter the possibility that reduced TauT expression is responsible for the reduction in TauT activity is investigated. Another possibility that post translational modification of existing transporters could mediate the reduced placental TauT activity in PE is investigated focussing on nitration as a potential modulatory factor.
Chapter 4: Investigating the mechanism of reduced placental TauT activity in pre-eclampsia

4.1 Introduction

The activity of the taurine transporter, TauT, was found to be significantly lower in placental villous tissue of women experiencing PE compared to normal pregnancy (Chapter 3). It was reported previously (Desforges et al., 2013a), and confirmed in the current study, that TauT activity is also significantly lower in placentas of obese women having otherwise normal pregnancy compared to their ideal weight counterparts (Chapter 3). However, placental TauT activity was lower in PE compared to normal pregnancy in women of ideal weight which suggests that PE lowers TauT activity per se. Although PE and maternal obesity are independently associated with reduced taurine uptake into STB, it is plausible that they have features in common which down regulate TauT activity.

The reduction in taurine uptake into STB in PE and maternal obesity, as assessed by accumulation of radiolabelled taurine into villous tissue, implicates a reduction in TauT activity at the MVM which could arise for at least four reasons: (a) lower protein expression in the MVM, following reduced mRNA expression and protein synthesis (b) altered trafficking of TauT so that existing transporter protein is not inserted into the MVM (c) altered affinity of TauT for taurine and/or (d) post translational modification of protein function. However, a reduction in placental TauT activity with no change in TauT protein expression has been demonstrated in FGR, maternal obesity and recently in women with gestational diabetes (Roos et al. 2004; Desforges et al. 2013; Ditchfield personal communication; Hey et al. 2014). Collectively these studies suggest that there is a post translational modification of TauT that inhibits TauT activity in pregnancy complications and/or altered TauT trafficking to the MVM. However, it remains to be determined whether TauT protein expression is lower in PE compared to normal pregnancy in parallel with the reduced TauT activity.

There are several studies of TauT regulation in human placenta. In CTBs, inhibition of mTOR with rapamycin showed a reduction in TauT activity but no change in protein expression. This led the authors to propose that mTOR regulates placental TauT activity by post translational modifications or by affecting transporter trafficking to the MVM (Roos et al., 2009). Phosphorylation inhibits TauT activity in several tissues (Brandsch et al., 1993; Han
et al., 1999; Loo et al., 1996; Voss et al., 2004) and activation of PKC, which phosphorylates TauT, inhibits TauT activity in JAr choriocarcinoma cells (Kulanthaivel et al., 1991) and placental villous fragments (Roos et al., 2004). Lastly, it has been established in many tissues that TauT is adaptively regulated by extracellular taurine concentration and in JAr cells this is achieved by altered activity of TauT (Jayanthi et al., 1995).

A potential mechanism of post translational down-regulation of TauT in STB, in the context of PE and maternal obesity, is through nitration of the transporter protein. Nitration of tyrosine residues, forming nitrotyrosine, is an important mechanism of post translational modification of proteins to regulate function. The TauT protein has 34 tyrosine residues which are potential targets for nitration (see Chapter 1, Figure 1.8). Kulanthaivel (1989) explored the post-translational regulation of TauT in STB MVM and demonstrated that the nitrating agent tetranitromethane, which targets tyrosine residues, significantly inhibited TauT activity. This showed the importance of tyrosine groups in regulating TauT and the potential for activity to be inhibited by nitration. Short term exposure in vitro of placental villous fragments (Khullar et al., 2004) and a rat STB cell line (Lee and Kang, 2010) to nitrative stress significantly reduced TauT activity implying that nitration of TauT could down regulate activity. In the placenta in situ, tyrosine groups on proteins are nitrated by ONOO\(^-\) which is generated under conditions of elevated nitrative stress (Myatt, 2010; Webster et al., 2008). Nitration of several proteins, including p38 MAPK and p2X(4), is elevated in the placenta in PE compared to normal pregnancy. Preliminary evidence, quoted in two review articles, suggests that TauT can be nitrated (Myatt, 2010) and that TauT nitration might be elevated in PE (Webster et al., 2008). There is also other evidence that supports chronically elevated placental nitrative stress in PE. Nitrotyrosine formation is a fingerprint of ONOO\(^-\) formation and action and is used as a marker of chronic exposure to ONOO\(^-\). Myatt et al. (1996) showed that nitrotyrosine protein expression in the villous endothelium, as indicated by immunohistochemistry, was greater in PE compared to normal pregnancy. However, increased expression of nitrotyrosine in STB remains to be shown. Increased placental nitrative stress is also a feature of obesity (Roberts et al., 2009) therefore TauT nitration could underlie reduced placental TauT activity in both PE and obesity.
Hypothesis and aims

The experiments in this chapter test the hypotheses that reduced placental TauT activity in PE is due to a) reduced TauT protein expression and/or b) increased nitration of TauT compared to normal pregnancy.

The main aims were:

1) To determine whether TauT protein expression is lower in placentas (in which TauT activity had previously been determined) of women with PE compared to normal pregnancy by Western blot analysis of placental membrane enriched homogenates and MVM isolates.

2) To determine whether nitrotyrosine protein expression is higher in placentas of women with PE compared to normal pregnancy by IHC and Western blot analysis of placental membrane enriched homogenates.

3) Explore whether placental TauT can be nitrated, and whether there is an increased proportion of nitrated TauT protein in placentas from women with PE compared to normal pregnancy, using Western blot analysis of nitrotyrosine following immunoprecipitation of TauT.

4.2 Methods

4.2.1 Western blot analysis of placental TauT expression

Placental TauT protein expression was assessed by Western blot analysis of membrane enriched homogenates from placentas in which TauT activity had been previously determined (with the exception of 2 in the PE obese group; for detailed methods see Chapter 2 sections 2.4). Membrane enriched homogenates were prepared from placentas of women having normal pregnancy who were ideal weight (n=9) or morbidly obese (i.e. BMI ≥40; n=8) and women having PE who were ideal weight (n=8) or obese (BMI>36; n=8). These BMI categories were chosen to examine TauT expression at the extremes of BMI. In addition, Western blot analysis of TauT expression was performed in isolated MVM from ideal weight women having normal pregnancy (n=4) and PE (n=4) from placentas with no matched TauT activity measurements to compare TauT expression in pure MVM to membrane enriched samples (for detailed methods see Chapter 2, section 2.4).
4.2.2 Analysis of nitrotyrosine expression using Western blotting and immunohistochemistry

Placental nitrotyrosine protein expression was assessed by Western blot analysis of membrane enriched homogenates from placentas in which TauT activity had previously been determined (for detailed methods see Chapter 2, section 2.4). Membrane enriched homogenates were prepared from placentas of women having normal pregnancy who were ideal weight (n=9) or morbidly obese (i.e. BMI ≥40; n=8) and women having PE who were ideal weight (n=8) or obese (BMI>36; n=8). Nitrotyrosine localisation and expression was assessed by immunostaining of placental villous tissue from ideal weight women having normal pregnancy (n=7) and PE (n=8) for nitrotyrosine (for detailed methods see Chapter 2, section 2.7.1-2.7.3).

4.2.3 Expression of results and statistical analyses

Following Western blot analysis of membrane-enriched placental homogenates and MVM samples, the density of TauT and nitrotyrosine immunoreactive species were normalised to β-actin expression in the same sample. Data are presented as scatter dot plots and analysed using Mann Whitney U or Kruskal Wallis statistical tests as appropriate. Semi-quantitative assessment of nitrotyrosine immunostaining at the STB in normal pregnancy and PE are presented as scatter dot plots and analysed using a Mann Whitney U test. All data analyses were performed using Image J image processing software version 1.44 (National Institutes of Health) and/or GraphPad Prism version 5.0 (GraphPad Inc, CA, USA). Statistical significance was set at p<0.05.

4.2.4 Immunoprecipitation of TauT and Western blot analysis of nitrotyrosine

To explore whether placental TauT can be nitrated and whether there is an increased proportion of nitrated TauT in PE compared to normal pregnancy, TauT protein was immunoprecipitated from placental membrane enriched homogenates using a protein G immunoprecipitation kit (for detailed methods see Chapter 2, section 2.5). Membrane enriched homogenates were prepared from placentas of women having normal pregnancy who were ideal weight (n=6) and women having PE who were ideal weight (n=8). TauT nitration was then assessed by Western blot analysis of immunoprecipitated samples. The
density of immunoreactive signals were normalised to an internal control; the same sample included on both blots to normalise for gel variations.

### 4.2.5 Expression of results and statistical analyses

Following Western blot analysis of immunoprecipitated samples data are presented as scatter dot plots and analysed using a Mann Whitney U test. All data analyses were performed using Image J image processing software version 1.44 (National Institutes of Health) and GraphPad Prism version 5.0 (GraphPad Inc, CA, USA). Statistical significance was set at p<0.05.
4.3 Results

4.3.1 Demographics

Table 4.1 shows the demographic data for women whose placentas were used in this study to investigate TauT protein expression. Membrane enriched homogenates were prepared from placentas in which TauT activity had been previously determined (with 2 exceptions in the PE obese group) to assess TauT protein expression in placentas from women having normal pregnancy or PE.

The demographic data for women whose placentas were used to prepare membrane enriched homogenates show some differences between groups. There were more vaginal deliveries in women with PE compared to normal pregnancy and maternal age was significantly lower in women of ideal weight having PE compared to normal pregnancy. There was no difference in gestational age, fetal weight or IBR between groups. None of the women having normal pregnancy delivered FGR infants but 25% (4/16) of the women with PE had babies below the 5th centile (FGR).

MVM was isolated from placentas of ideal weight women having normal pregnancy (n=4) or PE (n=4) (TauT activity was not determined in these placentas). The women whose placentas were used for studies of MVM were well matched for maternal age, mode of delivery, gestational age and fetal sex. However, babies from normal pregnancy were appropriately grown whereas the babies of women with PE were all FGR.
| Table 4.1: Demographic data for the samples used in TauT protein expression studies |
|---------------------------------|---------------------------------|---------------------------------|
| **BMI (kg/m²)**                 | **Membrane Enriched Homogenates** | **Microvillous Membrane Vesicles** |
| NP Ideal weight n=9            | NP Obese n=8                     | PE Ideal weight n=8              | PE Obese n=8 | P | NP Ideal weight n=4 | PE Ideal weight n=4 | P |
| **Maternal Age**               | **Ethnicity**                    | **Mode of delivery**             | **Gestation (Days)**             | **Smoking (#/day)** | **Fetal sex** | **Fetal weight (g)** | **Onset of PE** | **Mean TauT Activity (fmol/mg protein at 120 m)** |
|                               | **BMI (kg/m²)** | **Maternal Age** | **Ethnicity** | **Mode of delivery** | **Gestation (Days)** | **Smoking (#/day)** | **Fetal sex** | **Fetal weight (g)** | **Onset of PE** | **Mean TauT Activity (fmol/mg protein at 120 m)** |
|                               | 23 (21.7-24)² | **34.5 (25-43)²** | **7 (77%) Caucasian 1 (11%) Asian 1 (11%) African** | **CS** | **273 (267-276)** | - | 4 (100%) | 3 (100%) | **3422 (3040-3720)** |
|                               | 24.4 (19.8-24.3)² | **23 (19-27)²** | **7 (87.4%) Caucasian 1 (12.5%) Pakistani** | **VD** | **273 (266-277)** | 2 (25%) | 2 (100%) | 2 (50%) | **1306 (740-2350)** |
|                               | 37 (36-49)² | **24 (18-33)²** | **5 (12.5%) Caucasian** | **Descriptive data** | **249 (213-279)** | - | - | 2 (100%) | **69 (18-90)** |
|                               | **<0.0001** | **0.008** | **7 (87.5%) Caucasian 1 (12.5%) Caribbean** | **CS** | **278 (206-293)** | **0.13** | - | 2 (100%) | 4 (100%) |
|                               | **22.5 (21-24)** | **23.5 (20-24)** | **3 (75%) Caucasian 1 (25%) Pakistani 1 (25%) African** | **VD** | **273 (267-276)** | - | - | 2 (100%) | - |
|                               | **0.66** | **0.25** | **1 (25%) Caucasian 1 (25%) Pakistani 1 (25%) Indian** | **Descriptive data** | **3710 (3280-4140)** | **0.15** | - | 2 (100%) | - |
|                               | **1765 (1260-3380)** | **3805 (1357-4320)** | **2 (25%)** | **CS** | **1765 (1260-3380)** | **0.07** | - | 2 (100%) | **4 (100%)** |
|                               | **3805 (1357-4320)** | **3805 (1357-4320)** | **65 (50%)** | **CS** | **3805 (1357-4320)** | **0.03** | - | 2 (100%) | **4 (100%)** |
|                               | **2536.20** | **249 (19-27)%** | **4 FGR (50%)** | **CS** | **1306 (740-2350)** | - | - | 2 (100%) | - |
|                               | **2998.94** | **6 (0-59)%** | **4 FGR (50%)** | **CS** | **1306 (740-2350)** | - | - | 2 (100%) | - |
|                               | **2791.53** | **4 (50%)** | **4 FGR (50%)** | **CS** | **1306 (740-2350)** | - | - | 2 (100%) | - |
|                               | **3659.05** | **2719.53** | **4 FGR (50%)** | **CS** | **1306 (740-2350)** | - | - | 2 (100%) | - |
|                               | **2998.94** | **4 (50%)** | **4 FGR (50%)** | **CS** | **1306 (740-2350)** | - | - | 2 (100%) | - |
|                               | **2791.53** | **4 (50%)** | **4 FGR (50%)** | **CS** | **1306 (740-2350)** | - | - | 2 (100%) | - |
|                               | **3659.05** | **4 (50%)** | **4 FGR (50%)** | **CS** | **1306 (740-2350)** | - | - | 2 (100%) | - |
|                               | **2998.94** | **4 (50%)** | **4 FGR (50%)** | **CS** | **1306 (740-2350)** | - | - | 2 (100%) | - |
|                               | **2791.53** | **4 (50%)** | **4 FGR (50%)** | **CS** | **1306 (740-2350)** | - | - | 2 (100%) | - |
|                               | **3659.05** | **4 (50%)** | **4 FGR (50%)** | **CS** | **1306 (740-2350)** | - | - | 2 (100%) | - |
|                               | **2998.94** | **4 (50%)** | **4 FGR (50%)** | **CS** | **1306 (740-2350)** | - | - | 2 (100%) | - |
|                               | **2791.53** | **4 (50%)** | **4 FGR (50%)** | **CS** | **1306 (740-2350)** | - | - | 2 (100%) | - |
|                               | **3659.05** | **4 (50%)** | **4 FGR (50%)** | **CS** | **1306 (740-2350)** | - | - | 2 (100%) | - |
|                               | **2998.94** | **4 (50%)** | **4 FGR (50%)** | **CS** | **1306 (740-2350)** | - | - | 2 (100%) | - |
|                               | **2791.53** | **4 (50%)** | **4 FGR (50%)** | **CS** | **1306 (740-2350)** | - | - | 2 (100%) | - |
|                               | **3659.05** | **4 (50%)** | **4 FGR (50%)** | **CS** | **1306 (740-2350)** | - | - | 2 (100%) | - |
|                               | **2998.94** | **4 (50%)** | **4 FGR (50%)** | **CS** | **1306 (740-2350)** | - | - | 2 (100%) | - |
|                               | **2791.53** | **4 (50%)** | **4 FGR (50%)** | **CS** | **1306 (740-2350)** | - | - | 2 (100%) | - |
|                               | **3659.05** | **4 (50%)** | **4 FGR (50%)** | **CS** | **1306 (740-2350)** | - | - | 2 (100%) | - |
|                               | **2998.94** | **4 (50%)** | **4 FGR (50%)** | **CS** | **1306 (740-2350)** | - | - | 2 (100%) | - |
|                               | **2791.53** | **4 (50%)** | **4 FGR (50%)** | **CS** | **1306 (740-2350)** | - | - | 2 (100%) | - |
|                               | **3659.05** | **4 (50%)** | **4 FGR (50%)** | **CS** | **1306 (740-2350)** | - | - | 2 (100%) | - |
|                               | **2998.94** | **4 (50%)** | **4 FGR (50%)** | **CS** | **1306 (740-2350)** | - | - | 2 (100%) | - |
|                               | **2791.53** | **4 (50%)** | **4 FGR (50%)** | **CS** | **1306 (740-2350)** | - | - | 2 (100%) | - |
| Data are expressed as median and range unless otherwise specified. BMI=body mass index, CS=caesarean section, VD=vaginal delivery, INST=instrumental vaginal delivery, IBR=individualised birth weight ratio, FGR=fetal growth restriction, defined as an IBR of 0-5. Onset of PE defined as early=onset of symptoms before 34 weeks gestation, late=onset of symptoms after 34 weeks gestation. Data for the membrane enriched homogenates are compared between categories using a Kruskal Wallis test with a Dunn’s multiple comparison test, letters denote the columns between which statistical significance was found. Data for the microvillous membrane vesicles are compared using a Mann Whitney U test. Statistical significance was set at p<0.05.
4.3.2 Placental TauT protein expression in normal pregnancy and pre-eclampsia

Figure 4.1 a shows a Western blot for TauT protein expression in membrane enriched placental samples which reveals the presence of two immunoreactive species at 80 kDa and 70 kDa. 70 kDa is the expected size of the full length mature TauT protein (Iruloh et al., 2007; Jhiang et al., 1993; Liu et al., 1992; Roos et al., 2004). The mean signal intensity of these two immunoreactive species was normalised to β-actin mean signal intensity (immunoreactive species present at 45 kDa, Figure 4.1 b). There was no difference in β-actin protein expression between groups. There was no difference in normalised expression of the 80 kDa TauT protein species between groups (Figure 4.1 c). However, there was significantly higher expression of the 70 kDa protein species in PE compared to normal pregnancy in both ideal weight and obese women (Figure 4.1 d). There was no effect of maternal obesity on the expression of this 70 kDa TauT protein (Figure 4.1 d).

