

# **Angiogenesis Regulating Gene Polymorphisms in Adverse Pregnancy Outcomes**

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**Thesis submitted to the degree of  
Doctor of Philosophy**

**The Discipline of Obstetrics and Gynaecology  
Research Centre for Reproductive Health  
University of Adelaide  
Australia**

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# Abstract

**Introduction:** Both placental vascular defects and a genetic contribution are documented in pregnancies complicated by preeclampsia, small-for-gestational-age infants (SGA) and spontaneous preterm birth (sPTB). Our primary aim was to investigate the association between polymorphisms in genes regulating placental vascular integrity including vascular endothelial growth factor (*VEGFA*), placenta growth factor (*PGF*), kinase insert domain receptor (*KDR*), fms-like tyrosine kinase 1 receptor (*FLT1*), angiopoietin 1 (*ANGPT1*) and thrombospondin 1 (*TSP1*) and these pregnancy complications in a Caucasian cohort. The secondary aims were to investigate the association between these polymorphisms and (1) preeclampsia in Sri Lankan women (2) first trimester placental gene expression (3) abnormal uterine and umbilical artery Doppler (4) environment and lifestyle interactions that modify the risk of pregnancy complications and to (5) compare term placental angiogenic gene mRNA expression in complicated pregnancy with uncomplicated pregnancy.

**Methods:** Nulliparous pregnant women, their partners and infants (3234 trios) were recruited to a prospective multicenter cohort study (SCOPE study) in Adelaide, Australia and Auckland, New Zealand. Pregnancy outcomes were classified using international guidelines. Uterine and umbilical artery Doppler was performed at 20 weeks gestation. Mean uterine or umbilical artery resistance indices (RI) above the 90<sup>th</sup> percentile or the presence of bilateral notching of the uterine artery waveform were considered abnormal. A second Sri Lankan cohort comprised 175 nulliparous preeclamptic women and 171 matched controls. The polymorphisms in the Caucasian parent-infant trios, Sri Lankan women and first trimester placental tissue from elective pregnancy terminations (n = 74) were genotyped using the Sequenom Mass ARRAY system. Term placentae were collected from women with preeclampsia (n = 18), gestational hypertension (n = 15), normotensive SGA infants (n = 13), spontaneous

preterm birth (n = 10) and uncomplicated pregnancy (n = 30). Placental mRNA expression was analysed by quantitative RT-PCR.

**Results:** In the Caucasian cohort, maternal *ANGPT1 1414T/A* and paternal and infant *KDR -604T/C* polymorphisms were associated with preeclampsia; maternal *ANGPT1 1414T/A*, paternal and infant *KDR -604T/C*, paternal and infant *TSP1 2210A/G* and infant *VEGFA+936C/T* were associated with SGA. In the Sri Lankan cohort, *PGF 642C/A* was associated with preeclampsia. The *ANGPT1 1414T/A* was associated with abnormal uterine Doppler and the *VEGFA +936C/T* was associated with abnormal uterine and umbilical artery Doppler and reduced first trimester placental *VEGFA* mRNA expression suggesting that these polymorphisms may have a role in the pathogenesis of pregnancy complications. We also found that the maternal *ANGPT1 1414T/A* and *VEGFA -2578C/A* polymorphisms interact with maternal BMI to modify the risk of sPTB and that the maternal *KDR -604T/C* interacts with smoking to influence the risk of preeclampsia and SGA. In all these polymorphisms, genotypes associated with pro-angiogenic phenotypes reduced the risk and genotypes associated with anti-angiogenic phenotypes increased the risk of pregnancy complications. We were also able to demonstrate that term placental expression of *VEGFA*, *PGF*, *KDR* and *FLT1* mRNA were reduced in pregnancy complications compared to uncomplicated pregnancy.

**Conclusion:** This project demonstrates that inherited susceptibility to altered angiogenic gene expression in the placenta contributes to the risk of pregnancy complications.

# Declaration

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution to Prabha Andraweera and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

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2. PH Andraweera, GA Dekker, SD Thompson, LME McCowan, RA North, CT Roberts. A functional variant in the thrombospondin-1 gene and the risk of small for gestational age infants. *Journal of Thrombosis and Haemostasis* 2011;9:2221-8 – copyright resides with the International Society on Thrombosis and Haemostasis (ISTH)
3. PH Andraweera, GA Dekker, SD Thompson, LME McCowan, RA North, CT Roberts. A functional variant in *ANGPT1* and the risk of pregnancies with hypertensive disorders and small for gestational age infants. *Molecular Human*

*Reproduction*, 2011, doi: 10.1093/molehr/gar081- copyright resides with the European Society of Human Reproduction and Embryology

4. **PH Andraweera**, GA Dekker, SD Thompson, CT Roberts. A Single nucleotide polymorphisms in the KDR gene in pregnancies complicated by gestational hypertensive disorders and small for gestational age infants. *Reproductive Sciences*, 2012, doi: 10.1177/1933719111428520 – copyright resides with the Society for Gynecologic Investigation
5. **PH Andraweera**, GA Dekker, CT Roberts. Placental expression of VEGF family mRNA in adverse pregnancy outcomes. *Placenta*, In Press - copyright resides with Elsevier

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There are no conflicts of interest to declare for myself or my supervisors. This study was funded by the Premier's Science and Research Fund, Government of South Australia in Australia, the Foundation for Research Science and Technology, Health Research Council and Auckland District Health Board Charitable Trust in New Zealand, the National Health and Medical Research Council Australia and the Channel 7 Children's Research Foundation.

Prabha Andraweera

March 2012

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given in pursuing Doctoral studies. I would also like to thank Professor Vajira Dissanayake from the Department of Anatomy and Human Genetics of the Faculty of Medicine, University of Colombo, Sri Lanka for the help given in the Sri Lankan component of the study, for generously offering the Sri Lankan samples for the project and for the feedback on manuscript 7.

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Last but not least, I would like to thank my family for all the support, encouragement and love during my candidature, a special thank you to my parents and my brother for all the long distance calls and the encouraging words, to my husband Nalinda for the understanding and love and a very special thank you to my daughter Oshana for being such a great kid and helping me through all the lengthy periods of writing.

## Publications arising from this thesis

1. **PH Andraweera**, GA Dekker, SD Thompson, RC Nowak, VJ Zhang, LME McCowan, RA North, CT Roberts. Association of vascular endothelial growth factor +936 C/T single nucleotide polymorphism with pregnancies complicated by small for gestational age babies. *Archives of Pediatrics & Adolescent Medicine*, 2011;165(12):1123-1130 (IF 4.0)
2. **PH Andraweera**, GA Dekker, SD Thompson, LME McCowan, RA North, CT Roberts. A functional variant in the thrombospondin-1 gene and the risk of small for gestational age infants. *Journal of Thrombosis and Haemostasis* 2011;9:2221-8 (IF 5.4)
3. **PH Andraweera**, GA Dekker, SD Thompson, LME McCowan, RA North, CT Roberts. A functional variant in *ANGPT1* and the risk of pregnancies with hypertensive disorders and small for gestational age infants. *Molecular Human Reproduction*, 2011, Published Online; doi: 10.1093/molehr/gar081 (IF 3.5)
4. **PH Andraweera**, GA Dekker, SD Thompson, CT Roberts. A Single nucleotide polymorphisms in the KDR gene in pregnancies complicated by gestational hypertensive disorders and small for gestational age infants. *Reproductive Sciences*, 2012, Published online; doi: 10.1177/1933719111428520 (IF 2.6)
5. **PH Andraweera**, GA Dekker, CT Roberts. Placental expression of VEGF family mRNA in adverse pregnancy outcomes. *Placenta*, In Press (IF 2.9)

6. **PH Andraweera**, GA Dekker, CT Roberts. The Vascular endothelial growth factor family in adverse pregnancy outcomes. *Human Reproduction Update*, In Press (IF 8.7)
  
7. **PH Andraweera**, GA Dekker, SD Thompson, LME McCowan, RA North, CT Roberts. Interaction between maternal BMI and angiogenesis regulating gene polymorphisms associates with the risk of spontaneous preterm birth, Revision submitted to *Molecular Human Reproduction* – Manuscript ID – MHR-12-0007-R1
  
8. **PH Andraweera**, GA Dekker, VHW Dissanayake, TB Miotto, RW Jayasekara, CT Roberts. Vascular endothelial growth factor family gene polymorphisms in preeclampsia in Sinhalese women in Sri-Lanka. Under review by the *Journal of Maternal-Fetal and Neonatal Medicine* – Manuscript ID - DJMF-2012-0029