Western blot analysis of TauT protein expression in isolated MVM revealed a single immunoreactive species at 70 kDa (Figure 4.2 a) which was normalised to β-actin (present at 45 kDa, Figure 4.2 b). Figure 4.2 c shows that in this preliminary study, the expression of the 70kDa protein tends to be higher in PE compared to normal pregnancy, consistent with the observation in membrane enriched samples (Figure 4.1 d).
Figure 4.1: Western blot analysis of TauT protein expression in membrane enriched placental homogenates

(a) Representative Western blot for TauT expression in membrane enriched placental homogenates from ideal weight women having normal pregnancy (N IW) and with pre-eclampsia (PE IW), and obese women having normal pregnancy (N O) and pre-eclampsia (P O) previously shown to have reduced TauT activity.

(b) Corresponding β-actin reprobe following re-probing of nitrocellulose membranes.

(c) There was no difference in the normalised expression of 80 kDa TauT species between groups.

(d) There was significantly higher normalised expression of 70 kDa TauT species in P IW vs N IW and in P O vs N O. Lines represent the median. **p<0.005 Kruskal Wallis with Dunn’s post hoc test.
Figure 4.2: TauT protein expression in syncytiotrophoblast microvillous membrane vesicles

(a) Representative Western blot for TauT expression in microvillous membrane isolates from ideal weight women having normal pregnancy (N) and with pre-eclampsia (P). (b) Corresponding β-actin expression following re-probing of nitrocellulose membranes. (c) Normalised expression of TauT. Lines represent the median.
4.3.3 Placental TauT protein activity in relation to TauT protein expression in normal pregnancy and pre-eclampsia

To assess the relationship between MVM TauT activity and protein expression, TauT expression (normalised to β-actin) was plotted against TauT activity (measured at 120 minutes) in women of ideal weight having normal pregnancy or PE and obese women having normal pregnancy or PE. There was a significant positive relationship between placental TauT protein expression and TauT activity in women of ideal weight having normal pregnancy (Figure 4.3 a). However, there was no relationship between TauT protein expression and TauT activity in women of ideal weight having PE (Figure 4.3 b), in obese women having normal pregnancy (Figure 4.3 c) or in obese women experiencing PE (Figure 4.3 d).
Figure 4.3: Relationship between placental TauT activity and TauT protein expression in normal pregnancy and pre-eclampsia

(a) Placental TauT protein expression (normalised to β-actin) was significantly related to TauT activity in ideal weight women having normal pregnancy, $R^2=0.89$, $p<0.0002$; Least squares linear regression. There was no relationship between TauT protein expression and TauT activity in placentas of obese women ($BMI > 40 \text{ kg/m}^2$) having normal pregnancy (b), ideal weight women having pre-eclampsia (c) or obese women ($BMI > 36 \text{ kg/m}^2$) with pre-eclampsia (d).
4.3.4 Assessment of nitrotyrosine in syncytiotrophoblast of normal pregnancy and pre-eclampsia by immunohisotchemistry

To assess whether there is greater placental nitrotyrosine formation in STB in PE compared to normal pregnancy, villous tissue from placentas in which TauT activity had been determined was immunostained for nitrotyrosine. Figure 4.4 a-i shows representative images from normal pregnancy and PE. Nitrotyrosine staining is evident in stroma, endothelial cells and STB with some areas showing more intense staining at the MVM. STB staining was scored semi-quantitatively (see Chapter 2, section 2.7.6 for detailed methods) (Figure 4.4 j). Staining intensity was significantly higher in PE compared to normal pregnancy.
Figure 4.4: Immunostaining for nitrotyrosine in placental samples from normal pregnancy and pre-eclampsia

Representative images for nitrotyrosine immunostaining in placentas from normal pregnancy (a-c) and pre-eclampsia (d-f). (g) positive control tissue (incubation of tissue section with 10mM ONOO\(^{-}\) for 10 minutes) shows more intense staining compared to (h) an untreated control tissue section from the same placenta. (i) Negative control (non immune rabbit IgG). Black arrows indicate the syncytiotrophoblast, red arrows indicate intense MVM staining, FC = fetal capillaries. (j) Semi-quantitative analysis of syncytiotrophoblast nitrotyrosine staining intensity was significantly higher in pre-eclampsia compared to normal pregnancy. Line shows median, *p<0.05 Mann Whitney U test.
4.3.5 Assessment of nitrotyrosine protein modifications in normal pregnancy and pre-eclampsia by Western blotting

Nitrotyrosine protein expression was assessed in placental membrane enriched homogenates using Western blotting. Several distinct immunoreactive species of varying molecular weight were observed in all samples (Figure 4.5 a). There was no difference in the mean signal intensity of nitrotyrosine immunoreactive species observed between 250 and 60 kDa normalised to the corresponding β-actin signal intensity (present at 45 kDa, Figure 4.5 b) between groups (Figure 4.5 c). The intensity of the immunoreactive band detected at 70 kDa (i.e. the expected molecular weight of TauT) in each sample was analysed separately and normalised to the corresponding β-actin signal intensity. There was no difference in normalised nitrotyrosine protein expression at 70 kDa between groups (Figure 4.5 d).
Figure 4.5: Nitrotyrosine protein expression in membrane enriched placental homogenates

(a) Example Western blot for nitrotyrosine in membrane enriched homogenates from ideal weight women having normal pregnancy (N IW) and pre-eclampsia (PE IW), and obese women having normal pregnancy (N O) and pre-eclampsia (P O). (b) The immunoreactive product at the expected size when the samples were re-probed for β-actin. (c) The mean signal intensity of nitrotyrosine immunoreactive species observed between 250-60 kDa normalised to the corresponding β-actin signal intensity. (d) Nitrotyrosine immunoreactive signal intensity for the protein species present at 70 kDa normalised to β-actin signal intensity. Line represents the median.
4.3.6 Evaluation of immunoprecipitation to compare placental nitration in normal pregnancy and pre-eclampsia

Western blot analysis of nitrotyrosine in membrane enriched placental homogenates was performed following immunoprecipitation of TauT. Initial optimisation of the technique involved Western blot analysis of TauT following TauT immunoprecipitation and, as shown in Figure 4.6 a, an immunoreactive species was seen at ~70 kDa (i.e. the expected molecular weight of TauT). The next step involved Western blot analysis of nitrotyrosine using a rabbit polyclonal anti-nitrotyrosine antibody, previously used for IHC, following immunoprecipitation of TauT. An immunoreactive species was observed at 70 kDa, the expected molecular weight of TauT (Figure 4.6 b). Since both the antibodies used for immunoprecipitation and Western blotting were raised in rabbit, the heavy chain interaction between the antibodies is evident at 50 kDa (Figure 4.6 a-b). To avoid species-species interaction in future experiments, a mouse monoclonal anti-nitrotyrosine antibody was used for the Western blot analysis step. Figure 4.6 c-d shows the Western blots of placental homogenates of ideal weight women having normal pregnancy (c) or PE (d) probed for nitrotyrosine (raised in mouse). A single immunoreactive species was detected at 70kDa (i.e. the correct molecular weight for TauT) in all samples. However, an immunoreactive signal was also seen in the negative controls (anti-TauT antibody replaced with non immune rabbit IgG during immunoprecipitation, Figure 4.6 e) raising concerns over the specificity of the immunoprecipitation procedure. Nitrocellulose blots were therefore stripped and re-probed for TauT. Figure 4.6 f-g show the Western blots re-probed for TauT expression in placental homogenates for ideal weight women having normal pregnancy (f) or PE (g). An immunoreactive signal was observed at 70 kDa and encouragingly, no signal was observed in the negative controls (Figure 4.6 h). There was no difference in the intensity of the nitrotyrosine immunoreactive signal (samples normalised to an internal control) observed in normal pregnancy (n=6) and PE (n=8) (Figure 4.6 i). Figure 4.6 j shows the immunoreactive signal intensity (normalised to an internal control) for blots re-probed for TauT. There was significantly higher TauT signal intensity in PE compared to normal pregnancy. There was no difference in nitrotyrosine signal intensity normalised to TauT signal intensity in the corresponding sample (Figure 4.6 k).
Figure 4.6: Western blot analysis of membrane enriched placental homogenates following immunoprecipitation of TauT

(a) Western blot for TauT following immunoprecipitation of TauT in an optimisation experiment. TauT immunoreactive species is present at 70 kDa (black arrow). Antibody heavy chain interaction is evident at 50 kDa (red arrow). (b) Western blot analysis using a polyclonal rabbit anti-nitrotyrosine antibody following immunoprecipitation of TauT in an optimisation experiment. Immunoreactive species is present at 70 kDa (black arrow). Antibody heavy chain interaction evident at 50 kDa (red arrow).

Representative Western blots using a monoclonal mouse anti-nitrotyrosine antibody following immunoprecipitation of TauT from membrane enriched placental homogenates from (c) ideal weight women with normal pregnancy and (d) ideal weight women with pre-eclampsia. A single immunoreactive species is present at 70 kDa (black arrow). (e) Negative control (-ve) = anti-TauT antibody replaced with rabbit IgG during immunoprecipitation. N = immunoreactive signal from a normal pregnancy sample.

Matched Western blots re-probed for TauT following immunoprecipitation of TauT from membrane enriched homogenates from ideal weight women with normal pregnancy (f) and ideal weight women with pre-eclampsia (g). An immunoreactive species is present at 70 kDa (black arrow). (h) Negative control (-ve) = anti-TauT antibody replaced with rabbit IgG during immunoprecipitation. N = immunoreactive signal from a normal pregnancy sample.

(i) Nitrotyrosine immunoreactive signal intensity (using mouse anti-nitrotyrosine antibody) at 70 kDa normalised to an internal control. (j) TauT immunoreactive signal intensity normalised to an internal control is significantly higher in PE compared to normal pregnancy (*p<0.05, Mann Whitney U test). (k) Nitrotyrosine signal intensity normalised to internal control relative to the corresponding TauT signal intensity normalised to internal control.
4.4 Discussion

The main finding of this chapter was that placental TauT protein expression was higher in PE compared to normal pregnancy in both ideal weight and obese women. This elevated TauT expression was demonstrated in placentas from PE pregnancies in which TauT activity had been shown to be significantly reduced compared to normal pregnancy. Therefore, the reduced TauT activity in PE was not related to a reduction in TauT protein expression. In common with previous work from this laboratory (Ditchfield, personal communication) there was no difference in TauT protein expression in placentas of ideal weight and obese women having normal pregnancy, despite a significant reduction in TauT activity (Desforges et al., 2013a). This suggests that post-translational modification of TauT accounts for the reduction in activity in PE and obesity. In this context, the current study demonstrated significantly higher STB nitrotyrosine formation in PE compared to normal pregnancy when assessed by IHC. However, due to technical issues with immunoprecipitation methods it remains to be demonstrated that nitration of TauT is greater in PE or maternal obesity compared to normal pregnancy.

4.4.1 Placental TauT protein expression is higher in pre-eclampsia compared to normal pregnancy

In this study, Western blot analysis of TauT expression in placental membrane enriched homogenates from normal pregnancy and PE revealed the presence of an immunoreactive species at 70 kDa, the expected molecular weight of the full length TauT protein (Jhiang et al., 1993; Roos et al., 2004) and an additional immunoreactive species at 80. The expression of the 70 kDa, but not the 80 kDa, species was significantly higher in placentas of women with PE compared to normal pregnancy. This elevation of TauT expression in PE was observed in women who were either an ideal weight or obese. There was a greater range in placental TauT protein expression (70 kDa) in PE compared to normal pregnancy (Figure 4.1). The reasons for this are unclear, but the variation in TauT protein expression in PE did not relate to FGR, onset of PE, gestation or fetal sex in either the ideal weight or obese mothers.

TauT protein expression has been examined in a variety of human and animal cell types. An investigation into TauT protein expression by Iruloh and colleagues (2007) revealed the
presence of an immunoreactive species at 75 kDa and 80 kDa in fetal platelets and 114 kDa and 120 kDa in fetal T lymphocytes. The same study identified an immunoreactive signal at 82 kDa in JAr choriocarcinoma cells, and two signals at 44 kDa and 78 kDa in STB MVM. In contrast to the data of Iruloh et al (2007), a study by Roos and colleagues (2004) only identified one immunoreactive species at 70 kDa in MVM. Roos et al. (2004) also investigated TauT protein expression in the placental STB BM and identified a product at 48 kDa and a faint product at 70 kDa. Since the 48 kDa signal was not detected in the MVM, it was suggested that this could be a splice variant of the TauT gene that may be preferentially targeted to the BM. In the cat placenta, two species of TauT were observed at 86 kDa and 33 kDa (Champion et al., 2004). Membrane enriched homogenates contain both MVM and BM STB fractions as well as membranes of other cells including endothelial, smooth muscle, stromal and blood cells. Since the MVM has a large surface area it is probable that it made a substantial contribution to the protein species identified in this study. However, due to the heterogeneity of the membrane enriched fraction, Western blot analysis of TauT protein expression was also carried out on purified MVM. A single immunoreactive signal was identified at 70 kDa in the MVM fraction, consistent with the reports of Ramamoorthy et al. (1994b) and Roos et al. (2004). Analysis of the 70 kDa protein species in the MVM revealed a trend for increased TauT expression in PE, consistent with the data from the membrane enriched homogenates and supports the premise that MVM makes a major contribution to protein in membrane enriched homogenate.

It is clear from the discussion above that the molecular mass of TauT varies between species and tissue/cell type. It is not known what the 80 kDa species observed in the membrane enriched samples in this study may represent. The differences in molecular masses of TauT may be attributable to post-translational modifications of the protein such as phosphorylation or glycosylation. There are 6 PKC phosphorylation recognition sites on TauT, of which ser-322 was identified to be the critical site of PKC phosphorylation (Han et al., 1999). Phosphorylation of TauT by PKC has been demonstrated to inhibit TauT activity in several cell types including JAr cells (Jones et al., 1991; Kulanthaivel et al., 1991; Loo et al., 1996; Voss et al., 2004). In addition, there are 34 tyrosine residues which are potential nitration sites and 4 putative glycosylation sites on TauT (Uchida et al., 1991). Furthermore, Iruloh et al. (2007) demonstrated that de-glycosylating MVM resulted in a shift of 10 kDa of TauT molecular mass from 75 to 65 kDa. A similar difference in molecular mass was seen between products in the current study (80 and 70 kDa) raising the possibility that the 80kD
product is a glycosylated species of the 70kDa protein. A study by Voss et al. (2004) identified a TauT species at 67 kDa in fibroblasts which was mainly localised to the cytosol and a TauT species at 90 kDa in nuclear and plasma membrane fractions. It was suggested by the authors that subcellular localisation of TauT may influence its molecular mass (Voss et al., 2004). As the 80 kDa species was observed in membrane enriched placental homogenates but not in MVM in the current study, it must be predominantly expressed in non-MVM membranes and might be a consequence of glycosylation in non-STB cell types in the placental villous. Since only one TauT species at 70 kDa was identified in the MVM, it is likely that this species is contributing to taurine uptake into the STB.

4.4.2 Placental TauT protein expression is unaltered in maternal obesity compared to normal pregnancy

There was no difference in protein expression of either the 70 or 80 kDa TauT species in membrane-enriched homogenates of placentas from women who were ideal weight or obese, having otherwise uncomplicated pregnancy. Previous work from this laboratory examined TauT protein expression in membrane enriched placental homogenates of obese women (n=6) compared to women of ideal weight (n=7) and also showed (using a different anti-TauT antibody) that there was no difference in the expression of a TauT species identified at 69 kDa (Ditchfield, personal communication).

In summary, this study has demonstrated that placental TauT protein expression is significantly higher in women with PE compared to normal pregnancy and has extended previous reports that TauT protein expression is unaltered in maternal obesity compared to ideal weight women. Therefore, a reduction in TauT protein expression does not account for the lower STB TauT activity demonstrated in PE and maternal obesity compared to normal pregnancy. These expression studies suggest that there is post translational modification of existing TauT transporters in PE and maternal obesity to down-regulate their activity.

4.4.3 TauT activity is related to TauT expression in normal pregnancy

TauT Protein expression was explored in the same placentas that TauT activity was assessed (Chapter 3). In these placentas, STB TauT activity was significantly lower in obese women and women having PE compared to normal. This allowed, for the first time, the
relationship between TauT activity and expression to be explored. In women of ideal weight having normal pregnancy, there was a highly significant relationship between TauT activity and expression. This implies that under normal circumstances the uptake of taurine into the STB is directly related to the abundance of taurine transporters. However, this relationship was completely lost in obesity, and in women with PE who were of ideal weight or obese. This indicates that in these pregnancy complications, the reduction in TauT activity is unrelated to protein abundance and provides further support for the proposition that post-translational modification of the TauT inhibits its activity in PE and maternal obesity.

4.4.4 Placental nitrotyrosine in pre-clampsia and normal pregnancy

ONOO⁻ is the main RNS in the placenta in conditions of elevated nitrative stress and it nitrates tyrosine residues in proteins (Myatt, 2010). Tyrosine residues have been shown to be crucial for the optimal activity of TauT (Kulanthaivel et al., 1989) and there is some evidence to suggest that TauT can be nitrated and that nitration reduces function (Webster et al., 2008). Using IHC, Western blotting and enzyme-linked immunosorbant assay (ELISA) techniques increased placental nitrative stress has been reported in PE and maternal obesity (Myatt et al., 1996; Roberts et al., 2009). It has also been demonstrated that acute treatment of placental villous fragments and MVMs with SIN-1, a generator of ONOO⁻ reduces TauT activity (Khullar et al., 2004).

In the present study IHC on placental villous sections has shown nitrotyrosine immunostaining in stroma, endothelial cells and in the STB. Semi-quantitative analysis of STB nitrotyrosine staining showed significantly higher nitrotyrosine in placentas of PE (in which reduced STB TauT activity was confirmed) compared to normal pregnancy. A study by Myatt et al. (1996) investigated nitrotyrosine formation in placentas from women with PE (n=5) and matched controls (delivery before 37 weeks) and showed nitrotyrosine immunostaining in stroma, endothelial cell and faint staining in the STB. In contrast to the current study, although the authors found significantly higher nitrotyrosine staining intensity in villous endothelium in PE compared to controls, no difference was found in the intensity of staining at the STB. There were several methodological differences between the current study and the study of Myatt and colleagues (1996). Firstly, in the current study tissue sections were fixed in NBF and paraffin embedded prior to IHC whereas Myatt et al. (1996) snap froze tissue samples in liquid nitrogen and used frozen samples for IHC.
Secondly, this study used a polyclonal anti-nitrotyrosine antibody whereas the Myatt study used a monoclonal antibody. Either of these differences in IHC techniques or the patient demographics may contribute to the differences in nitrotyrosine staining observed between the two studies.

Placental nitration was also assessed in membrane enriched homogenates by Western blotting. Several immunoreactive signals were observed at varying molecular weights between 250 kDa and 60 kDa. This is consistent with a previous study which examined placental nitration in placental homogenates in obese women compared to ideal weight counterparts (Roberts et al., 2009). The study by Roberts et al. (2009) used an electrophoresis gel with a higher acrylamide content resulting in a lower degree of protein separation than the one used in this study; therefore it is difficult to tell whether an immunoreactive signal was observed at the correct molecular weight for TauT. The nitrocellulose membrane in the current study was cut at 50 kDa for the detection of β-alanine; therefore nitrated proteins with a smaller molecular weight (e.g. the SOD enzymes MnSOD (25 kDa), CuZnSOD (16 kDa) and ECSOD (36 kDa)) that were identified in the study by Roberts et al. (2009) were not detected in this study. It was noted by Roberts et al. (2009) that the number of bands detected in the Western blot for nitrotyrosine, made quantification of nitrotyrosine protein expression difficult. Therefore the authors used a quantitative nitrotyrosine ELISA and demonstrated an increase in nitrotyrosine residues in the placentas of obese women. There was no difference in mean signal intensity of the immunoreactive species identified between 250 kDa and 60 kDa between normal pregnancy and PE in the current study. However, there were some dense smears on the blots (as seen in Figure 4.5 a) which, when analysed by densitometry, will introduce a degree of error to the analysis. Similarly, there was no difference found in immunoreactive signal intensity for the 70 kDa species in normal pregnancy and PE. Due to the difficulty in assessing nitrotyrosine protein expression using Western blot analysis, a quantitative nitrotyrosine ELISA was tested as an alternative method. During optimisation, different methods of lysate preparation were tested. In addition, freshly isolated placental tissue was incubated in 1 mM SIN-1 or 0.1mM ONOO⁻ for 2 hours to act as positive controls. Different protein concentrations of samples were used for assay to assess which would be appropriate, however no signal was observed. It is unknown why detection of nitrotyrosine by ELISA was unsuccessful in this study when it was used successfully on similar samples in the study by Roberts et al. (2009), although the ELISA kits were obtained from different
sources. Future optimisation could include assaying higher protein concentrations of samples.

In summary, IHC analysis of nitrotyrosine demonstrated significantly increased nitrotyrosine formation at the STB in PE compared to normal pregnancy. IHC analysis allows for the assessment of localisation and semi-quantitative analysis of nitrotyrosine at the STB. In contrast, Western blot analysis of nitrotyrosine was performed using membrane enriched homogenates which contain several cells including endothelial, smooth muscle, stromal and blood cells. Due to the heterogeneity of the membrane enriched fraction it is impossible to determine nitrotyrosine formation at the STB. Future work could use MVM preparations to assess nitrotyrosine by Western blotting.

4.4.5 Evaluation of immunoprecipitation methods to compare placental TauT nitration in pre-eclampsia and normal pregnancy

Immunoprecipitation of TauT allows isolation from other proteins present in the tissue lysates. Once the TauT protein had been successfully immunoprecipitated from the tissue lysates the samples were used for Western blot analysis of nitrotyrosine i.e. to detect TauT protein that has been nitrated. In a pilot experiment, a polyclonal rabbit anti-nitrotyrosine antibody was used detected a protein species at the expected molecular weight for TauT (70 kDa). However, using a primary antibody in the Western which is raised in the same animal as the antibody used for the immunoprecipitation caused species-species interactions between the heavy chains of the antibodies. To avoid heavy chain interactions, a mouse anti-nitrotyrosine antibody was subsequently used to assess nitrotyrosine expression. Using a monoclonal mouse anti-nitrotyrosine antibody a protein species was detected at 70 kDa, the correct molecular weight for TauT. A negative control was included in the experiment in which the TauT antibody was replaced with rabbit IgG during the immunoprecipitation step. A faint signal was detected in the negative control samples when probed for nitrotyrosine which suggests a lack of specificity of the nitrotyrosine antibody. In addition, in a Western blot in which placental membrane enriched homogenates were probed using the mouse anti-nitrotyrosine antibody only one immunoreactive species was detected at 70 kDa. This suggests that the antibody might not be reliable as it has been demonstrated previously (Roberts et al., 2009) as well as in the current study (Figure 4.5) that there are several proteins of varying molecular weights that are nitrated in membrane enriched placental tissue homogenates.
Following detection of nitrotyrosine, membranes were stripped and re-probed using the anti-TauT antibody. The reason for this was two-fold; firstly to confirm that the immunoprecipitation had specifically pulled down TauT and secondly to attempt to normalise nitrotyrosine protein expression to TauT protein expression. Reassuringly there were no bands detected in the negative control samples when re-probed for TauT which lends confidence to the technique of immunoprecipitation of TauT and the specificity of the TauT antibody.

Using densitometry the immunoreactive species present following probing with the mouse nitrotyrosine antibody was quantified. There was no difference in nitrotyrosine protein expression between ideal weight women with normal pregnancy and PE. Quantification of the immunoreactive species present when the blot was re-probed for TauT revealed significantly higher expression of TauT protein in PE compared to normal pregnancy consistent with the increased TauT protein expression in PE compared to normal pregnancy (Figure 4.1; i.e. in samples that have not been immunoprecipitated). This lends confidence to the immunoprecipitation technique. Since TauT protein expression is higher in PE compared to normal pregnancy it is necessary to normalise nitrotyrosine expression to TauT expression. There was no difference in the proportion of TauT that was nitrated between membrane enriched homogenates from PE compared to NP.

Due to concerns over the reliability of the mouse nitrotyrosine antibody, firm conclusions could not be drawn as to whether the proportion of nitrated TauT is increased in PE compared to normal pregnancy. Future optimisation of this technique could include evaluation of different nitrotyrosine antibodies. However, the immunoprecipitation experiments present here do provide some supporting evidence that TauT can be modified by nitration in the placenta.

To further investigate TauT nitration in PE an alternative approach is to use mass spectrometry following immunoprecipitation of TauT from MVM isolations for normal pregnancy and PE. Using MVM isolations avoids any contamination with transporters from other cell types and allows for specific assessment of TauT nitration at the MVM. Proteomic analysis of MVM will also reveal other potential regulators of TauT involved in the down regulation of TauT in PE, such as phosphorylation and glycosylation. One method to address this would be to perform 2D gel electrophoresis followed by Coomassie staining on MVM samples following immunoprecipitation of TauT. The gel would then be imaged and analysed using Progenesis PG200 which enables the quantification of protein expression.
The same samples would be separated by 2D gel electrophoresis and Western blotted for nitrotyrosine. The nitrotyrosine immunoreactive band on the film obtained after Western blotting would be aligned to the Coomassie-stained gel and the protein of interest excised from the gel and digested with trypsin. The eluted peptides would then be analysed by tandem mass spectrometry. This technique has been used to assess post translational modifications of proteins including nitration of TauT by existing collaborators (Professor Leslie Myatt, personal communication; (Webster et al., 2006)).

In summary, this study has demonstrated that there is increased nitrotyrosine formation at the STB in PE in placentas shown to have reduced TauT activity. Western blot analysis of membrane enriched homogenates and immunoprecipitated samples provides supporting evidence that nitration is a modification of TauT that can occur in placenta. Whilst further work is required to fully elucidate the mechanism of reduced TauT activity in PE and maternal obesity it is important to consider the consequences of reduced TauT activity for STB turnover. The following chapter investigates the consequences of reduced TauT activity and intracellular taurine on STB turnover.
Chapter 5: Consequences of inducing nitrative stress on TauT nitration and syncytiotrophoblast turnover

5.1 Introduction

PE is associated with dysregulated renewal of STB, increased placental nitrative stress and heightened inflammation (Crocker, 2007; Gu et al., 2008; Myatt et al., 1996). It has been demonstrated in the current study that placental TauT activity is lower in PE compared to normal pregnancy. This reduced TauT activity was not associated with lower TauT protein expression which raises the possibility of down-regulation of TauT by post translational modification. Over 20 yrs ago Kulanthaivel et al. (1989) demonstrated the importance of tyrosine groups for TauT activity and showed that nitrating agents targeted these groups and inhibited TauT activity in STB MVM. It has been demonstrated in PE and maternal obesity that there is increased nitrotyrosine in the placenta (Myatt et al., 1996; Roberts et al., 2009) and an increase in nitrated proteins which could include TauT (Roberts et al., 2007; Webster et al., 2008, 2006). The acute treatment (2 hours) of MVM vesicles and placental villous fragments with SIN-1, used to generate ONOO⁻ which nitrates tyrosine residues on proteins to form nitrotyrosine, significantly reduces TauT activity (Khullar et al., 2004). It has also been shown using a rat STB cell line that treatment with SIN-1 reduces TauT activity (Lee and Kang, 2010). These studies support the possibility that in PE and obesity longer term exposure to ONOO⁻ inhibits TauT activity, however this has yet to be investigated.

Maintaining intracellular taurine is important for two reasons; firstly taurine promotes appropriate cell proliferation, apoptosis and differentiation (Desforges et al., 2013b; Fukuda et al., 2000; Ramos-Mandujano et al., 2014; Takatani et al., 2004b) and secondly taurine is cytoprotective (Jong et al., 2012; Oriyanhan et al., 2005). Therefore a reduction in intracellular taurine as a consequence of reduced TauT activity in PE could contribute to dysregulated STB renewal and increase susceptibility to damage by inflammatory cytokines or oxidative/nitrative stress. Indeed, cytokines and oxidative stress disrupt STB renewal in vitro (Crocker et al., 2004b; Heazell et al., 2009). Furthermore, reducing intracellular taurine with siRNA knockdown of TauT in CTB cells inhibited their differentiation/fusion to form multinucleate cells and reduced cytoprotection against TNFα-induced apoptosis (Desforges et al., 2013b).
We propose that taurine is required to sustain the renewal of STB and accordingly a reduction in intracellular taurine, following reduced TauT activity, could contribute to disrupted STB renewal in PE. It is plausible that in PE, the reduction in STB TauT activity is caused by nitration. This proposal also applies to maternal obesity which, in common with PE, is associated with altered STB turnover (Higgins et al., 2013), increased placental nitrative stress (Roberts et al., 2009), heightened inflammation (Challier et al., 2008), reduced STB TauT activity but unaltered TauT protein expression (Chapter 3 and 4; (Desforges et al., 2013a)). However, it is not yet known whether placental taurine depletion affects STB turnover.

Hypothesis and aims

In this chapter two hypotheses were tested:

1) Chronic exposure of placental villous fragments in vitro to RNS will down regulate TauT activity and disrupt STB cell turnover.