# Conference presentations and abstract publications arising from this thesis

## 2011

1. **PH Andraweera**, GA Dekker, SD Thompson, RA North, LME McCowan, CT Roberts. Single nucleotide polymorphisms in angiogenesis regulating genes and the risk of preeclampsia and small for gestational age birth. *12<sup>th</sup> International Congress of Human Genetics, October 2011, Montreal, Canada.*
2. **PH Andraweera**, GA Dekker, SD Thompson, RA North, LME McCowan, CT Roberts. Single nucleotide polymorphisms in angiogenesis regulating in preeclampsia and small for gestational age infants. *Postgraduate Research Conference, August 2011, University of Adelaide, Adelaide, Australia.*  
*“Awarded the best poster award”*
3. **PH Andraweera**, GA Dekker, SD Thompson, RA North, LME McCowan, CT Roberts. Single nucleotide polymorphisms in angiogenesis regulating genes and the risk of preeclampsia and small for gestational age birth: evidence from parent-infant trios, *ASMR June 2011, Adelaide, Australia*

## 2010

4. **PH Andraweera**, G.A. Dekker, S.D. Thompson, V.J. Zhang, C.T. Roberts. Association of maternal, paternal and fetal single nucleotide polymorphisms in vascular endothelial growth factor family genes with preeclampsia. *17<sup>th</sup> World Congress of the Society for the Study of Hypertension in Pregnancy, October 2010, Melbourne, Australia, Abstracted in Hypertension in Pregnancy 2010*

5. **PH Andraweera**, G.A. Dekker, S.D. Thompson, L.M.E. McCowan, R.A. North, C.T. Roberts. Association of single nucleotide polymorphism in the vascular endothelial growth factor gene with small for gestational age birth. *17<sup>th</sup> World Congress of the Society for the Study of Hypertension in Pregnancy, October 2010, Melbourne, Australia, Abstracted in Hypertension in Pregnancy 2010*
6. **PH Andraweera**, R.C. Nowak, S.D. Thompson, V.H.W. Dissanayake, R.W. Jayasekara, G.A. Dekker, C.T. Roberts. Association of single nucleotide polymorphisms in cytokine genes with preeclampsia in a Sinhalese population from Sri-Lanka. *17<sup>th</sup> World Congress of the Society for the Study of Hypertension in Pregnancy, October 2010, Melbourne, Australia, Abstracted in Hypertension in Pregnancy 2010*
7. **PH Andraweera**, S.D. Thompson, V.H.W. Dissanayake, R.W. Jayasekara, G.A. Dekker, C.T. Roberts. Insulin like growth factor family genes and the risk of preeclampsia in a Sinhalese population from Sri-Lanka. *17<sup>th</sup> World Congress of the Society for the Study of Hypertension in Pregnancy, October 2010, Melbourne, Australia, Abstracted in Hypertension in Pregnancy 2010*
8. GA Dekker, CT Roberts, DL Furness, **PH Andraweera**, Paternal Factors Involved in the Causation of Preeclampsia, *17<sup>th</sup> World Congress of the Society for the Study of Hypertension in Pregnancy, October 2010, Melbourne, Australia, Abstracted in Hypertension in Pregnancy 2010*

9. **PH Andraweera**, R.C. Nowak, S.D. Thompson, D.L. Furness, V.H.W. Dissanayake, R.W. Jayasekara, G.A. Dekker, C.T. Roberts. Single nucleotide polymorphisms in genes modulating inflammation and the risk of preeclampsia in a Sinhalese population from Sri-Lanka. *11<sup>th</sup> International Congress of Reproductive Immunology, August 2010, Cairns, Australia, Abstracted in the Journal of Reproductive Immunology 2010*
  
10. **P.H. Andraweera**, D.L. Furness , R.C. Nowak, S.D. Thompson, V.H.W. Dissanayake, R.W. Jayasekara, G.A. Dekker, C.T. Roberts. Single nucleotide polymorphisms in genes regulating inflammation and the risk of Recurrent Pregnancy Loss in a Sinhalese population from Sri-Lanka. *11<sup>th</sup> International Congress of Reproductive Immunology, August 2010, Cairns, Australia, Abstracted in the Journal of Reproductive Immunology 2010*
  
11. **PH Andraweera**, G.A. Dekker, S.D. Thompson , L.M.E. McCowan, R.A. North, C.T. Roberts. Vascular endothelial growth factor gene polymorphisms in placental impairment and small for gestational age birth. *Annual Scientific Conference of the Society for Reproductive Biology, August 2010, Sydney, Australia. "ANZPRA new Investigator Award finalist"*
  
12. **PH Andraweera**, S.D. Thompson, V.H.W. Dissanayake, R.W. Jayasekara, G.A. Dekker, C.T. Roberts. Hypoxia-Inducible factor-1 $\alpha$  gene polymorphisms and the risk of preeclampsia in a Sinhalese population from Sri-Lanka, *Annual Scientific Conference of the Endocrine Society of Australia, August 2010, Sydney, Australia*

13. **PH Andraweera**, G.A. Dekker, S.D. Thompson, L.M.E. McCowan, R.A. North, C.T. Roberts. Single nucleotide polymorphisms in the vascular endothelial growth factor gene and the risk of small for gestational age birth, *ASMR June 2010, Adelaide, Australia*
14. **PH Andraweera**, G.A. Dekker, S.D. Thompson, V.J. Zhang, C.T. Roberts. Maternal, paternal and fetal single nucleotide polymorphisms in vascular endothelial growth factor family genes associate with preeclampsia. *Annual Scientific Sessions of the Society for Maternal and Fetal Medicine, February 2010, Chicago, USA, Abstracted in American Journal of Obstetrics and Gynaecology 2010*

## **2009**

15. **PH Andraweera**, SD Thompson, RC Nowak, VJ Zhang, GA Dekker, CT Roberts. Single nucleotide polymorphisms in angiogenesis regulating genes are associated with pregnancy complications. *Annual Conference of the International Federation of Placental Associations (IFPA), October 2009, Adelaide, Australia, Abstracted in Placenta 2009*
16. **PH Andraweera**, SD Thompson, RC Nowak, VJ Zhang, GA Dekker, CT Roberts, Paternal and fetal single nucleotide polymorphisms in KDR gene associate with preeclampsia and intrauterine growth restriction, *Annual Scientific Conference of the Society for Reproductive Biology, August 2009, Adelaide, Australia*

17. **PH Andraweera**, SD Thompson, RC Nowak, VJ Zhang, GA Dekker, CT Roberts. Angiogenesis regulating gene polymorphisms in pregnancy complications. *Proceedings of the Annual Scientific Sessions of the Australian Society for Medical Research*. June 2009, Adelaide, Australia

# **Awards received for presentations arising from this thesis**

## **Conference Awards**

1. **Best Poster Award.** Awarded at the Research Day 2011, Robinson Institute, University of Adelaide, Australia, November 2011
2. **Best Poster Award.** Awarded at the Postgraduate Research Conference, Faculty of Health Sciences, University of Adelaide, August 2011
3. **Frederick P Zuspan Award.** This was awarded for the most outstanding Basic science work submitted to the 12<sup>th</sup> world congress of the International Society for the Study of Hypertension in Pregnancy, October 2010, Melbourne, Australia
4. **ISSHP Young Investigator Award.** This was awarded for excellence in abstracts submitted to the 12<sup>th</sup> world congress of the International Society for the Study of Hypertension in Pregnancy, October 2010, Melbourne, Australia

## **Travel Awards**

1. Healthy Development Adelaide (HDA) Postgraduate Travel Award 2011
2. Faculty of Health Sciences, University of Adelaide, Postgraduate Travelling Fellowship 2011
3. Research Centre for Reproductive Health, University of Adelaide, Travel Awards 2011, 2010 and 2009
4. Society for Reproductive Biology, Postgraduate Student Travel Award 2010
5. ANZPRA New Investigator Travel Award 2009. This was awarded by the Australian and New Zealand Placental Research Association

## Contribution made by the candidate

### Statement of Authorship: Prabha H Andraweera

I, Prabha H Andraweera declare that I am the principal author of the following manuscripts.

1. **PH Andraweera**, GA Dekker, SD Thompson, RC Nowak, VJ Zhang, LME McCowan, RA North, CT Roberts. Association of vascular endothelial growth factor +936 C/T single nucleotide polymorphism with pregnancies complicated by small for gestational age babies. *Archives of Pediatrics & Adolescent Medicine*, 2011;165(12):1123-1130
2. **PH Andraweera**, GA Dekker, SD Thompson, LME McCowan, RA North, CT Roberts. A functional variant in the thrombospondin-1 gene and the risk of small for gestational age infants. *Journal of Thrombosis and Haemostasis* 2011;9:2221-8
3. **PH Andraweera**, GA Dekker, SD Thompson, LME McCowan, RA North, CT Roberts. A functional variant in *ANGPT1* and the risk of pregnancies with hypertensive disorders and small for gestational age infants. *Mol Hum Reprod*;doi: 10.1093/molehr/gar081
4. **PH Andraweera**, GA Dekker, SD Thompson, CT Roberts. A Single nucleotide polymorphisms in the *KDR* gene in pregnancies complicated by gestational hypertensive disorders and small for gestational age infants. *Reproductive Sciences*, 2012, doi: 10.1177/1933719111428520
5. **PH Andraweera**, GA Dekker, JA Laurence, CT Roberts. Placental expression of VEGF family mRNA in adverse pregnancy outcomes. *Placenta*, In Press

6. **PH Andraweera**, GA Dekker, CT Roberts. The Vascular endothelial growth factor family in adverse pregnancy outcomes. *Human Reproduction Update* – In Press
7. **PH Andraweera**, GA Dekker, SD Thompson, LME McCowan, RA North, CT Roberts. Interaction between maternal BMI and angiogenesis regulating gene polymorphisms associates with the risk of spontaneous preterm birth. Revision submitted to *Molecular Human Reproduction*
8. **PH Andraweera**, GA Dekker, VHW Dissanayake, T Bianco-Miotto, RW Jayasekara, CT Roberts. Vascular endothelial growth factor family gene polymorphisms in preeclampsia in Sinhalese women in Sri Lanka. Under review by the *Journal of Maternal-Fetal and Neonatal Medicine*

I declare that I contributed to the design of the SCOPE candidate gene association study and the Sri Lankan candidate gene association study, performed the laboratory experiments for the evaluation of the first trimester and term placental gene expression, conducted the statistical analyses of the data and wrote the manuscripts. I declare that I have no conflicts of interest.

Prabha H Andraweera

# Contributions made by co-authors

## Statement of Authorship: Claire T Roberts

I, Claire T Roberts declare that I am a co-author of the following manuscripts.

1. PH Andraweera, GA Dekker, SD Thompson, RC Nowak, VJ Zhang, LME McCowan, RA North, **CT Roberts**. Association of vascular endothelial growth factor +936 *C/T* single nucleotide polymorphism with pregnancies complicated by small for gestational age babies. *Archives of Pediatrics & Adolescent Medicine*, 2011;165(12):1123-1130
2. PH Andraweera, GA Dekker, SD Thompson, LME McCowan, RA North, **CT Roberts**. A functional variant in the thrombospondin-1 gene and the risk of small for gestational age infants. *Journal of Thrombosis and Haemostasis* 2011;9:2221-8
3. PH Andraweera, GA Dekker, SD Thompson, LME McCowan, RA North, **CT Roberts**. A functional variant in *ANGPT1* and the risk of pregnancies with hypertensive disorders and small for gestational age infants. *Mol Hum Reprod*;doi: 10.1093/molehr/gar081
4. PH Andraweera, GA Dekker, SD Thompson, **CT Roberts**. A Single nucleotide polymorphisms in the *KDR* gene in pregnancies complicated by gestational hypertensive disorders and small for gestational age infants. *Reproductive Sciences*, 2012, doi: 10.1177/1933719111428520
5. PH Andraweera, GA Dekker, JA Laurence, **CT Roberts**. Placental expression of VEGF family mRNA in adverse pregnancy outcomes. *Placenta*, In Press
6. PH Andraweera, GA Dekker, **CT Roberts**. The Vascular endothelial growth factor family in adverse pregnancy outcomes. *Human Reproduction Update* – In Press

7. PH Andraweera, GA Dekker, SD Thompson, LME McCowan, RA North, **CT Roberts**. Interaction between maternal BMI and angiogenesis regulating gene polymorphisms associates with the risk of spontaneous preterm birth. Revision submitted to *Molecular Human Reproduction*
8. PH Andraweera, GA Dekker, VHW Dissanayake, T Bianco-Miotto, RW Jayasekara, **CT Roberts**. Vascular endothelial growth factor family gene polymorphisms in preeclampsia in Sinhalese women in Sri Lanka. Under review by the *Journal of Maternal-Fetal and Neonatal Medicine*

I wish to attest the contribution made by Prabha Andraweera. I declare that I contributed to the design and supervision of the SCOPE clinical trial and the candidate gene association study and critically reviewed these manuscripts for important intellectual content. I declare that I have no conflicts of interest.

I give consent for these manuscripts to be included in the thesis submitted to the degree of Doctor of Philosophy by Prabha Andraweera.

Claire T Roberts

## Statement of Authorship: Gustaaf A Dekker

I, Gustaaf A Dekker declare that I am a co-author of the following manuscripts.

1. PH Andraweera, **GA Dekker**, SD Thompson, RC Nowak, VJ Zhang, LME McCowan, RA North, CT Roberts. Association of vascular endothelial growth factor +936 C/T single nucleotide polymorphism with pregnancies complicated by small for gestational age babies. *Archives of Pediatrics & Adolescent Medicine*, 2011;165(12):1123-1130
2. PH Andraweera, **GA Dekker**, SD Thompson, LME McCowan, RA North, CT Roberts. A functional variant in the thrombospondin-1 gene and the risk of small for gestational age infants. *Journal of Thrombosis and Haemostasis* 2011;9:2221-8
3. PH Andraweera, **GA Dekker**, SD Thompson, LME McCowan, RA North, CT Roberts. A functional variant in *ANGPT1* and the risk of pregnancies with hypertensive disorders and small for gestational age infants. *Mol Hum Reprod*;doi: 10.1093/molehr/gar081
4. PH Andraweera, **GA Dekker**, SD Thompson, CT Roberts. A Single nucleotide polymorphisms in the *KDR* gene in pregnancies complicated by gestational hypertensive disorders and small for gestational age infants. *Reproductive Sciences*, 2012, doi: 10.1177/1933719111428520
5. PH Andraweera, **GA Dekker**, JA Laurence, CT Roberts. Placental expression of VEGF family mRNA in adverse pregnancy outcomes. *Placenta*, In Press
6. PH Andraweera, **GA Dekker**, CT Roberts. The Vascular endothelial growth factor family in adverse pregnancy outcomes. *Human Reproduction Update* - In Press

7. PH Andraweera, **GA Dekker**, SD Thompson, LME McCowan, RA North, CT Roberts. Interaction between maternal BMI and angiogenesis regulating gene polymorphisms associates with the risk of spontaneous preterm birth. Revision submitted to *Molecular Human Reproduction*
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I give consent for these manuscripts to be included in the thesis submitted to the degree of Doctor of Philosophy by Prabha Andraweera.

Gustaaf A Dekker

## Statement of Authorship: Lesley ME McCowan

I, Lesley ME McCowan declare that I am a co-author of the following manuscripts.

1. Andraweera PH, Dekker GA, Thompson SD, North RA, **McCowan LME** and Roberts CT. A functional variant in the thrombospondin-1 gene and the risk of small for gestational age infants. *J Thromb Haemost.* 2011;9(11):2221-8.
2. Andraweera PH, Dekker GA, Thompson SD, Nowak RC, Zhang JV, **McCowan LME**, North RA and Roberts CT. Association of Vascular Endothelial Growth Factor +936 C/T Single-Nucleotide Polymorphism With Pregnancies Complicated by Small-for-Gestational-Age Babies. *Arch Pediatr Adolesc Med.* 2011;165(12):1123-30..
3. Andraweera PH, Dekker GA, Thompson SD, North RA, **McCowan LME** and Roberts CT. A functional variant in *ANGPT1* and the risk of pregnancies with hypertensive disorders and small for gestational age infants. *Mol Hum Reprod.* 2011; doi: 10.1093/molehr/gar081.
4. Andraweera PH, Dekker GA, Thompson SD, **McCowan LME**, North RA, Roberts CT. Interaction between maternal BMI and angiogenesis regulating gene polymorphisms associates with the risk of spontaneous preterm birth. Revision submitted to *Molecular Human Reproduction*

I wish to attest the contribution made by Prabha Andraweera. I declare that I contributed to the design and supervision of the SCOPE clinical trial and critically reviewed these manuscripts for important intellectual content. I declare that I have no conflicts of interest. I give consent for these manuscripts to be included in the thesis submitted to the degree of Doctor of Philosophy by Prabha Andraweera.