2) Maintaining STB taurine is required for appropriate STB renewal.

The aims of this study were to:

- Determine if treatment of placental villous explants with ONOO⁻/SIN-1:
  1) Increases STB nitrotyrosine formation
  2) Reduces TauT activity
  3) Alters proliferation, apoptosis and STB regeneration

- Determine if reducing intracellular taurine in placental villous explants by (a) siRNA knockdown of TauT or (b) competitively inhibiting taurine uptake using the TauT substrate β-alanine, alters proliferation, apoptosis and STB regeneration.
5.2 Methods

5.2.1 The effect of RNS on TauT activity, syncytiotrophoblast nitrotyrosine and syncytiotrophoblast turnover

The effect of long term application of RNS on placental TauT activity, STB nitrotyrosine and STB turnover (proliferation, apoptosis and regeneration) was assessed using placental villous explants in vitro (see Chapter 2, Methods section 2.6).

5.2.1.1 Preparation of ONOO\(^{-}\), SIN-1 and H\(_2\)O\(_2\) solutions

A 10 mM stock solution of ONOO\(^{-}\) (Millipore) was prepared in a 1:1 ratio of 0.3 M NaOH and 0.3 M NaCl and stored at -20°C prior to use. A 100 mM stock of SIN-1 (Enzo Life Sciences UK Ltd) was prepared in sterile PBS and stored at -80°C. A 100 mM stock solution of H\(_2\)O\(_2\) was prepared immediately prior to treatment by diluting a 32.63 M stock of H\(_2\)O\(_2\) in sterile PBS. On days 5 and 6 of culture, explants were treated with either 0.1 mM ONOO\(^{-}\), 1 mM SIN-1 or 1 mM H\(_2\)O\(_2\) by diluting stock solutions 1:100 in explant culture medium.

5.2.1.2 Treatment of placental villous explants with reactive nitrogen species

Villous fragments from normal term placentas (n=6) were cultured for 7 days and treated on days 5 and 6 with 0.1 mM ONOO\(^{-}\), 1 mM SIN-1 or 1 mM H\(_2\)O\(_2\). ONOO\(^{-}\) is a potent nitrating agent which nitrates tyrosine residues which have been shown to be crucial for TauT activity (Kulanthaivel et al., 1989). The half life of ONOO\(^{-}\) is very short; approximately 100 ms in vivo (Pacher et al., 2007). SIN-1 is a compound that generates O\(_2^{-}\) and NO, which spontaneously combine to produce ONOO\(^{-}\), and has a half-life of between 60-90 minutes (Rosenkranz et al., 1996). The concentrations of ONOO\(^{-}\) and SIN-1 were chosen as they are physiologically relevant. 0.1 mM ONOO\(^{-}\) added in vitro is equivalent to a physiological concentration of 2.8 µM (Radi et al., 1991). ONOO\(^{-}\) is formed by the reaction of NO and O\(_2^{-}\) and levels of NO are estimated to be between 2-5 µM in disease states; therefore it is assumed that resulting concentrations of ONOO\(^{-}\) in vivo would also be in the 2-5µM range (Nossuli et al., 1998). It has been shown that 1 mM SIN-1 generates 7.02 µM/min O\(_2^{-}\) and 3.68 µM/min NO, concentrations of which are physiologically relevant (Hogg et al., 1992). H\(_2\)O\(_2\) is the product of the reaction between O\(_2^{-}\) and superoxide dismutase. Placental villous fragments were also treated with 1 mM H\(_2\)O\(_2\) to elucidate whether O\(_2^{-}\) was the radical responsible for any effects on TauT activity or STB turnover produced by SIN-1. 1 mM H\(_2\)O\(_2\) was chosen based on a studies which found that a range of concentrations (including 1
mM) induced altered trophoblast turnover in term villous explants (Heazell et al., 2009, 2007). Treatment days were chosen based on evidence that over the first few days in culture the STB degenerates and is shed followed by regeneration of a new STB by day 5 (Simán et al., 2001).

5.2.1.3 Measurement of $^3$H-taurine uptake into placental villous fragments following treatment with reactive nitrogen species

On day 7, Na$^+$ dependent $^3$H-taurine uptake at 90 minutes was measured to determine the effect of ONOO$^-$, SIN-1 and H$_2$O$_2$ on TauT activity. 90 minutes was chosen to represent the activity of TauT at initial rate as previous work from this study (Chapter 3; Figure 3.1) showed that TauT activity was linear over 30-120 minutes.

5.2.1.4 Assessment of nitrotyrosine formation using immunohistochemistry in placental villous fragments following treatment with reactive nitrogen species

To confirm that nitrative stress was induced by ONOO$^-$ and SIN-1 treatment, 6 explants per explant culture were fixed on day 7 of culture and nitrotyrosine formation was assessed using IHC (see Chapter 2, Methods, section 2.7).

5.2.1.5 Effect of reactive nitrogen species on syncytiotrophoblast turnover

To determine the effects of RNS on STB regeneration, 6 explants per explant culture were fixed on day 7 of culture for IHC analysis of M30 to assess apoptosis, ki67 and E-cadherin dual staining to identify proliferating CTBs and cytokeratin 7 as a marker of STB regeneration (see Chapter 2, Methods, section 2.7).

5.2.2 The effect of reducing intracellular taurine on syncytiotrophoblast turnover

5.2.2.1 Treatment of placental villous explants with TauT siRNA

Initially experiments were performed to reduce intracellular taurine by siRNA knockdown of TauT. However, although this approach has been used successfully in CTB cells (Desforges et al., 2013b), treatment of placental villous tissue with TauT specific siRNA failed to produce a consistent reduction in TauT activity and the non-targeting sequences affected TauT activity. These pilot experiments are described in the Appendix: Chapter 7.
5.2.2.2 Treatment of placental villous explants with β-alanine

To assess whether intracellular taurine contributes to STB turnover, taurine depletion was induced in placental villous explants by competitively inhibiting taurine uptake by TauT using β-alanine, a substrate for the transporter.

Experiments were performed to confirm that β-alanine treatment reduced intracellular taurine. Intracellular taurine can be assessed as the steady state accumulation of $^{3}$H-taurine over time. Pilot experiments were performed to determine the time at which steady state was achieved. Fragments of villous tissue from normal pregnancy were incubated in a 12 well plate on Netwell supports in 1.5 ml CO$_2$-free culture media (Gibco, Life Technologies, UK) containing trace amounts (0.5μCi/ml; 25nM) $^{3}$H-taurine at 37°C and 100μM unlabelled taurine to mimic maternal plasma taurine concentration (Philipps et al., 1978). At several time points over a 24 hr period, tissue was removed from the incubator and washed with 2 x 25 ml ice cold Tyrode’s buffer over 1 minute to remove extracellular isotope and stop any further taurine uptake. The fragments were lysed in 0.3 M NaOH overnight at 37°C. 1 ml of lysate was added to 12 ml of Scintisafe scintillation fluid which was counted in a β-scintillation counter. The $^{3}$H radioactivity of the incubation medium was determined by counting 100 μl + 900 μl 0.3 M NaOH + 12 ml scintillation fluid in duplicate as standards. The samples and standards were counted for 10 minutes along with background counts (1 ml 0.3 M NaOH + 12 ml scintillation fluid). The remaining lysates were assayed for protein content using a Biorad protein assay (Chapter 2, Methods, section 2.3.3) and total taurine accumulation expressed as pmol/mg protein: i.e. the sum of radiolabelled plus unlabeled taurine (calculated by dividing the counts in the tissue by the counts/fmol in the incubation medium: the latter was calculated by dividing the counts/ml of incubation solution by the labelled (25nM) plus unlabeled (100μM) taurine in the incubation solution).

Figure 5.1 a shows the time course of $^{3}$H-taurine accumulation into villous fragments over 24 hours. Equilibrium (representing intracellular taurine at steady state) was achieved between 20-24 hours. Therefore, to confirm that β-alanine reduced intracellular taurine in explants in subsequent experiments, $^{3}$H-taurine accumulation was measured over 20 hours between days 6-7 of explant culture with β-alanine.

A previous study has shown that 10 mM β-alanine inhibits taurine uptake by >90% (Champion et al. 2004), confirming that high concentrations of β-alanine will reduce taurine uptake by competitive inhibition. A potential complication of using β-alanine is that the cell accumulates this amino acid at the expense of taurine which could induce off-target
effects. Therefore, initial optimisation experiments were performed to determine the lowest concentration of β-alanine that would significantly deplete intracellular taurine but minimise the cellular accumulation of β-alanine. Explants were maintained in CMRL 1066 culture media containing 25 μM taurine and β-alanine (0.1 mM-10 M) for the duration of culture (day 0-day 7). Between days 6 and 7, ³H-taurine (0.5 μCi/ml; 25 nM) was added to the culture medium for 20 hr. Explants were then removed from the incubator, washed in ice-cold Tyrode’s buffer and lysed in 0.3 M NaOH as described above. Total taurine accumulation (i.e. radiolabelled plus unlabelled taurine) was expressed in nmol/mg protein (calculated as described above).

Figure 5.1 b shows intracellular taurine in response to increasing concentrations of β-alanine in the explant culture medium. 2.5 mM β-alanine reduced intracellular taurine by ~85%. Therefore to assess the effect of low intracellular taurine on cellular turnover, explants were cultured for 7 days in the presence of 2.5 mM β-alanine.

5.2.2.3 Effect of β-alanine on syncytiotrophoblast turnover

To determine the effects of 2.5 mM β-alanine on STB regeneration, explants were fixed on day 7 of culture for IHC analysis of M30 to assess apoptosis, ki67 and E-cadherin dual staining to identify proliferating CTBs and cytokeratin 7 as a marker of STB regeneration (see Chapter 2, Methods section 2.7).

5.2.3 Expression of results and statistical analysis

TauT activity in placental villous explants following treatment with ONOO⁻, SIN-1 and H₂O₂ was expressed as a percent of untreated control and analysed by a Wilcoxon signed rank test vs 100%.

Semi-quantitative scores of nitrotyrosine staining in control, ONOO⁻ and SIN-1 treated explants were expressed as a scatter dot plot and medians compared using a Mann Whitney U test.

To determine any differences in apoptosis, proliferation or regeneration between control, SIN-1 and β-alanine treated explants, data were expressed as a scatter dot plot and medians compared using a Mann Whitney U test.

All data analyses were carried out using GraphPad Prism version 5.0 (GraphPad Inc, CA, USA). Statistical significance was set at p<0.05.
Figure 5.1: $^3$H-taurine accumulation into placental villous fragments.
(a) $^3$H-taurine uptake into placental villous fragments from normal pregnancy over 24 hours. A steady state (representing intracellular taurine) was achieved between 20 and 24hr. Mean±SE; n=5 placentas. (b) Intracellular taurine (assessed by steady-state taurine uptake over 20hr) was reduced following culture for 7 days with increasing concentrations of β-alanine. Median and interquartile range (n=1 placenta with measurements taken in duplicate).
5.3 Results

5.3.1 TauT activity in placental villous explants treated with ONOO\(^{-}\), SIN-1 and H\(_2\)O\(_2\)

TauT activity at 90 minutes in untreated controls was 2994 ± 2320-6218 fmol/mg protein/90 minutes (median and range: data not shown). Figure 5.2 shows the effect of ONOO\(^{-}\), SIN-1 and H\(_2\)O\(_2\) on explant TauT activity (measured at 90 minutes) compared to the corresponding control (100%). TauT activity was not affected by 0.1 mM ONOO\(^{-}\) or 1 mM H\(_2\)O\(_2\) but was significantly reduced (18%) by 1 mM SIN-1 compared to control.

![Figure 5.2: Effect of peroxynitrite (ONOO\(^{-}\)), SIN-1 and hydrogen peroxide (H\(_2\)O\(_2\)) on TauT activity in placental explants](image)

Effect of 0.1 mM ONOO\(^{-}\), 1 mM SIN-1 and 1 mM H\(_2\)O\(_2\) (treated on days 5 and 6) on TauT activity (Na\(^{+}\)-dependent uptake of \(^{3}\)H-taurine) measured on day 7. Data are % of corresponding control (dotted line at 100%). Line represents the median.*p<0.05 vs control (100%); Wilcoxon signed rank test; n=6 placentas.
5.3.2 Nitrotyrosine staining in placental villous explants treated with ONOO$^-$ and SIN-1

To confirm that treatment of villous explants with 0.1 mM ONOO$^-$ or 1 mM SIN-1 for 48hr (days 5 and 6 of culture) induced nitrative stress, explants were fixed on day 7 and nitrotyrosine was examined by IHC and compared with untreated controls (Figure 5.3). Staining was observed in the stroma, endothelial cells and the STB. The intensity of staining in the STB was scored by 2 blinded observers using a scale of 0-4.

ONOO$^-$ did not alter STB nitrotyrosine staining compared to control (Figure 5.4). However, 5 out of 6 explant cultures treated with SIN-1 showed increased nitrotyrosine staining in the STB compared to controls (Figure 5.4).
Figure 5.3: Immunostaining for nitrotyrosine in placental explants

Representative images for nitrotyrosine staining in control (a-b), ONOO$^-$ (c-d) and SIN-1 (e-f) treated explants. (g) Negative control = non immune rabbit IgG. Scale bar in (a) applies to all images. Black arrows indicate STB, blue arrows indicate fetal capillaries.
Figure 5.4: Syncytiotrophoblast nitrotyrosine staining intensity in placental explants

Syncytiotrophoblast nitrotyrosine staining (day 7) in explants maintained in control conditions and following treatment with 0.1 mM ONOO⁻ and 1mM SIN-1 (days 5 and 6). Line represents the median (Data not significant).
5.3.3 Assessment of cell turnover in SIN-1 treated explants

Since there was no effect of ONOO⁻ on TauT activity or nitrotyrosine formation at the STB the effect of ONOO⁻ on STB turnover was not investigated.

To assess whether treatment with SIN-1 and reduced TauT activity affected cell turnover, control and SIN-1 treated placental villous explants were fixed on day 7 and immunostained for M30, dual stained for Ki67 and E-cadherin and immunostained for cytokeratin 7 (Figures 5.5, 5.6 and 5.7 respectively).

Figure 5.5 a-d shows M30 staining in control and SIN-1 treated placental explants. M30 staining was localised to the trophoblast layer of the villi. There was significantly more M30 positive staining in SIN-1 treated explants compared to matched controls indicative of increased apoptosis (Figure 5.5 f).

Figure 5.6 a-b shows images of control and SIN-1 treated placental explants dual stained for Ki67 and E-cadherin. There were significantly fewer proliferating CTBs in SIN-1 treated explants compared to controls (Figure 5.6 c).

Figure 5.7 a-d shows example images of control and SIN-1 treated placental explants stained with cytokeratin 7 used to assess STB integrity on day 7 of culture. The number of villi with intact STB were significantly lower (24%) with SIN-1 treatment compared to control (Figure 5.8 a); accordingly there were significantly more villi with shed STB in SIN-1 treated explants compared to control (Figure 5.8 a). There was a trend for the number of villi with a complete cellular STB on day 7 (i.e intact villi plus villi with shed STB and complete regeneration) to be lower in SIN-1 treated explants compared to control (Figure 5.8 c). There was no difference in the percent of villi with shed STB undergoing regeneration between control and SIN-1 treated explants on day 7 (Figure 5.8 b).
Figure 5.5: M30 staining for apoptosis in control and SIN-1 treated placental explants

Representative images for M30 staining at day 7 of culture in (a-b) control explants and (c-d) explants treated for 48 hours with 1 mM SIN-1. (e) Negative control = non immune mouse IgG. Scale bar in negative (a) applies to all images.

(f) M30 staining expressed as a percent of total villous area in control explants and explants treated with 1mM SIN-1. Line represents median. *p<0.05 vs control; Wilcoxon matched pairs signed rank test.
Figure 5.6: Dual staining for proliferating cytotrophoblasts (CTBs) in control and SIN-1 treated placental explants

Example images for control (a) and SIN-1 treated (1mM for 48hr) (b) explants on day 7 of culture dual stained for Ki67 (a marker of proliferation) and E-cadherin (a marker of CTBs). Yellow arrows indicate examples of non-proliferating CTBs, black arrows indicate examples of proliferating CTBs. Scale bar in (a) applies to both images. (c) There were significantly fewer proliferating CTBs in SIN-1 treated explants compared to control. *p<0.03 Wilcoxon matched pairs signed rank. Line represents the median.
Figure 5.7: Cytokeratin 7 staining in control and SIN-1 treated placental explants

Cytokeratin 7 staining in control (a-b) and SIN-1 (1mM for 48hr) (c-d) treated explants on day 7 of culture. (e) Negative control = non immune mouse IgG. Scale bar in (a) applies to all images. (in) = villi with intact STB and no evidence of shedding, (s) = shed STB, arrows indicate STB regeneration.
Figure 5.8: Quantification of cytokeratin 7 staining in control and SIN-1 treated placental explants

(a) There are significantly fewer villi with intact STB and significantly more villi with shed syncytiotrophoblast (STB) in SIN-1 treated explants compared to control; *p<0.03, Wilcoxon matched pairs signed rank test. (b) There is no difference in the percentage of villi with shed STB that are undergoing regeneration. (c) The number of villi with a complete STB (intact villi plus villi with shed STB and complete regeneration) tends to be lower in explants treated with SIN-1 compared to control. Line represents the median.
5.3.4 Effect of β-alanine on intracellular taurine

Figure 5.9 shows intracellular taurine at 20 hours measured on day 7 in untreated and β-alanine treated placental explants. 2.5 mM β-alanine depletes intracellular taurine to ~10% of untreated controls at day 7 of culture.

Figure 5.9: Intracellular taurine in placental explants treated with 2.5 mM β-alanine as a percent of control

Line represents the median. *p<0.03 Wilcoxon matched pairs signed rank test (vs control at 100%: dotted line).
5.3.5 Assessment of cell turnover in β-alanine treated explants

To assess whether STB taurine depletion, achieved by treating placental explants with 2.5 mM β-alanine affected cell turnover, control and β-alanine treated placental villous explants were fixed on day 7 and immunostained for M30 (apoptosis), dual stained for Ki67 and E-cadherin (proliferation) and immunostained for cytokeratin 7 (STB regeneration) (Figures 5.10, 5.11 and 5.12 respectively).

Figure 5.10 a-d shows representative images of M30 staining in control and β-alanine treated explants on day 7 of culture. There was significantly more M30 positive staining, indicative of increased apoptosis, in β-alanine treated placental explants compared to untreated control (Figure 5.10 e).

Figure 5.11 shows representative images for control (a) and β-alanine treated (b) explants dual stained for Ki67 and E-cadherin. There was no effect of β-alanine treatment on CTB proliferation (Figure 5.11 c).

Figure 5.12 a-d shows example images of control and β-alanine treated placental explants stained with cytokeratin 7. The number of villi with intact STB were significantly lower following treatment with β-alanine compared to control (Figure 5.13 a). Accordingly, there were significantly more villi with shed STB in β-alanine treated explants compared to control (Figure 5.13 a). There was no difference in the number of villi with a complete cellular STB on day 7 (i.e intact villi plus villi with shed STB and complete regeneration) in β-alanine treated explants compared to control (Figure 5.13 a). There was a trend for the percent of villi with shed STB undergoing regeneration to be higher in β-alanine treated explants on day 7 compared to control (Figure 5.13 b).
Figure 5.10: M30 staining for apoptosis in control and β-alanine treated placental explants

Representative images for M30 staining in (a-b) control explants and (c-d) explants treated with 2.5 mM β-alanine. Negative control (non immune mouse IgG) shown in Figure 5.4. Scale bar in (a) applies to all images.

(e) M30 staining expressed as a percent of total villous area in control explants and explants treated with 2.5 mM β-alanine. Line represents median. *p<0.05 vs control; Wilcoxon matched pairs signed rank test.
Figure 5.11: Dual staining for proliferating cytotrophoblasts (CTBs) in control and SIN-1 treated placental explants

Example images for control (a) and β-alanine treated (b) explants on day 7 of culture dual stained for Ki67 (a marker of proliferation) and E-cadherin (a marker of CTBs). Yellow arrows indicate non proliferating CTBs. Scale bar in (a) applies to both images. (c) There was no difference in the number of proliferating CTBs in β-alanine treated explants compared to control. Line represents the median.
Figure 5.12: Cytokeratin 7 staining for syncytiotrophoblast (STB) integrity in control and β-alanine treated placental explants

Representative images for cytokeratin 7 staining in (a-b) control explants and (c-d) explants treated with 2.5 mM β-alanine. Negative control (non immune mouse IgG) is shown in figure 5.6. Scale bar in (a) applies to all images. In= villi with fully intact STB, s=shed STB, arrows indicate STB regeneration.
Figure 5.13: Quantification of cytokeratin 7 staining in control and β-alanine treated placental explants

(a) There are significantly fewer villi with intact syncytiotrophoblast (STB) and significantly more villi with shed STB in β-alanine treated explants compared to control. *p<0.03, Wilcoxon matched pairs signed rank test. (b) There is a trend for the number of villi with shed STB that are undergoing regeneration to be higher in β-alanine treated placental explants compared to control. (c) There is no difference in the number of villi with a complete STB (intact villi plus villi with shed STB and complete regeneration) in explants treated with β-alanine compared to control. Line represents the median.
5.4 Discussion

In this chapter the impact of inducing placental nitrative stress on TauT activity and STB regeneration was assessed in vitro. Treatment of placental villous explants with the nitrating agent SIN-1 significantly reduced TauT activity compared to untreated controls. Explants treated with SIN-1 also demonstrated raised nitrotyrosine formation at the STB. The consequences of reduced TauT activity for STB turnover were also investigated. Experimentally reducing TauT activity by treatment with SIN-1 significantly increased apoptosis and decreased CTB proliferation. In addition, the number of villi with an intact STB was reduced and shedding of the STB increased in SIN-1 treated placental explants compared to control. There was a trend for fewer villi with a complete STB (whether intact or shed with complete regeneration) that should be capable of transporting taurine, in SIN-1 treated explants. The loss of STB integrity suggests a dysregulation of STB turnover in culture in response to SIN-1.

The consequences of a marked reduction in intracellular taurine (to 10% of normal), induced by competitive inhibition of taurine uptake with β-alanine, were also assessed. CTB proliferation was unaffected by β-alanine treatment but apoptosis was significantly increased in explants treated with β-alanine compared to untreated controls. β-alanine significantly reduced the number of villi with an intact STB and an increased the number with shedding STB. There was a tendency for increased cellular regeneration in the villi with shed STB in response to β-alanine treatment. Therefore, the evidence presented here suggests that maintaining intracellular taurine is important for appropriate cell turnover to renew the STB.

5.4.1 Term placental villous explants

The placental villous explant preparation is useful to investigate placental function by enabling the study of nutrient transport, cell turnover processes involved in renewal of STB, endocrine function and metabolism in normal pregnancy and pregnancy pathologies. Explants are also a useful tool to study the effects of long-term interventions on placental function. Villous explants have been used in many studies to assess placental function and STB renewal in vitro (Audette et al., 2010; Crocker et al., 2004a, 2004b; Heazell et al., 2009; Moll et al., 2007; Rosario et al., 2013). CTB’s isolated from the term placenta and maintained in primary culture are also useful to assess some of the processes that renew STB in situ, such as migration, fusion and differentiation (Desforges et al., 2013b), but these
cells do not proliferate under culture conditions and interaction with non-trophoblast cells is lost. A major benefit of placental villous explants is the maintenance of tissue architecture.

The placental villous explant preparation is often used to assess the cellular kinetics involved in renewal of STB (Audette et al., 2010; Crocker et al., 2004a, 2004b; Heazell et al., 2008b, 2007). However, it is important to note that, in contrast to the situation in vivo, in villous explants the STB completely detaches over the first few days of culture and a new STB is then regenerated in a period of days. This does not mimic the dynamics of STB renewal in situ, although CTB proliferation, fusion and apoptosis are involved in both regeneration and renewal. In explants, the STB is shed over the first few days of culture, probably in response to damage incurred during dissection, and a new STB is then produced by CTBs by day 5, as confirmed by electron microscopy (Simán et al., 2001). Once reformed, the maintenance of this new layer is thought to reflect renewal processes of STB in vivo as the newly formed STB remains viable for at least 11 days of culture. During this period, solute transport function is maintained (Simán et al., 2001). The release of hCG by explants during culture has been used to indicate their endocrine viability as well as STB differentiation (Simán et al., 2001). hCG is produced and secreted from terminally differentiated STB in vivo; however, the relationship between the characteristic temporal changes that are observed in hCG secretion from explants (Simán et al., 2001) and STB regeneration is not fully understood and there is significant variability in inter and intra placental hCG secretion from term placental explants (Turner et al., 2006). Turner et al. (2006) consider that hCG secretion could reflect normal placental function, release from damaged tissues in response to stress or be a result of tissue generation.

In summary, the term placental villous explant preparation has enabled studies of the mechanisms of STB renewal in normal pregnancy and in pregnancy complications, and how renewal is regulated by external stimuli.

5.4.2 The effect of reactive nitrogen species on TauT activity

Placental explants from normal pregnancy were cultured for 7 days and treated with 0.1 mM ONOO\(^-\) or 1 mM SIN-1 on day 5 and 6, to mimic placental nitrative stress that is evident in PE and maternal obesity, and TauT activity was assessed on day 7. SIN-1 significantly reduced TauT activity compared to controls but there was no effect of ONOO\(^-\).
treatment on taurine uptake. The effect of SIN-1 is consistent with previous findings that acute treatment with SIN-1 (2 hours) reduces TauT activity in placental villous fragments (Khullar et al, 2004). The lack of effect of ONOO⁻ on TauT activity may be due to its short half-life (~100ms in vivo). Consistent with the lack of effect of ONOO⁻ on TauT activity, ONOO⁻ did not increase the formation of nitrotyrosine in STB. In vivo, ONOO⁻ is able to diffuse 1-2 cell lengths by passing readily through membranes and has significant interactions with biomolecules in its relatively short half-life. However, the lack of formation of nitrotyrosine with ONOO⁻ suggests that the daily administration of 0.1mM for 2 days was insufficient to cause protein nitration. SIN-1 has a half-life of 60-90 minutes (Rosenkranz et al., 1996), during which time it produces NO and O₂⁻ which spontaneously combine to produce ONOO⁻ in a diffusion limited reaction. NO has a half-life of 0.5 seconds and a large diffusion distance of 100 µm; in contrast O₂⁻ is limited to a diffusion distance of 0.4 µm in its half-life of <1ms (Myatt, 2010). Villous tissues express antioxidant enzymes such as SOD and as such have the capacity to sequester ROS. However, NO has a much faster rate of reaction with O₂⁻ (~10 x 10⁹ M⁻¹ s⁻¹) than the rate of reaction between O₂⁻ and SOD (1-2 x 10⁹ M⁻¹ s⁻¹). Therefore the production of NO, O₂⁻ and ONOO⁻ over an extended period of time may account for the reduction in TauT activity achieved through treatment with SIN-1. 5 out of 6 SIN-1 treated explants showed an increase in STB nitrotyrosine staining, suggesting that SIN-1 generated ONOO⁻ which induced nitration of tyrosine groups on proteins over 48 hours. Increased nitrotyrosine formation was also evident in the villous stroma and endothelial cells in SIN-1 treated explants.

As discussed above, SIN-1 generates NO, O₂⁻ and ONOO⁻ and either of these have the potential to reduce STB TauT activity. O₂⁻ could be responsible for the reduction in TauT activity, either directly or indirectly through conversion to H₂O₂ (by SOD). However, a direct effect of H₂O₂ generated by SIN-1 is unlikely there was no effect of treatment with 1 mM H₂O₂ on TauT activity (Figure 5.2). As NO sequesters O₂⁻ to form ONOO⁻, and SIN-1 increased STB nitrotyrosine formation (5/6 experiments) a possible explanation for the reduction in TauT activity with SIN-1 is through nitration of TauT by ONOO⁻. In support of this, a study conducted by Khullar et al. (2004), demonstrated a reduction in TauT activity in MVM vesicles following acute treatment with SIN-1 (2 hours); effects which were attributed to ONOO⁻. In the latter study the effect of SIN-1 on TauT activity was prevented by SOD (to sequester O₂⁻) or haemoglobin (to sequester NO). Therefore a direct effect of NO or O₂⁻ was ruled out as SOD/haemoglobin treatment would have exacerbated the effect of SIN-1 on TauT activity (Khullar et al., 2004). In addition, the authors demonstrated
increased nitrotyrosine concentrations in SIN-1 treated MVMs which confirmed the formation of ONOO'. Using rat renal brush border membrane vesicles, Koo et al. (2012) demonstrated a similar reduction in TauT activity following treatment with sodium nitroprusside (a slow releasing NO donor which leads to ONOO' formation). Treatment with rapid releasing NO donors did not produce a functional impairment of TauT and treatment with SOD reversed the impairment on TauT activity by pre-incubation with sodium nitroprusside. This lead the authors to conclude that ONOO' was the species responsible for the reduction in TauT activity (Koo et al., 2012). Khullar et al. (2004), also demonstrated a reduction in TauT activity in placental villous fragments treated with SIN-1 for 2 hours. However, in contrast to the effect of SIN-1 on MVM, this reduction was not prevented by SOD or haemoglobin. In fact, haemoglobin caused a decrease in TauT activity. However, the reduction of TauT activity by haemoglobin was not necessarily attributable to increased O$_2^-$ as haemoglobin binds oxygen leading to hypoxia which can affect free radical generation and scavenging (Khullar et al., 2004). Therefore, it was not resolved which species was responsible for the reduction in TauT activity in response to SIN-1 in villous tissue (Khullar et al., 2004). This demonstrates the complexity of experiments in tissue which has endogenous antioxidant and free radical generating capacity.

The Khullar study (2004) demonstrated a reduction in system A activity and altered Na$^+$ permeability in MVM vesicles following treatment with SIN-1. However, in PE, where there is evidence for increased placental nitrative stress, STB system A activity is not different from normal pregnancy (Shibata et al., 2008). In obesity, also associated with elevated placental nitrative stress, in those placentas where TauT activity was significantly reduced with increasing BMI, system A activity was increased (Ditchfield, personal communication. Therefore in PE and maternal obesity, there appears to be a selective effect of the in utero environment to decrease TauT activity and it is possible that this is by nitration.