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2. Andraweera PH, Dekker GA, Thompson SD, Nowak RC, Zhang JV, McCowan LM, **North RA** and Roberts CT. Association of Vascular Endothelial Growth Factor +936 C/T Single-Nucleotide Polymorphism With Pregnancies Complicated by Small-for-Gestational-Age Babies. *Arch Pediatr Adolesc Med.* 2011;165(12):1123-30.
3. Andraweera PH, Dekker GA, Thompson SD, **North RA**, McCowan LM and Roberts CT. A functional variant in *ANGPT1* and the risk of pregnancies with hypertensive disorders and small for gestational age infants. *Mol Hum Reprod.* 2011; doi: 10.1093/molehr/gar081.
4. Andraweera PH, Dekker GA, Thompson SD, McCowan LME, **North RA**, Roberts CT. Interaction between maternal BMI and angiogenesis regulating gene polymorphisms associates with the risk of spontaneous preterm birth. Revision submitted to *Molecular Human Reproduction*

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2. PH Andraweera, GA Dekker, **SD Thompson**, LME McCowan, RA North, CT Roberts. A functional variant in the thrombospondin-1 gene and the risk of small for gestational age infants. *Journal of Thrombosis and Haemostasis* 2011;9:2221-8
3. PH Andraweera, GA Dekker, **SD Thompson**, LME McCowan, RA North, CT Roberts. A functional variant in *ANGPT1* and the risk of pregnancies with hypertensive disorders and small for gestational age infants. *Mol Hum Reprod*;doi: 10.1093/molehr/gar081
4. PH Andraweera, GA Dekker, **SD Thompson**, CT Roberts. A Single nucleotide polymorphisms in the *KDR* gene in pregnancies complicated by gestational hypertensive disorders and small for gestational age infants. *Reproductive Sciences*, 2012, doi: 10.1177/1933719111428520
5. PH Andraweera, GA Dekker, **SD Thompson**, LME McCowan, RA North, CT Roberts. Interaction between maternal BMI and angiogenesis regulating gene polymorphisms associates with the risk of spontaneous preterm birth. Revision submitted to *Molecular Human Reproduction*

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I wish to attest the contribution made by Prabha Andraweera. I declare that I contributed to the RNA extraction from the term placentae. I declare that I have no conflicts of interest.

I give consent for this manuscript to be included in the thesis submitted to the degree of Doctor of Philosophy by Prabha Andraweera.

Jessica A Laurence

## **Thesis explanation**

This thesis is arranged as a portfolio of published/accepted or submitted manuscripts.

Manuscript 1 provides a comprehensive review of literature on the role of the vascular endothelial growth factor family of angiogenic growth factors and a brief overview of the role of the angiopoietin family and the thrombospondin family in normal and complicated pregnancies. This is followed by the hypotheses and aims of the project.

The work described in the thesis mainly arises from the Adelaide, Australia and Auckland, New Zealand arms of the SCOPE (Screening for Pregnancy Endpoints) study. The SCOPE study is an international, multicenter, prospective cohort study with the aim of developing screening tests to predict the risk of pregnancy complications namely preeclampsia, small for gestational age infants and spontaneous preterm birth. The SCOPE study is registered in the Australian and New Zealand clinical trial registry and the details are given below.

**Trial Registry Name:** Screening nulliparous women to identify the combinations of clinical risk factors and/or biomarkers required to predict preeclampsia, small for gestational age babies and spontaneous preterm birth.

**URL:** <https://www.anzctr.org.au>

**Registration number:** ACTRN12607000551493

Manuscripts 2 - 6 describe the associations of polymorphisms in candidate genes that regulate angiogenesis in the SCOPE cohort. As these papers are from the same study there is some repetition in the methods sections of the manuscripts.

Manuscript 7 describes the same polymorphisms in a case-control study population comprising preeclamptic women and matched controls from Sri Lanka.

Manuscript 8 describes the association of placental mRNA expression of the VEGF family in complicated compared to uncomplicated pregnancy. This study population is a randomly selected subset of those recruited to the SCOPE study in Adelaide.

The final section of the thesis comprises a general discussion based on the overall significance of the findings, the problems encountered and future directions of the work.

## Abbreviations

cDNA	Complementary dioxyribonucleic acid
CI	Confidence interval
DNA	Dioxyribonucleic acid
<i>FLT1</i>	Fms-like tyrosine kinase receptor 1 gene
FLT-1	Fms-like tyrosine kinase receptor 1 protein
<i>KDR</i>	Kinase-insert domain receptor gene
KDR	Kinase-insert domain receptor protein
OR	Odds ratio
PCR	Polymerase chain reaction
<i>PGF</i>	Placenta growth factor gene
PIGF	Placenta growth factor protein
RNA	Ribonucleic acid
SNP	Single nucleotide polymorphism
<i>TSP1</i>	Thrombospondin 1 gene
TSP-1	Thrombospondin 1 protein
<i>VEGFA</i>	Vascular endothelial growth factor gene
VEGF-A	Vascular endothelial growth factor protein

# Manuscript 1

## **The Vascular endothelial growth factor family in adverse pregnancy outcomes**

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### **Abstract**

**Background:** Preeclampsia, small for gestational age infants, preterm birth and recurrent miscarriage complicate a significant number of pregnancies. Vascular endothelial growth factor family of angiogenic growth factors are implicated in the pathophysiology of these complications. We aimed to elucidate the role of these angiogenic factors in placentation and evaluate the predictive value of these angiogenic proteins and genetic variations in the genes that encode them in pregnancy complications.

**Methods:** We performed a systematic search of PubMed, and retrieved original articles. Search terms were a combination of VEGF-A, PlGF, KDR, FLT-1, sFLT-1, preeclampsia, small for gestational age infants, preterm birth, recurrent miscarriage, placenta, prediction and polymorphisms.

**Results:** This review summarizes current knowledge on the role of the VEGF family in early placentation and the abnormalities in maternal plasma and placental expression of angiogenic proteins in adverse pregnancy outcomes compared to normal pregnancy. PlGF and sFLT-1 in combination with other clinical and biochemical markers in late

first or second trimester appear to predict early onset preeclampsia with a high sensitivity and specificity. These angiogenic proteins do not appear to have sufficient power to accurately predict late onset preeclampsia, small for gestational age pregnancies or preterm birth. Functional polymorphisms in these angiogenic genes are implicated in pregnancy complications but their contribution appears to be minor.

**Conclusions:** The VEGF family has an important role in normal and complicated pregnancy. The predictive value of the VEGF family as biomarkers appears to be limited to a subset of preeclampsia.

## **Introduction**

Preeclampsia, small for gestational age pregnancy, preterm birth and recurrent miscarriage together complicate approximately 17-29% of all pregnancies [1-4]. Early placentation defects including impaired trophoblast invasion, spiral artery remodelling and angiogenesis are implicated in the pathogenesis of these complications [5-9]. The vascular endothelial growth factor (VEGF) family of angiogenic growth factors are important molecules regulating early placental vascular changes. The key molecules VEGF-A, placenta growth factor (PlGF) and the receptors VEGF receptor 1 (fms-like-tyrosine kinase receptor 1, FLT-1) and VEGF receptor 2 (kinase insert domain receptor, KDR) are expressed in the human placenta throughout gestation. The VEGF family is known to regulate placental angiogenesis and maternal spiral artery remodelling [10, 11]. In addition to the two main membrane bound receptors, a splice variant of FLT-1 designated soluble FLT-1 (sFLT-1) is expressed in the placenta and is known to have potent anti-angiogenic properties. Soluble FLT-1 antagonises both VEGF-A and PlGF and induces symptoms of preeclampsia in animal models [12].

In humans, placental expression and maternal serum sFLT-1 are up-regulated and maternal serum free VEGF-A and PlGF are reduced in preeclamptic women compared to normotensive pregnant women. Consistent evidence exists that these changes are detected several weeks prior to the clinical onset of symptoms of preeclampsia [13]. Therefore, sFLT-1 and PlGF are considered as biomarkers in predicting preeclampsia early in pregnancy. Although these biomarkers in combination with other clinical and biochemical markers are demonstrated to have strong predictive values for early onset preeclampsia and severe preeclampsia, their value in predicting the more common late onset disease is questionable.

These biomarkers are also not restricted to preeclampsia, as several groups have shown their involvement in pregnancies complicated by small for gestational age infants and to a lesser extent in the pathophysiology of preterm birth and recurrent miscarriage.

This review examines the role of the VEGF family angiogenic growth factors in placentation and the implications of derangements in these molecules in the development of preeclampsia, pregnancies complicated by small for gestational age infants, preterm birth and recurrent miscarriage. We also evaluate the role of VEGF family proteins, as well as genetic variations in genes encoding them in predicting these pregnancy complications.