In summary, the data are consistent with SIN-1 inhibiting TauT activity through nitration of tyrosine residues. However, to confirm that TauT was nitrated under these experimental circumstances necessitates further experiments including immunoprecipitation of TauT followed by mass spectrometry (see Chapter 4, Section 4.4.5).
5.4.3 Consequences of reduced TauT activity for syncytiotrophoblast turnover

To investigate whether a reduction in TauT activity led to disrupted STB renewal, explants treated with SIN-1 that had reduced TauT activity were processed for IHC. M30 staining was increased in SIN-1 treated explants, indicative of increased apoptosis. M30 detects caspase-cleaved cytokeratin-18 within the cytoplasm and is specific for epithelial apoptosis events and therefore produces discrete staining within the trophoblast of placenta. Since the STB is a multinucleated layer and M30 is a cytoplasmic stain (i.e. it does not detect nuclei) it is not possible to attribute a M30 positive event to particular nuclei within the STB. For this reason, M30 staining was not expressed in terms of number of trophoblast nuclei but instead as a percent of total villous area. Although apoptosis has been detected within the CTB and STB of the placenta (Sharp et al., 2010), a recent paper using a marker of CTB cells concluded that apoptosis is in fact limited to the CTB cells (Longtine et al., 2012). Another way to assess apoptosis would be to use TUNEL to identify apoptotic nuclei which could be used in conjunction with E-cadherin as a marker of CTBs. TUNEL detects DNA fragmentation, a very late stage apoptosis event. Kadyrov and colleagues (2001) have compared the application of TUNEL and M30 staining as markers of apoptosis and concluded that M30 is superior in the detection of apoptotic trophoblast cells. This was based on the fact that (a) M30 detects a larger number of cells within the apoptotic cascade as caspase cleavage is an earlier event than DNA cleavage (b) some trophoblast cells can also bypass DNA cleavage and would therefore not stain positive for TUNEL (c) TUNEL staining did enable reproducible quantification of data and (d) there are reports that necrotic cells may also express TUNEL positivity (Kadyrov et al., 2003, 2001). Therefore in this study M30 was used as a marker of trophoblast apoptosis.

Dual staining analysis for proliferating CTBs demonstrated fewer proliferating CTBs in SIN-1 treated placental explants. CTBs are responsible for maintaining the STB layer by proliferating, differentiating and fusing into the STB (Huppertz et al., 2002). CTB nuclei can be very hard to distinguish from other placental nuclei present (e.g. stromal and STB nuclei) without the use of a CTB marker (Longtine et al., 2012). For this reason, in addition to identifying proliferating nuclei with ki67, E-cadherin (a cell-cell adhesion protein) was used to identify CTBs. The expression of E-cadherin is lost as CTBs differentiate and fuse with the STB (Brown et al., 2005). Other studies assessing proliferation have expressed it in terms of
total number of nuclei present in placental villi or a field of view (Chan et al., 1999; Higgins et al., 2013) or as the BrdU:Ki67 ratio which represents the fraction of cells in S phase of the cell cycle compared to total proliferating cells (Arnholdt et al., 1991). However, in the current study dual staining for Ki67 and E-cadherin facilitated accurate identification of CTB’s and proliferating CTB’s were expressed as a % of non-proliferating CTB’s.

Cytokeratin 7 is expressed by epithelial cells and was used to demark the STB in placental explants to facilitate assessment of STB integrity and regeneration. In placental explants, the STB is shed and regenerated over a 5 day period (Simán et al., 2001) and so by day 7 in culture it is expected that the placental villi are covered by a renewed intact STB. Therefore, cytokeratin 7 staining can be used to assess whether reduced TauT activity affected the ability of the STB to regenerate. In SIN-1 treated explants there were significantly fewer villi with an intact STB (and therefore more villi with shedding STB) compared to controls. SIN-1 also significantly reduced the number of villi with a complete cellular STB (intact villi plus villi with shed STB and complete cellular regeneration) that could be transporting nutrients normally.

In summary, SIN-1 increases nitrotyrosine formation, decreases TauT activity and disrupts cellular regeneration in villous explants. The effect of SIN-1 on STB regeneration could be mediated by a fall intracellular taurine following a reduction in TauT activity through nitration. However, it is also possible that SIN-1 alters STB regeneration by mechanisms that are independent of a reduction in TauT activity.

5.4.4 Consequences of reduced intracellular taurine for syncytiotrophoblast renewal

The possibility that a reduction in taurine uptake following TauT inhibition can alter STB regeneration was assessed using β-alanine to competitively inhibit taurine uptake. However, a more specific method to reduce intracellular taurine would be the knockdown of TauT with TauT specific siRNA. In isolated primary CTBs knockdown of TauT with siRNA significantly reduced TauT mRNA expression, TauT protein expression, and TauT activity which led to a marked reduction in intracellular taurine (70%) compared to untransfected controls (Desforges et al., 2013b). However, in the current study, siRNA knockdown of TauT in term placental explants was not achieved (see Appendix, Chapter 7). Measuring TauT activity as the functional end point of TauT knockdown demonstrated inconsistent and variable results with no significant reduction in TauT activity following treatment with TauT.
specific siRNA. In addition, treatment with non-targeting siRNA negative controls also reduced TauT activity casting doubt on the specificity of TauT siRNA (Appendix; Chapter 7). Differing success of siRNA knockdown in primary CTBs and term placental explants has also been shown for the system A transporters (SNATs). In primary CTBs, knockdown of SNAT1 with siRNA demonstrated reduced mRNA and protein expression and reduced transporter activity (Desforges et al., 2010). However, siRNA knockdown of SNAT1 in term placental explants was unsuccessful, with no reduction in mRNA/protein expression or transporter activity achieved compared to untransfected controls (Desforges, personal communication). Disparity between siRNA knockdown of TauT in primary CTBs and term placental explants may be due to the complexity of the villous explant model. In placental explants tissue architecture is maintained therefore several cell types are present. In addition, placental explants represent a dynamic model of cell turnover in which the STB, the target tissue in which siRNA knockdown of TauT should be achieved, is shed and regenerated. Assessment of transfection success by siGLO and fluorescence imaging indicated high levels of fluorescence in shed STB with some punctuate staining in the underlying layer over which TauT activity would be measured. Therefore the complexity of STB turnover events in placental villous explants may be one contributing factor for the failure of TauT knockdown by siRNA. Consequently, treatment of villous explants with β-alanine was used as an alternative approach to reduce intracellular taurine.

β-alanine is substrate of TauT, and when present in excess of taurine, caused a dose-dependent inhibition of intracellular taurine in villous fragments (Figure 5.1 b). Although this is an effective means to deplete STB of taurine, the STB will accumulate β-alanine with possible off-target effects. To minimise this, experiments were performed to determine the minimum concentration of β-alanine necessary to produce a marked reduction in intracellular taurine. It is not known what effects elevated intracellular β-alanine might have on STB. β-alanine is the rate limiting precursor to carnosine, therefore it is possible that any off-target effects of β-alanine treatment could be due to the conversion of β-alanine to carnosine. Indeed, supplementation with β-alanine in humans leads to an increase in skeletal muscle cell carnosine concentrations (Sale et al., 2010). The physiological functions of carnosine have been studied in skeletal muscle where it acts as an effective pH buffer (Sale et al., 2010). Other proposed functions of carnosine include protection of proteins against glycation, prevention of the formation of protein-protein cross links, chelation of ions, a role as an antioxidant and as a neurotransmitter (Sale et al., 2010).
Intracellular taurine was significantly reduced by approximately 85% following treatment of placental villous explants with 2.5 mM β-alanine. M30 positive staining was significantly increased in β-alanine treated explants compared to control, indicative of an increase in apoptosis. In contrast to treatment with SIN-1, there was no effect of β-alanine on CTB proliferation. Using cytokeratin 7 to assess STB integrity, fewer villi with intact STB (and more villi with shed STB) were evident in β-alanine treated explants. There was a trend for more villi with shed STB to have cellular regeneration in β-alanine treated explants compared to control. This indicates that although the β-alanine treated explants have the ability to regenerate a new STB layer, regeneration might have been delayed. Previous work from our laboratory which supports this proposal (Greenwood, personal communication) assessed STB regeneration in explants by cytokeratin 7 staining at each day of culture during treatment with 10mM β-alanine. Explants treated with β-alanine showed a significantly higher degree of STB shedding and fewer regenerating villi on days 2 and 3 of culture compared to the control. In the current study there was no difference in the number of villi with a complete cellular STB (intact plus shed STB with complete regeneration) between explants untreated explants and explants treated with β-alanine. Osmotic controls (the addition of 10 mM mannitol) have been carried out previously in this laboratory and showed no effect on villous regeneration of the STB in culture (Greenwood personal communication). In summary, results show that marked reduction in intracellular taurine disrupts the processes of STB cellular regeneration.

5.4.5 Summary

Table 5.1 summarises the effects of reduced TauT activity (treatment with SIN-1) and reduced intracellular taurine (treatment with β-alanine) on STB turnover on day 7 of villous explant culture. Similarities on STB turnover between the two treatments include an increase in apoptosis, a reduction in the number of villi with intact STB (and an increase in the number of villi with shed STB). There were different effects of SIN-1 and β-alanine on CTB proliferation (Table 5.1); this could suggest that SIN-1 may be having effects not attributable to reduced TauT activity, although notably a reduction in proliferation of renal embryonic 293 cells following TauT knockdown by RNAi has been demonstrated (Han and Chesney, 2013).
It has been reported in PE that apoptosis is increased (reviewed by: Sharp et al. 2010), proliferation is increased (Unek et al., 2014) or unchanged (Tomas et al., 2011) and CTB differentiation/fusion is decreased (Langbein et al., 2008; Lee et al., 2001; Vargas et al., 2011) compared to normal pregnancy. Recent evidence demonstrated alterations in STB renewal in maternal obesity; notably a reduction in proliferation index and apoptotic index (Higgins et al., 2013). Mechanisms of STB renewal are not thoroughly understood in normal pregnancy (Chapter 1: section 1.3.3 and reviewed by Mayhew (2014). There has been debate over the involvement of apoptosis in the fusion of CTBs into the STB, the phenotype of trophoblast which is undergoing apoptosis and the involvement of apoptosis in the removal of aged nuclei from the CTB. One difficulty in assessing the mechanism of STB renewal is that cellular turnover is a dynamic and continuous process and fixed tissue can only provide a snap shot of the events that occur in vivo. However, the current study has identified some similarities in altered turnover events in SIN-1/β-alanine treated villous tissue and PE. Treatment of villous explants with 2.5 mM β-alanine reduced intracellular taurine by 85% compared to controls. It has yet to be determined whether intracellular taurine is reduced by SIN-1 although it is unlikely that a reduction in TauT activity of 18% would reduce intracellular taurine to the same extent as 2.5 mM β-alanine (15% of normal). Intracellular taurine following SIN-1 treatment will be important to determine in future experiments.
Therefore these data support the possibility that reduced intracellular taurine as a result of reduced TauT activity in PE and maternal obesity could be a causal factor in altered STB renewal. It will be important for future work to elucidate the intracellular mechanisms by which taurine brings about its effects on apoptosis, proliferation and differentiation which are largely unknown (Chapter 1, Introduction, sections 1.6.2.2 – 1.6.2.3). One approach to address this would be to carry out arrays on tissue with experimentally reduced intracellular taurine to identify changes in markers of apoptosis/proliferation. Using a cell stress array preliminary data demonstrated altered stress-related protein expression in primary CTB cells with siRNA mediated TauT knockdown following exposure to TNF-α. Taurine deplete cells failed to elicit a pro-survival response (i.e. increased expression of anti-apoptotic proteins Bcl-2 and COX-2) compared to taurine replete cells (Desforges and Greenwood, 2014).

In summary, the results presented in this chapter have indicated that reduced TauT activity induced by SIN-1 is associated with dysregulated events that are involved in renewing STB. Reducing intracellular taurine with β-alanine also demonstrated disruption to normal cell turnover processes. Taken together, this provides supporting evidence that maintaining intracellular taurine may be important for maintaining appropriate STB renewal.
Chapter 6: General Discussion

PE is a severe disease that affects approximately 5% of pregnancies globally per year (Abalos et al., 2013) and has serious adverse consequences for the immediate and long term health of the mother and infant. PE represents an important clinical challenge as there is no way to reliably predict which women will develop PE and delivery of the baby and placenta remains the only effective treatment. For reasons which are unknown, obesity is a key risk factor for developing PE. Both conditions are associated with disrupted renewal of STB, increased placental nitritative stress and heightened inflammation.

Taurine is a conditionally essential amino acid during pregnancy and the supply of taurine to the placenta and fetus must be met by uptake from the maternal blood. Studies have demonstrated the importance of maintaining intracellular taurine for facilitating appropriate cell turnover processes and cytoprotection from free radicals and inflammatory cytokines in several tissues (Hanna et al., 2004; Oriyanhan et al., 2005) including CTB (Desforges et al., 2013b). It has previously been reported that the uptake of taurine into the placenta is reduced in obese women compared to their ideal weight counterparts (Desforges et al., 2013a). A reduction in intracellular taurine following a reduction in STB TauT activity could have several implications for the placenta and the fetus including (a) reduced delivery of taurine to the fetus (b) disrupted maintenance of STB and (c) loss of cytoprotection. Therefore, this thesis focused on investigating whether placental taurine transport is altered in PE compared to normal pregnancy. Furthermore, potential causes and consequences of reduced taurine transport into the STB were explored.

6.1 Syncytiotrophoblast TauT activity but not protein expression is reduced in pre-eclampsia

This study demonstrated that STB TauT activity is significantly lower in PE compared to normal pregnancy. The reduction in TauT activity could not be attributed to an effect of maternal obesity as reduced TauT activity was demonstrated in women of ideal weight experiencing PE compared to normal pregnancy. There was also no influence of FGR, onset of disease or gestation on TauT activity in PE and therefore reduced TauT activity is a feature of the disease itself. Two potential mechanisms for the reduction in TauT activity in PE were explored; a reduction in TauT protein expression at STB MVM and increased nitration of TauT. In membrane enriched placental homogenate, TauT protein expression at
70 kDa (the expected size of the full length mature protein) was significantly higher in PE compared to normal pregnancy. The 70 kDa TauT species was also expressed in MVM preparations from normal pregnancy and PE. STB TauT activity was positively related to TauT protein expression in membrane enriched homogenates in ideal weight women having normal pregnancy. However, this relationship was lost in ideal weight women experiencing PE. As there is more TauT protein in the STB MVM in PE compared to normal pregnancy but reduced taurine uptake, the activity of the transporters could be down-regulated by post translational modification. It could also be speculated that increased TauT protein expression could be a compensatory mechanism to attempt to overcome down-regulation by post translational modification. However, the mechanism of the elevated TauT protein expression in PE compared to normal pregnancy remains to be determined.

Recent data have shown reduced taurine concentration in the serum of women who later develop PE (Kuc et al., 2014); indeed, plasma taurine has been advocated as an early biomarker of disease. The underlying cause of the reduction in plasma taurine was not explored in this study, but aside from its potential as a biomarker, low plasma taurine could have profound implications for STB health and fetal growth and development in early pregnancy. It is possible that STB taurine depletion occurs in the first trimester and that TauT expression is up-regulated as a consequence. However, as disease progresses, the activity of TauT is inhibited by in-utero factors which could include nitratative stress.

6.2 Downregulation of TauT activity by nitration in pre-eclampsia

This study investigated the possibility that down-regulation of TauT in PE may be due to post translational modification by nitration. Initial experiments aimed to confirm and extend earlier reports of increased nitrotyrosine staining in placental villous tissue in PE compared to normal pregnancy (Myatt et al., 1996). IHC was performed to determine nitrotyrosine protein expression in placentas of women with PE (in which TauT activity was reduced) compared to normal pregnancy. IHC demonstrated increased nitrotyrosine formation in the positive control and revealed a greater staining intensity in STB in PE than in normal pregnancy. While IHC has the advantage of localising the sites of nitrotyrosine
expression in villous tissue, the technique only allowed nitrotyrosine expression to be assessed semi-quantitatively.

In contrast, Western blot analysis of nitrotyrosine in this study did not identify any differences in mean signal intensity of protein species identified between 250 and 60 kDa or the 70 kDa species between PE and normal pregnancy. However, as noted by Roberts and colleagues (2009) the presence of multiple bands on Western blots of membrane enriched placental homogenates with the anti-nitrotyrosine antibody makes the quantification of nitration difficult. It is highly likely that several proteins with a similar molecular weight to TauT (e.g. COX-2 (Wetzka et al., 1997)) will be nitrated and appear at the same molecular weight as TauT. Therefore to elucidate whether there is an increased proportion of nitrated TauT in PE compared to normal pregnancy, Western blot analysis for nitrotyrosine was carried out following immunoprecipitation of TauT from placental membrane enriched homogenates. However, optimisation experiments identified some concerns over the reliability of the monoclonal mouse antibody used for Western blotting and time constraints did not allow further evaluation with alternative anti-nitrotyrosine antibodies. Therefore mass spectrometry analysis on STB MVM would be an improved approach to assess TauT nitration in PE.