## **Methods**

An extensive online search of published articles on VEGF family in normal and complicated pregnancy was undertaken. We used the Pubmed database using combinations of the following search terms: pregnancy, vascular endothelial growth factor, placenta growth factor, FLT-1, KDR, sFLT-1, placenta, trophoblast invasion, spiral artery remodelling, angiogenesis, vascularisation, preeclampsia, small for gestational age infants, preterm birth, recurrent pregnancy loss, single nucleotide polymorphism, biomarker and prediction. The search was mainly focused on publications involving human experiments but experimental studies with animal or cell culture models were included where appropriate. In addition, references cited in the selected articles and reviews were searched. Only articles written in English were considered. The review was conducted according to the role of the VEGF family in the pathophysiology of pregnancy which was classified as trophoblast invasion and spiral artery remodelling, vascularisation and the role in pregnancy complications classified as preeclampsia, small for gestational age pregnancy, preterm birth and recurrent

pregnancy loss. Finally we reviewed articles on polymorphisms in VEGF family genes in pregnancy complications.

## **Results**

### **The biology of the VEGF family of angiogenic growth factors**

The VEGF family consists of VEGF-A, VEGF-B, VEGF-C, VEGF-D and their receptors VEGFR-1 also called fms-like tyrosine kinase-1 (FLT-1), VEGFR-2 also called kinase insert domain receptor (KDR) in humans and fetal liver kinase (Flk) in mice [14], VEGFR-3 (FLT-4) and the co-receptors neuropilin-1 (NRP-1) and neuropilin-2 (NRP-2).

### **VEGF-A**

The human *VEGFA* gene has been assigned to chromosome 6p12-p21.1 [15] and is organized as eight exons separated by seven introns [16]. Alternative exon splicing results in six different isoforms VEGF-A<sub>121</sub>, VEGF-A<sub>145</sub>, VEGF-A<sub>165</sub>, VEGF-A<sub>183</sub>, VEGF-A<sub>189</sub> and VEGF-A<sub>206</sub> having 121, 145, 165, 189 and 206 amino acids respectively. VEGF-A<sub>165</sub> is the predominant isoform and native VEGF-A closely resembles VEGF-A<sub>165</sub> [16]. VEGF-A binds with high affinity to two related receptor tyrosine kinases expressed on vascular endothelial cells [17, 18], FLT-1 and KDR [14]. In addition, VEGF-A also binds to neuropilin 1 (NRP-1) and NRP-2.

VEGF-A mediates many functions in endothelial cells. VEGF-A promotes angiogenesis, induces the growth of vascular endothelial cells [19], reduces apoptosis [20] mediated via KDR/Flk1 receptor through the PI3-kinase/Akt signal transduction pathway [21] and increases vascular permeability [22]. In addition, VEGF-A promotes vasodilatation via the endothelial derived nitric oxide pathway. Hypoxia is a potent stimulus for the expression of *VEGFA* mRNA and is mediated via hypoxia-inducible-

factor-1 $\alpha$  [23, 24]. In addition, several growth factors including fibroblast growth factor, transforming growth factor (TGF- $\alpha$  and TGF- $\beta$ ), keratinocyte growth factor, insulin-like growth factor 1 (IGF-1), platelet derived growth factor (PDGF) and the inflammatory cytokines (interleukin-1 $\alpha$  and interleukin-6 are also known to up-regulate *VEGFA* expression [19, 25]

### **Placenta Growth Factor (PlGF)**

Placenta Growth Factor (PlGF) demonstrates 42% amino acid sequence identity with VEGF-A [26]. Placental growth factor gene (*PGF*) has been assigned to human chromosome 14 and consists of seven exons [27]. Alternative mRNA splicing of the *PGF* primary transcript results in four isoforms, PGF-1 (PGF<sub>131</sub>), PGF-2 (PGF<sub>152</sub>), PGF-3 (PGF<sub>203</sub>), and PGF-4 (PGF<sub>224</sub>) [27] differing in secretion properties and binding affinities [28]. PlGF homodimers bind FLT-1 and NRP-1 while PlGF/VEGF-A heterodimers bind KDR and FLT-1/KDR heterodimers *in vitro*. PlGF is predominantly expressed in the placenta, heart and lungs. The exact physiological actions of PlGF are still not clear, however, evidence suggests a pivotal role for PlGF in regulating VEGF-A dependent angiogenesis under pathological conditions [29]. A few proposed mechanisms by which PlGF potentiates angiogenesis are, by (i) stimulating endothelial cells via FLT-1, (ii) separating VEGF-A from FLT-1, allowing VEGF-A to activate KDR, (iii) recruiting monocytes/macrophages which have a crucial role in vessel growth [28] and (iv) inducing the secretion of VEGF-A from monocytes [30].

### **VEGF-B**

VEGF-B has structural similarities to VEGF-A and PlGF, and *VEGFB* gene is localized to chromosome 11q13 [31]. It is expressed as two isoforms VEGF-B<sub>167</sub> and VEGF-B<sub>186</sub>, and is abundant in heart and skeletal muscle [32]. VEGF-B forms stable heterodimers

with VEGF-A [33] and is generally co-expressed with VEGF-A. VEGF-B binds to two of the VEGF receptors, FLT-1 [34] and NRP-1 [35]. VEGF-B is reported to behave as an endothelial cell mitogen [32] but part of the mitogenic activity may be due to VEGF-A/VEGF-B heterodimers.

### **VEGF-C**

VEGF-C has been recognised as a lymphatic system regulator during embryogenesis as well as in adult life and the *VEGFC* gene is localized to chromosome 4q34 [31]. VEGF-C has a high affinity for both KDR and FLT-4 [36]. VEGF-C induces selective lymphangiogenesis without accompanying angiogenesis and is mainly expressed together with FLT-4 predominantly in regions where lymphatic vessels develop.

### **VEGF-D**

VEGF-D is also a primarily lymphangiogenic growth factor and the *VEGFD* gene has been localized to chromosome Xp22.31 [37]. VEGF-D binds to both KDR and FLT-4 and is present in many human tissues, most abundantly in skin and lung during embryogenesis. Although mainly a mediator of lymphangiogenesis, animal experiments have shown VEGF-D to possess strong angiogenic properties [38].

## **VEGF Receptors**

### **FLT-1**

Fms-like tyrosine kinase receptor is composed of seven extracellular immunoglobulin homology domains, a single transmembrane region and an intracellular tyrosine kinase sequence that is interrupted by a kinase-insert domain [39]. FLT-1 is localized to chromosome 13q12-q13 [37]. FLT-1 binds VEGF-A, VEGF-B and PlGF with high affinity, is expressed in many human tissues including monocytes/macrophages and

placental trophoblasts and its expression is up-regulated by hypoxia [40]. Alternative splicing of the pre-mRNA which encodes FLT-1 results in the production of soluble FLT-1 (sFLT-1) comprising the ligand binding domain of FLT-1 but lacking the membrane-spanning and intracellular domains [41]. Soluble FLT-1 is secreted by endothelial cells, monocytes and the placenta [42]. Soluble FLT-1 acts as a potent antagonist of VEGF-A and PlGF by inhibiting their binding to cell surface receptors [43], as well as by forming heterodimers with KDR [44] and is considered an anti-angiogenic factor. Recently a human-specific splice variant of FLT-1 was discovered producing a soluble receptor designated sFLT-14 [45]. Soluble FLT-14 is primarily expressed in non-endothelial cells, notably vascular smooth muscle cells. Placental expression of the two isoforms of sFLT changes over time with sFLT-1 being the dominant form during the first trimester to almost exclusive sFLT-14 production by term. Major sites of placental sFLT-14 expression are degenerative syncytiotrophoblasts known as syncytial knots. Soluble FLT-14 is qualitatively different to sFLT-1 but is a potent inhibitor of VEGF-A signalling, its inhibitory activity being comparable to that of sFLT-1 [45].