Previous studies of acute treatment of MVM vesicles and villous fragments with SIN-1 demonstrated a reduction in STB TauT activity (Khullar et al., 2004; Roos et al., 2004). To assess the effect of chronic induction of nitrative stress (through an ONOO⁻ donor) in the current study, TauT activity was assessed in vitro using the placental villous explant preparation. Chronic exposure of explants to the nitrating agent SIN-1 significantly reduced TauT activity. Exposure to H₂O₂, which induces oxidative stress, did not have any effect on TauT activity and therefore an effect of SIN-1 through generation of O₂⁻ is unlikely. Using IHC to determine the expression and localisation of nitrotyrosine staining, the fingerprint of ONOO⁻ formation and action, an increase in nitrotyrosine formation in the STB in SIN-1 treated explants was demonstrated in PE compared to controls. In MVMs, Khullar et al. (2004) showed that treatment with SOD (to sequester O₂⁻) and haemoglobin (to sequester NO) prevented impairment of TauT activity by SIN-1, indicating that ONOO⁻ is the species responsible for TauT inhibition. In the current study the species responsible for the reduction in TauT activity was not identified. However as the generation of nitrotyrosine indicated that ONOO⁻ was elevated with SIN-1 treatment, it is probable that TauT activity was reduced through the nitration of tyrosine residues by ONOO⁻. ONOO⁻ was used to test this more directly but daily treatment with 1 mM ONOO⁻ failed to generate nitrotyrosine or
inhibit TauT activity. Bearing in mind the short half-life ONOO’ (Chapter 1; section 1.8.1) these experiments should be repeated with increased frequency of ONOO’ application.

6.3 Decreased TauT activity is associated with dysregulated syncytiotrophoblast turnover

An important consequence of reduced TauT activity at the MVM could be a fall in intracellular taurine (discussed further in section 6.5.1) and in turn this could lower cytoprotection (section 6.5.2), reduce delivery of taurine to the fetus (section 6.5.3) and disrupt STB turnover. Altered STB turnover is an important feature of PE and there is evidence that maintenance of STB is also altered in maternal obesity (Higgins et al., 2013). Several studies have demonstrated increased apoptosis in the placenta in PE (reviewed by: (Heazell and Crocker, 2008; Sharp et al., 2010)). It is also evident that proliferation, differentiation and fusion of CTBs is disrupted in PE (Langbein et al., 2008; Unek et al., 2014). It has been reported that STB area is reduced in PE compared to normal pregnancy (Daayana et al., 2004). Finally, PE is associated with elevated syncytial knots (Calvert et al., 2013) as well as increased deportation of necrotic and inflammatory material from STB into maternal blood (Y. Chen et al., 2012; Goswami et al., 2006). In common with PE, placental apoptosis and proliferation have been shown to be increased in maternal obesity compared to ideal weight women however (Higgins et al., 2013).

The co-ordination of proliferation, fusion/apoptosis and shedding of obsolete material is incompletely understood and hampered by the snapshot nature of observations made on fixed tissue (Mayhew, 2014). Assessment has been hindered in part by the difficulty in identifying the phenotype of trophoblast demonstrating alterations in apoptosis. Identification of CTBs is important for an accurate assessment of alterations to CTB proliferation and to address this, dual staining for Ki67 and E-cadherin was used in the current study. Recently the definition of syncytial knots and the hypothesis that they contain apoptotic nuclei that are shed into maternal blood has been challenged (Burton and Jones, 2009; Coleman et al., 2013). Since the significance of syncytial nuclear aggregates is currently uncertain, they were not used as a measure of STB turnover in this study. Further work is required to establish the mechanisms of trophoblast turnover and deportation in normal pregnancy and the disruption to these events in pregnancy pathologies.
Following successful inhibition of TauT activity by treatment of placental explants with SIN-1, apoptosis, CTB proliferation and STB regeneration were assessed. In placental explants with reduced TauT activity, apoptosis was increased, CTB proliferation decreased and integrity of the STB disrupted. There were fewer villi with intact STB (and more villi with shedding STB) on day 7 of culture following SIN-1 treatment compared to control. Taken together these data suggest that the normal processes of STB renewal are disrupted when TauT activity is reduced. This could be due to reduced intracellular taurine in the STB, following reduced TauT activity, or another direct/indirect effect of the free radicals donated by SIN-1. The ideal experiment to address this would be the knock-down of TauT with siRNA; however when assessed in term placental explants this approach was not successful (Appendix; Chapter 7). No consistent reduction in TauT activity could be achieved and in some instances transfection with non-targetting siRNA controls reduced TauT activity meaning that any effect following treatment with TauT-siRNA could not be considered specific. Therefore, an alternative approach was adopted to reduce intracellular taurine by treatment with β-alanine. It was demonstrated that reducing intracellular taurine with β-alanine also disrupted the maintenance of the STB. Consistent with the effects of SIN-1, treatment with β-alanine significantly increased apoptosis and decreased the number of villi with intact STB (increased the number of villi with shed STB). Out of the villi with shedding STB, there was a trend in the β-alanine treated villous explants for the number of villi with cellular regeneration to be increased. This suggests that regeneration of the STB layer may be delayed following β-alanine treatment (Chapter 5; section 5.4.4). In contrast to treatment with SIN-1, treatment of villous explants with β-alanine demonstrated no difference in CTB proliferation. The effects of SIN-1 and β-alanine on STB renewal could be attributed to (a) reduced intracellular taurine (b) a direct effect of free radicals generated by SIN-1 or (c) elevated intracellular β-alanine. A previous study demonstrated that siRNA knockdown of TauT in isolated primary CTB cells successfully reduced intracellular taurine to 25% of normal which lead to compromised differentiation and increased apoptosis in response to TNF-α (Desforges et al., 2013b).

Taken together, these results suggest that maintaining intracellular taurine is important for facilitating appropriate STB cell turnover and raises the possibility that a reduction in intracellular taurine could contribute to disrupted STB renewal in pregnancy complications such as PE and obesity; it is plausible that reduced TauT activity may be a cause, and not consequence, of dysregulated STB turnover.
6.4 Syncytiotrophoblast TauT activity but not protein expression is reduced in maternal obesity

This study confirmed previous reports that STB TauT activity is lower in obese women compared to women of ideal weight (Desforges et al., 2013a). Reduced TauT activity in maternal obesity was not associated with a reduction in TauT protein expression which also confirming and extending previous observations (Ditchfield, personal communication). The highly significant relationship between STB TauT activity and TauT protein expression demonstrated in ideal weight women in this study (Chapter 4, Figure 4.3) was lost in placentas from obese women. Therefore, the reduced TauT activity and lack of relationship between TauT activity and TauT protein expression is common to both PE and maternal obesity. However, in PE TauT protein expression was increased compared to normal pregnancy whereas no change was detected in maternal obesity. Although not explored directly in this study there is evidence for increased placental nitric stress in obesity (Roberts et al., 2009) raising the possibility that in common with PE, the reduced TauT activity is a consequence of post translational modification by nitration and that this contributes to altered STB renewal.

6.5 Further implications of reduced TauT activity in pre-eclampsia and maternal obesity

The reduced TauT activity in PE and maternal obesity demonstrated in this study could have several implications for the placenta and the developing fetus including reduced intracellular taurine, disrupted STB renewal (discussed above), reduced protection from inflammatory cytokines and reduced delivery of taurine to the fetus.

6.5.1 Reduced intracellular taurine

Firstly it is important to determine whether reduced TauT activity at the MVM results in reduced intracellular taurine in the STB as it has been demonstrated previously that maintaining intracellular taurine concentration is important for maintaining CTB differentiation/multinucleation and cytoprotection (Desforges et al., 2013b). There are some data supporting the proposal that a reduction in TauT activity at the STB in PE will cause a stable reduction in intracellular taurine. Firstly siRNA knockdown ofTauT in CTBs and competitive inhibition of taurine uptake by β-alanine results in a significant decrease in
intracellular taurine. In addition, TauT activity at STB MVM is reduced in FGR compared to normal pregnancy, and fetal plasma taurine is lower in the FGR compared to normally grown fetus (Cetin et al., 1990; Economides et al., 1989; Norberg et al., 1998). This implies that the reduction in taurine uptake depletes STB taurine and lowers the concentration gradient that drives taurine diffusion from STB to the fetus. Therefore it is possible that STB taurine will be lower following inhibition of TauT activity. It can be further speculated that up-regulation of TauT expression may occur in response to reduced intracellular taurine but due to an environment of increased oxidative/nitrative stress in PE, uptake of taurine cannot be enhanced.

One approach that could be used to estimate intracellular taurine is to measure taurine levels homogenised placental villous tissue lysates using HPLC. However, placental tissue homogenates contain several cell types (endothelial, smooth muscle and stromal cells, as well as fetal blood), which will contribute to the measured taurine concentration. For example, constituents of blood e.g. plasma, erythrocytes, leukocytes and platelets have a high intracellular taurine concentration (Fukuda et al., 1984; Kanemori et al., 1992). Taurine uptake into platelets has been shown to be increased in women with PE compared to normal pregnancy (Otani et al., 1992). Therefore contamination of placental homogenates with fetal blood, as well as other cell types makes it impossible to determine how much of the lysate taurine concentration is contributed from the STB. Another possibility to indirectly assess STB taurine is to immunostain villous sections from normal pregnancy and PE with an anti-taurine antibody followed by semi-quantitative assessment of the STB compartment. This antibody to taurine has been used in several tissues including rat kidney, retina, pituitary gland, brain, mouse cerebellum, cardiac muscle cells, adipose tissue and male reproductive organs (Lobo et al., 2000; Pow et al., 2002; Terauchi et al., 1998; Trachtman et al., 1993). Therefore, it could be useful to detect taurine content specifically in the STB compartment although it might not have the sensitivity to detect differences in PE compared to normal pregnancy if any changes in intracellular taurine content are small. Another method to estimate intracellular taurine, used in the current study, is to measure the steady state accumulation of $^{3}$H-taurine by placental villous tissue or CTB’s. Steady state was shown to be achieved in 20 hrs in tissue (Chapter 5, Figure 5.1 a) and cells (Desforges et al., 2013b). Although this technique does not measure intracellular taurine concentration directly, it can be used to assess differences in intracellular taurine in relation to pregnancy pathology or intervention. However, any difference in STB volume due, for example, to a pregnancy complication compared to normal pregnancy, would
contribute to the steady state measurement. In addition, when considering tissue, this measure does not assess taurine levels specifically in STB, but will reflect the steady state in all accessible cell types within the villous. Therefore, due to limitations of each method, a combination of the three measures may be a more useful approach.

6.5.2 Reduced cytoprotection

Both PE and maternal obesity are associated with increased inflammation (Challier et al., 2008; Gu et al., 2008; Rinehart et al., 1999) and increased oxidative and nitrative stress (Myatt et al., 1996; Roberts et al., 2009). Taurine has cytoprotective properties and plays an important role in protecting cells against ischaemia-reperfusion injury, inflammation and free radicals (Desforges et al., 2013b; Hanna et al., 2004; Jong et al., 2012; Takatani et al., 2004a; Zhang et al., 2008). It has been demonstrated in isolated CTBs that reducing intracellular taurine by siRNA knockdown increases susceptibility to TNF-α induced apoptosis (Desforges et al. 2013). The mechanisms by which taurine mediates its cytoprotective effect are poorly understood but there is increasing evidence that it may be related to the ability of taurine to preserve mitochondrial function (reviewed by: (Jong et al., 2012)). Preliminary evidence indicates that depletion of intracellular taurine in BeWo cells is associated with mitochondrial dysfunction (Desforges and Greenwood, 2014). Mitochondria not only provide cells with energy, but also have a role in intracellular signalling, cell differentiation and cell death. Using a TauT knockout mouse, Warskulat et al. (2006), demonstrated that liver mitochondria have a reduced respiratory control ratio which is indicative of mitochondrial dysfunction. Placental mitochondrial dysfunction has also been reported in PE (Muralimanoharan et al., 2012).

In common with the observations at term, STB TauT activity is lower in obese women compared to women of ideal weight, and negatively correlated to BMI, in the first trimester of pregnancy (Desforges et al. 2013). In view of the cytoprotective role of taurine discussed above, this could have important consequences for the ongoing pregnancy. Obesity is a major risk factor for developing PE but the reasons for this are unclear. Obesity and PE have several features in common which include endothelial dysfunction, hypertension, heightened inflammation and increased oxidative/nitrative stress (Challier et al., 2008; Gu et al., 2008; Myatt et al., 1996; Roberts et al., 2009). The possibility that both conditions independently dysregulate STB turnover and function but can combine to have additive adverse effects seems plausible but has not been addressed. In obese women, the reduction in STB TauT activity in early pregnancy could lead to STB taurine depletion,
mitochondrial dysfunction and loss of cytoprotection to inflammatory cytokines/free radicals which are elevated in obesity and PE thereby increasing the susceptibility to developing PE.

6.5.3 Reduced delivery of taurine to the fetus

If taurine is lowered in STB following a reduction in TauT activity in PE and maternal obesity, delivery of taurine to the fetus may be compromised through a reduction in the concentration gradient that drives taurine efflux across BM (Chapter 1, Figure 1.5). Reduced delivery of taurine to the fetus by the placenta leading to fetal taurine depletion will be detrimental to the developing fetus as taurine is essential for normal fetal growth and development (Aerts and Van Assche, 2002; Heller-Stilb et al., 2002; Ito et al., 2010; Roysommuti et al., 2009; Sturman, 1993; Sturman et al., 1984). Furthermore, animal studies have demonstrated that taurine depletion in the perinatal period alters renal function and increases risk of hypertension in adult offspring (Roysommuti et al., 2009).

Although transport across the MVM is crucial to accumulate solutes in STB, efflux transporters on the BM are essential to deliver nutrients to support fetal growth and development. Compared to the MVM, there have been fewer studies of BM, in part because of the difficulties in accessing this membrane in intact tissue. Using the perfused human placental cotyledon preparation, several amino acid transport systems have been identified on the BM, including accumulative and amino acid exchangers and facilitated transporters (Cleal and Lewis, 2008; Cleal et al., 2011). TauT is present on the BM, where its activity is only 6% of the TauT present on the MVM, and will transport taurine from fetal plasma into STB in accordance with the prevailing $\text{Na}^+$ electrochemical gradient (Norberg et al., 1998). It has been suggested that taurine efflux across the BM could occur through volume regulated anion channels as taurine efflux is stimulated by cell swelling (Riquelme, 2009). There have been no studies of taurine efflux from STB in either PE or maternal obesity and the possibility remains that this might be altered to influence both intracellular taurine and delivery of taurine to the fetus in these pregnancy conditions.

Whilst further investigation is required to establish the mechanism of taurine efflux across the BM, fetal plasma taurine concentration has been measured in cord blood using HPLC. Umbilical venous plasma taurine concentration is lower in FGR compared to normal pregnancy (Cetin et al., 1990; Economides et al., 1989); however there are conflicting reports for taurine concentration in fetal cord blood in PE. Cockburn et al. (1971) measured taurine in cord plasma and showed no difference in PE but Evans et al. (2003) reported an
increase in taurine in the serum in PE compared to normal pregnancy. The disparity in these results could be due to the different constituents of blood used for measurement (plasma and serum) or due to differing methods of sample preparation. The study by Cockburn et al. (1971) collected and processed blood samples within 20 minutes of delivery whereas the study by Evans et al. (2003) left the blood to stand for 1 hour at room temperature before processing. This is potentially very important as the method of collection and storage has been shown to affect taurine levels (Davis et al., 2009; Mou et al., 2002; Trautwein and Hayes, 1990). Therefore fetal plasma taurine concentrations in PE require confirmation. Umbilical vein whole blood/plasma and maternal urine and whole blood/plasma samples have been collected from normal pregnancy and PE for measurement of amino acid concentrations by HPLC during the course of the current study. To understand the significance of the reduction in STB TauT activity that is evident in PE it is essential to determine (a) STB taurine uptake and efflux and (b) the concentrations of taurine in maternal plasma, fetal plasma and STB in normal pregnancy and PE.