## **KDR**

Kinase insert domain receptor has a structure similar to FLT-1 and the KDR gene is localized to chromosome 4q11-q12 [37]. KDR binds VEGF-A, VEGF-C and VEGF-D. Although the binding affinity of VEGF-A for KDR is lower than that for FLT-1, it has been shown that KDR is the primary receptor transmitting VEGF-A signals in endothelial cells [46]. Despite this, other cell types including neuronal cells and megakaryocytes also express KDR. The expression of KDR is auto-regulated, being up-regulated by VEGF-A, VEGF-C and VEGF-D. A soluble form of the KDR receptor has also been detected in human plasma and is suggested to be secreted by endothelial cells

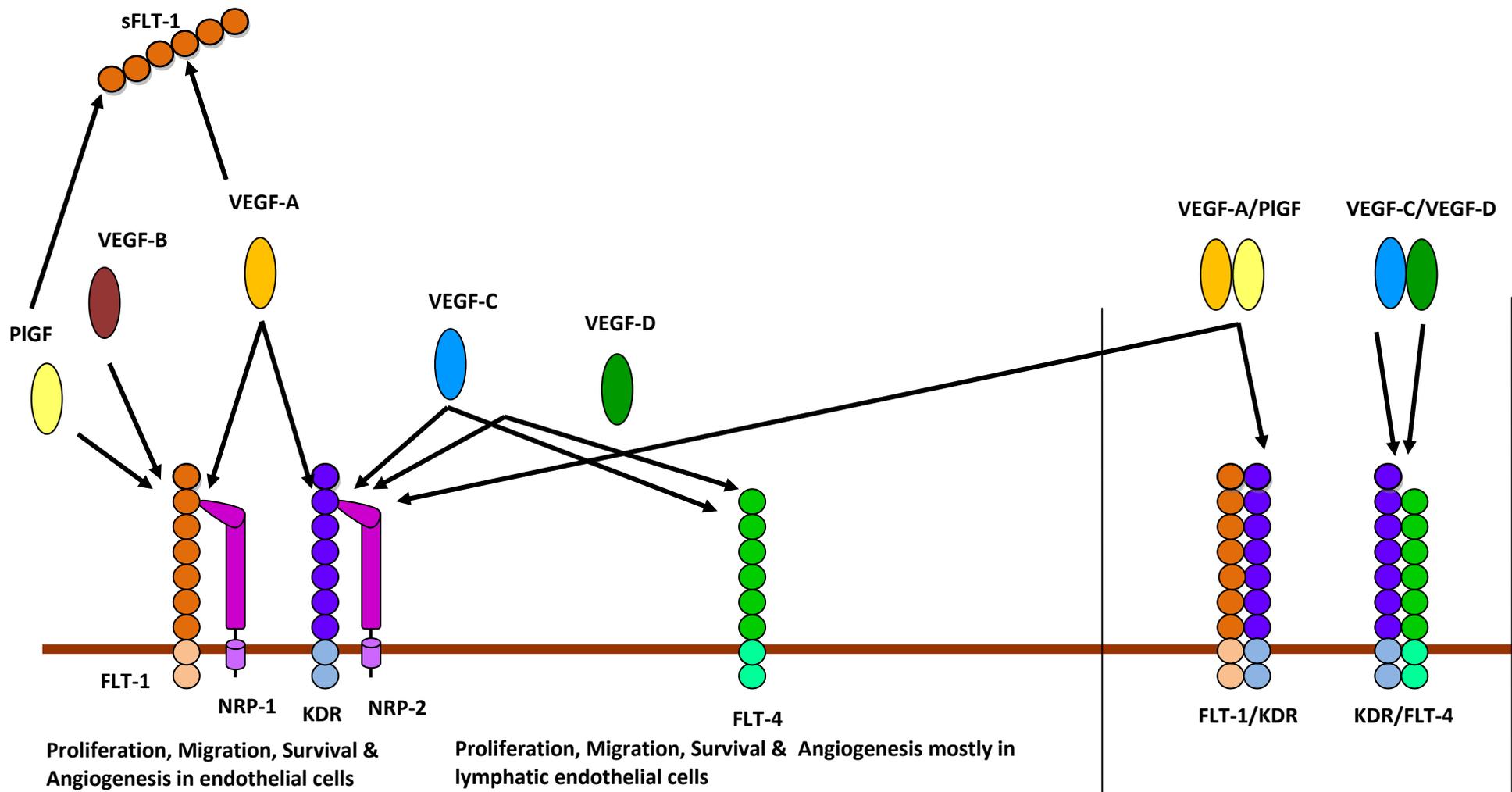
[47]. Alternative mRNA splicing or proteolytic cleavage of the membrane-bound receptor is hypothesized as the probable mechanism of generation of this receptor but the mechanism remains to be elucidated [47]. Soluble KDR is also considered an anti-angiogenic protein [12] but its mechanism of action still remains unclear.

#### **FLT-4**

Fms-like tyrosine kinase-4 has only six immunoglobulin-like domains and the FLT-4 gene is localized to chromosome 5q33-qter [37]. FLT-4 binds VEGF-C and VEGF-D. FLT-4 is present on all endothelia during development but becomes restricted to lymphatic endothelial cells and certain fenestrated vascular endothelial cells in adult life [48, 49].

#### **Neuropilins (NRPs)**

Neuropilin-1 binds VEGF-A, VEGF-B and PlGF while NRP-2 binds VEGF-A, VEGF-C and PlGF [50]. In endothelial cells, NRPs are thought to increase VEGF signalling by ensuring optimal presentation of ligands to the receptors and by stabilizing VEGF/VEGF-R complexes. Interaction of VEGF-A with NRP-1 is required for VEGF-A binding to KDR, activation of KDR and the downstream signalling and biological actions. Similarly, interaction of VEGF-A or VEGF-C with NRP-2 increases the phosphorylation threshold of KDR and promotes endothelial cell survival and motility [51].



**Figure 1** VEGF family angiogenic growth factors: interactions and functions

## **VEGF family and pregnancy**

### **Potential role in implantation**

Implantation is a complex process where the developing embryo establishes contact with the maternal endometrium initiating the development of the placenta. At present knowledge is limited on the role of angiogenesis in implantation but considering the importance of vascular development to the receptive endometrium, angiogenesis is likely to be critical for successful implantation [52]. The most frequently expressed VEGF-A splice variants VEGF-A<sub>121</sub> and VEGF-A<sub>165</sub> are expressed in the endometrium [53]. Uterine expression of VEGF-A appears to be cycle dependant with increased levels of both *VEGFA* messenger RNA and protein levels reported during the mid secretory period [54, 55]. In contrast, increased mid secretory VEGF-A expression is not evident in women experiencing repeated IVF failure [56] suggesting that VEGF-A may have a role in successful implantation. In support of this hypothesis, Hannan and colleagues recently demonstrated that VEGF-A levels were significantly reduced in uterine fluid during the mid secretory phase in women with unexplained infertility compared with fertile women. They also demonstrated that culturing mouse embryos with either mid secretory phase uterine fluid from fertile women or recombinant human VEGF-A enhanced blastocyst outgrowth and that treatment of human endometrial epithelial cells with uterine fluid from fertile women or recombinant human VEGF-A increased endometrial epithelial cell adhesion [57]. These findings suggest novel mechanisms by which VEGF-A may regulate implantation.

### **VEGF family and the placenta**

During pregnancy the placenta expresses VEGF family angiogenic growth factors but the literature is somewhat controversial. While some report that *VEGFA* mRNA is expressed in villous and extravillous trophoblasts, Hofbauer cells and maternal decidual

cells [58-62] throughout pregnancy, others report that the expression is seen in villous mesenchyme, decidual macrophages and decidual glands but not in trophoblasts [63, 64]. In the first trimester of pregnancy, PlGF is mainly expressed in extravillous trophoblast cells within the maternal decidua but towards term the expression is shown to be abundant in villous trophoblasts [62-64]. The inconsistent results on VEGF-A expression are attributed to the probes used in *in-situ* hybridization studies that may cross react with PlGF in the trophoblast layer resulting in false positive results [63]. The expression pattern of FLT-1 is similar to that of VEGF-A while abundant KDR expression is localized to areas of endothelial cells [58, 65]. These findings suggest that VEGF-A and PlGF may have vital role in the development of the placental vasculature. At present data on the expression and function of the other members of the VEGF family on pregnancy and placentation are limited. Recently it was shown that VEGF-C was expressed in all EVT populations and that VEGF-D was expressed in trophoblasts, decidual stromal cells, endothelial cells and vascular smooth muscle cells of spiral arteries [66]. However, their role in placentation is yet to be determined. Additionally, uterine natural killer cells (NK cells) express high levels of mRNAs for VEGF-C and PlGF while the expression of VEGF-A has been demonstrated to be low [67, 68].

### **Regulation of placental vasculogenesis and angiogenesis**

Vasculogenesis and angiogenesis are two processes that are essential in the establishment of the utero-placental circulation. Human placental vascular development begins as early as 21 days post conception by formation of haemangioblastic cords and is observed at the stage of early tertiary chorionic villi [69]. The initial period of vasculogenesis is followed by a phase of branching angiogenesis (day 32 to week 25 post conception) during which the haemangioblastic cords develop into a richly branched capillary bed [70]. Placental expression of VEGF-A, FLT-1 and KDR are

intense and the expression of PlGF is moderate during this period [70]. It has been shown that during the organization of the first vessels, angiogenic factors required for the commencement of angiogenesis are supplied by the cytotrophoblast cells, and that as pregnancy advances and villous maturation occurs, additional VEGF-A is supplied by stromal cells including Hofbauer cells (fetal placental macrophages) [71, 72]. *In vitro* experiments on the chorioallantoic membrane of the chick have shown that binding of VEGF-A to FLT-1 and KDR stimulate branching angiogenesis [73].

From 25 weeks gestation onwards angiogenesis switches from branching to non-branching and is accompanied by a decline in VEGF-A and KDR and an increase in the expression of PlGF, FLT-1 and sFLT-1. PlGF which is expressed in trophoblasts throughout gestation acting on FLT-1 may, in early gestation, have a supplementary role in vasculogenesis and branching angiogenesis by recruiting macrophages, and from then onwards, have a role in the regulation of non-branching angiogenesis that continues to term.

### **Regulation of trophoblast invasion and spiral artery remodelling**

During normal development of the placenta, extravillous trophoblast cells (EVT) invade the uterine decidua, the inner third of the myometrium (interstitial invasion) and the spiral arteries (endovascular invasion). The process of trophoblast invasion is highly controlled so that the depth of invasion of the uterus is sufficient but not so excessive as to penetrate the myometrium and adjacent organs. For successful invasion, EVT cells need to both increase their motility and secrete specific proteases to breakdown the extracellular matrix. The urokinase plasminogen activator/plasminogen pathway and the matrix metalloproteinases play a key role in cellular invasion by degrading the extracellular matrix. In addition, to its well researched action of stimulating endothelial cell proliferation and migration, VEGF-A is known to stimulate metalloproteinase

activity of endothelial cells [74]. Most *in vitro* studies have primarily focused on endothelial cells in examining the biologic actions of the VEGF family. The fact that VEGF-A, PlGF and their receptors are expressed in non-endothelial cells, including trophoblast cells raises the possibility that VEGF-A and PlGF may exert similar biological actions on trophoblast cells.

However, *in vitro* studies to date report results contrary to this hypothesis. One group has shown that VEGF-A [75] and PlGF [76] promote EVT cell proliferation without influencing their migratory or invasive behaviours while others have shown that VEGF-A and PlGF do not stimulate EVT proliferation [77, 78] or invasiveness [77, 78] but increase motility of trophoblast cells [77-79]. One study has also shown that VEGF-A inhibits the invasion of first trimester trophoblast cells and decreases the cell surface expression of urokinase plasminogen activator, a molecule required for trophoblast invasion [77]. This VEGF-A induced reduction in invasion is inhibited by the addition of a VEGF-A neutralizing antibody [77]. The results of these *in vitro* studies indicate that the increased motility of trophoblast cells in response to VEGF-A may be an initial response to attract trophoblast cells to the decidua, and that VEGF-A may then limit the degree to which the cells invade [78].

During endovascular invasion, the endothelium and the underlying vascular smooth muscle cells are replaced by EVT embedded in a fibrinoid rich matrix [80]. This spiral artery remodelling, results in the conversion of narrow calibre high resistance vessels into wide calibre low resistance vessels capable of supplying enough maternal blood to the placenta to accommodate the increasing demands of the rapidly growing fetus [81-83]. In addition to the trophoblast mediated spiral artery remodelling, subtle changes in spiral artery structure are observed early in pregnancy in the decidua, and are termed trophoblast-independent or decidua-associated remodelling events [80]. Early pregnancy is associated with an influx of leukocytes into the decidua including uterine

NK cells and macrophages. Uterine NK cells isolated from first trimester decidua secrete many angiogenic growth factors including VEGF-A, VEGF-C, PIGF and un-remodelled spiral arteries express KDR [67]. Uterine NK cells are proposed to be a major source of angiogenic growth factors at the fetal-maternal interface responsible for decidua-associated spiral artery remodelling [84].

The molecular mechanisms controlling spiral artery remodelling are still not clear but it is known that during invasion the cytotrophoblasts lose their ability to divide [85] and that the cells which interdigitate between maternal endothelial cells lose their epithelial characteristics and acquire an endothelial phenotype in a transition process called pseudovasculogenesis. This includes changes in the expression of cell adhesion molecules. During differentiation, cytotrophoblasts down-regulate adhesion molecules highly characteristic of epithelial cells including integrin  $\alpha_6\beta_4$ ,  $\alpha_5\beta_6$  and epithelial cadherin and up-regulate those that are expressed on endothelial cells including integrins  $\alpha_v\beta_3$ ,  $\alpha_1\beta_1$ , vascular endothelial cadherin, vascular cell adhesion molecule 1 (VCAM-1) and platelet endothelial cell adhesion molecule 1 (PECAM-1) [86]. These changes in cell adhesion molecules are seen only in the invasive cytotrophoblasts suggesting that changes in the microenvironment alter gene expression [87]. Integrin  $\alpha_v\beta_3$  is known to play a key role in angiogenesis [88]. VEGF-A induces endothelial cells to express increased levels of mRNA encoding  $\alpha_v$  and  $\beta_3$  integrin subunits which lead to increased protein expression of  $\alpha_v\beta_3$  on the endothelial cell surface. VEGF-A also induces mRNA encoding Osteopontin (OPN), an  $\alpha_v\beta_3$  ligand. Cell migration assays report that OPN promotes migration of microvascular endothelial cells in a concentration dependent manner and that continuous VEGF-A stimulation, leading to increased induction of  $\alpha_v\beta_3$  expression, results in increased cell migration which is inhibited by an  $\alpha_v\beta_3$  neutralising antibody [11]. VEGF-A is also known to stimulate the expression of intercellular adhesion molecule 1 (ICAM-1) and VCAM-1 in HUVECs *in*

*vitro* via activation of NF- $\kappa$ B [89]. These findings suggest that the VEGF family has a role in trophoblast invasion and spiral artery remodelling but more extensive studies are needed to elucidate the exact role.

### **VEGF family gene ablation**

Gene ablation studies in mice have demonstrated that *VEGFA*, *FLT1* and *Flk* are crucial for embryonic angiogenesis and homozygous gene mutations in all result in embryonic death (Table 1) [29, 90-93]. However, *VEGFA* appears to be the most critical, as embryonic lethality occurs even in the heterozygous state. *PGF* does not appear to be essential for embryonic angiogenesis but can be considered a potent stimulator of this process. PlGF stimulates VEGF-A secretion by monocytes [30], increases VEGF-A mRNA in peripheral blood mononuclear cells [94] and over expression of PlGF in mice results in up-regulation of *FLT1* and *Flk* transcription [95]. PlGF deficiency impairs endothelial cell response to VEGF-A which is restored by the administration of exogenous PlGF [29]. Therefore, although ineffective in being a strong angiogenic stimulator on its own, PlGF appears to amplify the effects mediated via VEGF-A. The aforementioned gene ablation studies in mice have focused on defects in embryonic angiogenesis. At present there is a paucity of literature on the effects of VEGF family gene ablation on placental vascularisation which may provide new knowledge on the role of these genes in the pathogenesis of pregnancy complications of placental origin.

**Table 1 Effects of VEGF family gene ablation in mice**

<b>Gene</b>	<b><i>Gene</i><sup>+/-</sup></b>	<b><i>Gene</i><sup>-/-</sup></b>
<i>VEGFA</i>	Die E11-E12, displaying defects in early vascular development	Die E8-E9 from defects in blood island formation
<i>PGF</i>		No apparent vascular defects Reduced ability to respond to ischemic damage
<i>FLT1</i>	Normal vessels	Formation of endothelial cells not affected, but assembled to form abnormal vessels Die by E8
<i>Flk</i>	Normal endothelial cells	Endothelial precursors form Mature cells do not form Die E8.5-E9.5

E, Embryonic day

## **VEGF family in adverse pregnancy outcomes**

### **VEGF family and Preeclampsia**

Preeclampsia is a multiorgan disorder affecting 2-7% of pregnant women leading to substantial maternal and perinatal morbidity and mortality [3]. Preeclampsia is characterized by new onset hypertension and proteinuria after 20 weeks of gestation. In some women it can progress to HELLP (*haemolysis, elevated liver enzymes and low platelets*) syndrome and eclampsia (seizures). Preeclampsia occurs only in the presence of the placenta [96]. Therefore, the only successful treatment remains the delivery of the placenta and hence the fetus, which involves significant morbidity and even death of the baby in case of early-onset preeclampsia.