6.6 Other potential down-regulators of placental TauT activity in pre-eclampsia/maternal obesity

Aside from the potential of STB TauT activity to be down-regulated by nitration (section 6.2), there are several other regulators of TauT in the placenta that could have a role in reducing STB TauT activity in PE and maternal obesity. There is some evidence that oxidative stress may regulate TauT activity as treatment of a rat STB cell line with H$_2$O$_2$ led to a reduction in TauT activity (Lee and Kang, 2010). However, this study found no effect of 1mM H$_2$O$_2$ on TauT activity in placental villous explants.

mTOR is a Ser/Thr protein kinase that has a wide variety of functions including regulating cell growth, proliferation, motility, survival, protein synthesis and transcription (Watanabe et al., 2011). Inhibition of mTOR in cultured primary CTBs reduces activity of TauT (Roos et al., 2009). It has also been demonstrated that mTOR reduces System A and System L transport on CTBs by reducing the insertion of the transporters into the plasma membrane without altering overall protein expression (Rosario et al., 2013). Placental mTOR expression is lower in FGR compared to normal pregnancy; however, the same study found no difference in mTOR expression in PE (Aiko et al., 2014). mTOR signalling has been shown to be increased in placentas from obese women that had LGA babies (Jansson et al., 2013). Although it is unknown how obesity with AGA offspring affects mTOR signalling compared to normal pregnancy. Furthermore, in baboons fed a low protein diet to induce FGR, mTOR
signalling was decreased and was associated with a reduction in STB TauT activity (Kavitha et al., 2013). Further investigation is necessary to determine whether mTOR has a role in regulation of TauT in PE.

There are several putative phosphorylation and glycosylation sites on TauT (Chapter 1, Introduction, Figure 1.7-1.8). Ser-322 has been identified as the crucial site of PKC phosphorylation of TauT (Han et al., 1999) and regulation of TauT by PKC has been demonstrated in JAr cells (Kulanthaivel et al., 1991). Roos et al. (2004) showed that PMA, an activator of PKC reduced TauT activity in placental fragments. Furthermore, an activator of PKC which is increased in PE (Khatun et al., 2000) and maternal obesity (Baranowska et al., 2005), neuropeptide-Y, has been shown to reduce TauT activity by 20% in villous fragments (Desforges et al., 2013a). Therefore it is possible that phosphorylation of the transporter contributes to the down-regulation of TauT in PE. Future experiments using mass spectrometry (Chapter 4, section 4.4.5) could provide information on post translational modifications of TauT in PE.

Extracellular taurine concentration has been shown to regulate TauT activity and mRNA expression in a variety of cells including JAr cells (Han et al., 2006; Jayanthi et al., 1995). In low extracellular taurine, TauT mRNA and activity is up-regulated and when extracellular taurine concentration is high TauT is down-regulated. Maternal plasma taurine concentration has been shown to be reduced in normal pregnancy compared to non-pregnant women (Di Giulio et al., 2004) and in obese non pregnant individuals compared to ideal weight counterparts (Rosa et al., 2014). The plasma taurine concentration in women with PE has been measured in two studies and shown to be similar to women having normal pregnancy (Cockburn et al., 1971; Evans et al., 2003). However a study by Kuc et al. (2014), demonstrated significantly lower maternal serum taurine concentration in the first trimester in women who went on to develop early onset PE compared to women who had a normal pregnancy. Reduced maternal serum taurine predicted early onset PE with a detection rate of 55% when combined with first trimester mean arterial pressure measurements (Kuc et al., 2014). It could be speculated that reduced serum taurine in the first trimester may up-regulate TauT expression in PE but that the in utero environment of increased oxidative/nitrative stress in later pregnancy could result in post translational modification and inhibition of TauT activity. In addition, TauT activity in placentas from obese women in the first trimester has been shown to be significantly lower compared to ideal weight women (Desforges et al., 2013a). Therefore taurine could be a potential biomarker of PE useful in first trimester screening for the disease.
6.7 Therapeutic application of taurine

Taurine is used therapeutically in a range of conditions including cardiovascular disease, hypercholesterolemia, epilepsy, Alzheimer’s, alcoholism and cystic fibrosis without contraindications (Birdsall, 1998). It is not known at this stage whether there is a potential role for taurine supplementation in improving placental function in women suffering from PE or women at increased risk of developing PE. It is possible that taurine supplementation might not be of benefit as this study has shown that STB TauT activity is reduced in PE despite an increase in TauT protein expression. This suggests that TauT may be post translationally modified in PE and so increasing taurine availability could fail to increase intracellular taurine if the transporter activity is hindered. Further work is required to determine the mechanism/s of down-regulation of STB TauT activity in PE and obesity as well as intracellular signalling pathways that both regulate TauT activity and are regulated by taurine in normal pregnancy and pregnancy complications before the therapeutic value of taurine supplementation can be evaluated.

6.8 Conclusions

Figure 6.1 presents a working model which combines the main result of this thesis with published findings and highlights important information to be determined in the future.

This study demonstrated that STB TauT activity is reduced in PE compared to NP and also confirmed that STB TauT activity is negatively related to maternal BMI. Placental TauT protein expression was significantly higher in PE and unaffected in maternal obesity compared to normal pregnancy and therefore did not account for the reduced TauT activity. Nitration, a potential post translational modification of TauT, was explored. STB nitrotyrosine formation was significantly higher in PE compared to normal pregnancy and inducing nitrative stress in vitro reduced TauT activity. Furthermore, reduced TauT activity by nitrative stress was associated with increased apoptosis, decreased CTB proliferation and disrupted STB regeneration. Finally, this study has shown that experimentally reducing intracellular taurine in placental villous explants is associated with increased apoptosis and disrupted STB regeneration. Taken together these results demonstrate a role for taurine in facilitating STB renewal in normal pregnancy and provide evidence that the reduction in TauT activity in PE could contribute to the dysregulated STB turnover that is evident in this disease.
In the long term, further understanding of the regulatory mechanisms of TauT in placenta and how taurine mediates its intracellular effects are necessary. In mouse neuronal progenitor cells, DNA microarray analysis demonstrated taurine induced changes in transcription of genes involved in proliferation, mitochondrial functions, cell adhesion and cell death (Ramos-Mandujano et al., 2014). However microarray analysis to give information on intracellular signalling of taurine in placenta has yet not been performed. Fully understanding the role of taurine in placental development and function and fetal growth in PE will be important in identifying novel diagnostic tools and treatments to improve the wellbeing of mother and baby.

6.9 Future Directions

Suggestions for future work into further investigating the relationship between taurine transport and PE in the placenta are as follows:

- Collection of maternal plasma/urine, fetal cord blood, and placental villous tissue from women with normal pregnancy, those experiencing PE, and obese women to measure the concentration of taurine. This would determine (a) substrate availability of taurine in women with PE or obese women (b) whether there is decreased delivery of taurine to the fetus in PE or maternal obesity and (c) whether placenta intracellular taurine is decreased in PE or maternal obesity.

- Using tandem mass spectrometry following immunoprecipitation of TauT from MVM isolations from normal pregnancy and PE to investigate whether there is an increased proportion of nitrated TauT in PE compared to normal pregnancy. This technique could also offer information on other potential regulators of TauT in the placenta including phosphorylation and glycosylation.

- Using placental villous fragments to determine if a reduction in TauT activity/intracellular taurine is associated with an increased susceptibility to inflammatory cytokines (e.g. TNF-α).

- Using microarray analysis to identify changes in genes involved in proliferation, apoptosis differentiation and mitochondrial function to investigate intracellular signalling pathways of taurine in placenta.

- Using the placental perfusion model to investigate taurine efflux across the BM in normal pregnancy and to investigate differences in taurine efflux in PE.
Figure 6.1: Proposed causes and consequences of altered placental taurine uptake in pre-eclampsia

It is proposed that syncytiotrophoblast (STB) TauT activity is reduced by post translational modification of transporters by nitration in PE compared to normal pregnancy. Reduced TauT activity at the STB leads to reduced intracellular taurine which (a) leads to disrupted STB turnover (b) reduced cytoprotection and (c) reduced delivery of taurine to the fetus.

? = information is unknown and requires further investigation. Points highlighted in red indicate findings demonstrated in the present study.
References


CESDI: Confidential enquiry into stillbirths and deaths in infancy. 5th Annual report. Maternal and child health consortium, 1998.


190


Appendix: Knockdown of TauT in placental villous explants using siRNA

1. Optimisation of methodology

To test the hypothesis that reducing intracellular taurine will compromise the renewal of STB, a protocol was evaluated to knock-down the expression of TauT in placental villous explants with TauT-specific siRNA.

Previous work in this laboratory using fluorescently labelled non-targetting siRNA has demonstrated that STB spontaneously takes up siRNA without the need for a transfection reagent. Placental villous explants were cultured for 7 days in 1.5 mls explant culture medium (see Chapter 2, section 2.6.1). In initial optimisation experiments, 50 nM and 100 nM of siGLO transfection indicator (GE healthcare Dharmacon, UK) was added to the culture medium to determine which concentration was most successful in transfection. To determine the optimum time for transfection, explants were treated with siGLO transfection indicator on day 2, day 3, day 5, day 2 + 5 and day 3 + 5 of explant culture. Day 5 was chosen as a treatment day to match the protocol for treatment of explants with RNS/ROS (Chapter 4) and days 2 and 3 were chosen as alternative treatment days to investigate whether transfection was more efficient during regeneration STB before its completion (by day 5) (Simán et al., 2001). Treatment with siGLO on days 2 + 5 and days 3 + 5 were chosen as a combination strategy to assess whether transfection was more efficient before and after shedding occurred. On day 7 placental explants were harvested and placed in optimal cutting temperature (OCT) and frozen overnight at -20°C before being stored at -80°C prior to sectioning. Placental explants were sectioned using a CM 1850 cryostat (Leica Biosystems, UK) and fixed onto slides with methanol. Immunofluorescence was observed using an Axio Observer.Z1 microscope (Carl Zeiss Ltd, UK) and images photographed using an Axiocam MR microscope camera (Carl Zeiss, Ltd, UK).

Following assessment of the siGLO experiments, 100 nM of TauT specific siRNA (Qiagen, West Sussex, UK) was added to 1.5 mls of culture medium and TauT activity (over 90 minutes) measured at day 7 as described in Chapter 2, section 2.3. This TauT specific siRNA sequence has been demonstrated to effectively knock down TauT mRNA, protein and activity in isolated primary CTBs [Desforges et, al 2013]. To determine the optimal time for transfection, explants were treated with TauT-specific siRNA for 48 hours on day 3 (culture
medium changed 48 hours after treatment and all subsequent days of culture), day 3 + day 5 (culture medium changed on day 5) or from day 5 of culture (culture medium not changed on the 2 remaining days after treatment). Two non-targeting siRNA sequences (Invitrogen, Life Technologies, Paisley, UK and Ambion, Life Technologies, UK) were included as negative controls.

2. Results

2.1 Transfection of placental villous explants with siGLO transfection indicator

Figure 1 and 2 show fluorescence images for placental explants treated with 50 nM and 100 nM siGLO on day 2, day 3, day 5 (Figure 1) and day 2 + 5 and day 3 + 5 (Figure 2). siGLO (red fluorescence) transfection is mainly localised to the STB with some faint punctate fluorescence in the stroma. Nuclei are stained with 4',6-diamidino-2-phenylindole (DAPI; blue fluorescence). Shed STB are indicated by the white arrows and punctate fluorescence of the underlying lying layer can be seen in Figure 1 D3 (indicated by a white dot). Intact STB (identified by yellow arrows) shows strong fluorescence.

Fluorescence appears more intense in placental villous explants with siGLO added on day 3 compared to addition on day 2 (Figure 1). Day 5 shows successful transfection with consistent red fluorescence throughout the STB and faint fluorescence in the stroma (Figure 1). Addition of siGLO on day 3 + 5 is more consistent and intense compared to day 2 + 5 (Figure 2). Therefore, day 3, day 5 and day 3 + 5 were chosen as the siRNA treatment days. Fluorescence is similar between 50 nM and 100 nM siGLO in most images. However, Day 2 + 5 (Figure 2) demonstrates one instance where fluorescence intensity was greater following addition of 100 compared to 50 nM siGLO and accordingly siRNA experiments were conducted with 100 nM siRNA.
Figure 1: siGLO fluorescence images for placental villous explants

Representative images for treatment of placental villous explants with 50 nM (left hand side) or 100 nM (right hand side) siGLO transfection indicator (red fluorescence) on day 2 (D2), day 3 (D3) and day 5 (D5) of culture. White arrows indicate shed STB and white dot indicates punctuate staining in the underlying layer. Yellow arrows indicate intact STB. Negative is shown in Figure 7.1 b.
Figure 2: siGLO fluorescence images for placental villous explants

Representative images for treatment of placental villous explants with 50 nM (left hand side) or 100 nM (right hand side) siGLO transfection indicator on day 2 + day 5 (D2 + 5) and day 3 + day 5 (D3 + 5) of culture. White arrows indicate shed STB and yellow arrows indicate intact STB. Negative=untransfected control.
2.2 siRNA knockdown of TauT in placental villous explants

Figure 3 shows $^3$H-taurine uptake on day 7 for placental explants treated for 48 hours on day 3, day 5 or day 3 + 5 with 100nM TauT siRNA, and non-targeting (NT) siRNA, as a percent of their matched untransfected controls in two experiments. Table 1 summarises the effects of TauT specific siRNA and NT siRNA and on TauT activity. TauT specific siRNA had inconsistent effects on TauT activity both within and between explant cultures. Furthermore, treatment with two non-targeting siRNA sequences as negative controls, markedly and variably, altered taurine uptake.

<table>
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<tr>
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<th>Experiment 1</th>
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<th>Experiment 2</th>
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<tbody>
<tr>
<td></td>
<td>Ambion NT siRNA</td>
<td>Invitrogen NT siRNA</td>
<td>TauT specific siRNA</td>
<td>Ambion NT siRNA</td>
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<tr>
<td>Day 3</td>
<td>↓ 7%</td>
<td>↓ 13%</td>
<td>↓ 3%</td>
<td>↑ 57%</td>
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<tr>
<td>Day 5</td>
<td>-</td>
<td>↓ 8%</td>
<td>↓ 7%</td>
<td>-</td>
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<tr>
<td>Day 3 + 5</td>
<td>↓ 16%</td>
<td>↓ 9%</td>
<td>↑ 8%</td>
<td>↑ 61%</td>
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Table 1: Summary of changes in TauT activity following treatment of placental villous explants with siRNA or non-targeting (NT) siRNA

Changes in TauT activity expressed as a percent of matched un-transfected controls following treatment of placental villous explants on day 3, day 5 or day 3 + 5 of culture with 100 nM siRNA or NT-siRNA in two pilot experiments.
Figure 3: Effect of TauT specific siRNA and non-targeting siRNA on taurine uptake into placental villous explants

Effect of 100 nM TauT siRNA and non-targeting (NT) siRNA applied for 48hr on day 3, day 5 or day 3 + 5 of explant culture on taurine uptake (% of corresponding untreated control). Blue and black symbols represent two different explant cultures.