Early placentation defects, including impaired trophoblast invasion and inadequate maternal spiral artery remodelling have been demonstrated in preeclampsia [6]. *In vitro* and *in vivo* studies have shown that preeclamptic placentae retain the adhesion molecules  $\alpha_6\beta_4$ ,  $\alpha_5\beta_6$  and E-Cadherin and fail to up-regulate  $\alpha_v\beta_3$ ,  $\alpha_1\beta_1$ , VE Cadherin, VCAM-1 and PECAM-1 that are normally expressed by the most differentiated and invasive trophoblasts [97, 98]. Therefore, it is postulated that the failure of cytotrophoblasts to switch to a vascular phenotype could tip the balance of molecules that permit invasion in favour of those that restrain it leading to a net effect of shallow endovascular invasion [98].

Evidence from many laboratories suggests that the absence of the normal repertoire of VEGF family members at the maternal-fetal interface may result in these deficits in cytotrophoblast differentiation observed in preeclampsia. Extensive studies from Dr. Susan Fisher's group have shown that cytotrophoblasts respond to the VEGF ligands they produce, and blocking the ligand binding significantly decreases their expression of integrin  $\alpha_1$ , and increases apoptosis. They have also shown that in severe preeclampsia and HELLP syndrome VEGF-A and FLT-1 expression are decreased [99].

Studies on placental VEGF-A protein expression using immunohistochemistry have reported a decrease in VEGF-A immunostaining in preeclampsia [99-101], severe preeclampsia and HELLP syndrome [99] compared to normotensive pregnancies, while some others have reported an increase in VEGF-A protein expression in preeclampsia [102, 103].

Investigations on placental *VEGFA* mRNA have shown that its level is reduced [104, 105] in preeclamptic placentae compared to normal placentae while others have demonstrated an increase [106] and no difference [107]. A recent study found placental *VEGFA* mRNA to be higher in gestational hypertension, where hypertension occurs in the absence of proteinuria, lower in preeclampsia with HELLP syndrome and no difference in preeclamptic placentae compared to normal placentae [108]. The authors propose that the high *VEGFA* expression in the gestational hypertension group may be a compensatory mechanism in an attempt to restore placental blood flow to normal and that in more severe states as in preeclampsia and HELLP syndrome there is an attempt at compensation with only some components of the placenta being able to produce *VEGFA* [108].

Initially, impaired invasion and spiral artery remodelling were thought to lead to defective utero-placental circulation and subsequent placental ischaemia [109]. More recently, damage to chorionic villi by the failure of remodelling has been elegantly modelled by Burton *et al.* and the consequences described [110]. True ischaemia probably only occurs in the more advanced stages of the disease. Various stressors, like oxidative stress, inflammation, and possibly even mechanical shear stress are now thought to contribute to the release of soluble factors that enter the maternal circulation inducing endothelial dysfunction leading to the clinical features of preeclampsia. There is considerable ongoing research on circulating factors that contribute to this maternal endothelial dysfunction. Many studies have demonstrated that increased placental

expression and secretion of sFLT-1 that circulates in maternal plasma antagonises both VEGF-A and PlGF contributing to endothelial dysfunction [12]. Soluble FLT-1 over-expression in rats is known to result in hypertension, proteinuria and glomerular endotheliosis characteristic of preeclampsia [12]. The anti-angiogenic state induced by excess placental production of sFLT-1 can be “rescued” by administering VEGF-A and PlGF [12]. The hypothesis that excess placental sFLT-1 may contribute to the pathogenesis of preeclampsia is further supported by the increased incidence of preeclampsia in mothers carrying trisomy 13 fetuses [111]. The genes for sFLT-1 and FLT-1 are localized to chromosome 13. Therefore, fetuses with an extra copy of this chromosome are likely to produce more of the gene products compared to their normal counterparts [111]. It has been shown that the ratio of circulating sFLT-1 to PlGF is significantly increased in women carrying trisomy 13 fetuses, possibly contributing to the increased risk of preeclampsia seen in these women [112]. The molecular mechanisms leading to excess placental sFLT-1 in preeclampsia and the role of sFLT-1 in placentation are not yet clear. As mentioned earlier, up to recently, it was believed that hypoxia was the major trigger for the release of sFLT-1 [113-115]. Redman and Sargent recently hypothesized that the primary placental defect that triggers preeclampsia is likely to be oxidative stress rather than hypoxia. They propose that this inflammatory stimulus provokes the release of sFLT-1 to a similar or greater extent than hypoxia [116]. Recently four splice variants of sFLT-1 were also discovered of which three are known to be up-regulated in preeclamptic placentae [117]. The sFLT-14 variant which has anti-angiogenic effects similar to sFLT-1 is primate specific and is known to be up-regulated in syncytial knots in preeclamptic placentae [45].

There is consistent evidence that placental expression [12, 99, 118] and maternal serum [13, 118, 119] sFLT-1 are increased in preeclamptic women compared to normal pregnant women. The majority of studies have demonstrated that there is no significant

difference in maternal serum sFLT-1 levels prior to 20 weeks of gestation in women who develop preeclampsia compared to normal pregnancies [120]. A few studies have shown a significant difference prior to 20 weeks (Table 2). In women who subsequently develop preeclampsia, maternal serum sFLT-1 levels are known to rise at 20 weeks gestation and the levels are significantly increased 5 weeks prior to the onset of hypertension and proteinuria [13]. The sFLT-1 level is observed to be directly proportional to the degree of proteinuria [121] and in preeclamptic women the concentration is known to be higher in those with earlier onset disease [13, 121], severe disease [12, 13, 121] and in those who deliver small for gestational age infants [13, 122].

Maternal serum PlGF is known to be the reciprocal of sFLT-1; the higher the sFLT-1, the lower the PlGF [13]. During normal pregnancy, there is a steady increase in serum PlGF during the first two trimesters, a peak at 29-32 weeks and a decline thereafter [13, 123]. In women who subsequently develop preeclampsia, serum PlGF concentrations are lower as early as 10-13 weeks gestation [13, 123-130] with considerable diminution 5 weeks prior to the clinical onset of the disease [13]. Serum PlGF levels at 21-32 weeks of gestation are known to be lower in early preeclampsia (prior to 37 weeks) compared to late onset, severe disease compared to mild disease and in preeclampsia associated with a small for gestational age infant compared to appropriate size for gestational age infant [13]. Urinary PlGF is known to parallel the serum level with a rise in the first two trimesters, reaching a peak at 29-32 weeks and a steady decline thereafter. In preeclamptic women, the pattern is similar but substantially lower [131] with the most pronounced difference observed 5 weeks prior to the clinical onset of the disease [132]. The development of preeclampsia, however, is not preceded by altered urinary PlGF in the first trimester of pregnancy [133].

Current evidence suggests that low circulating PIGF and high circulating sFLT-1 early (11-13 weeks) and in mid pregnancy, can distinguish women who subsequently develop preeclampsia from those who remain normotensive throughout pregnancy (Table 2). It has also been demonstrated that an algorithm that uses PIGF in combination with other markers [130, 134-140] and sFLT-1/PIGF ratio [141-144] are better screening tools than when either PIGF or sFLT-1 is used on its own (Table 2).

Although VEGF-A plays an important role in normal pregnancy and in the pathogenesis of preeclampsia, maternal serum VEGF-A has a limited clinical role in the prediction of preeclampsia. Soluble FLT-1 binds VEGF-A with a higher affinity than PIGF resulting in extremely low circulating levels of free VEGF-A. A few studies have reported reduced serum VEGF-A in preeclampsia [145] and have proposed VEGF-A as a promising marker for prediction of severe, early-onset preeclampsia [126] but others have failed to observe this due to the concentration being below the detection level of currently available ELISA kits [123, 128, 146]. Contrary to the theory of reduced serum VEGF-A in preeclampsia, several investigators have shown that maternal serum VEGF-A is increased in preeclamptic pregnancies compared to normotensive pregnancies [147-150]. This discrepancy is believed to be due to the type of VEGF-A measured. It is proposed that total serum VEGF-A may be increased in preeclamptic pregnancies and that the free level is reduced due to binding to sFLT-1. VEGF-A is produced by many cells at the maternal-fetal interface. A recent study demonstrated that the proportions of VEGF-A expressing peripheral blood T and NK cells were markedly decreased in preeclamptic women compared to healthy pregnancies suggesting that the reduced maternal serum VEGF-A is not only the result of antagonism by sFLT-1 [151].

The strong evidence supporting the use of PIGF and sFLT-1 as predictive screening tests for preeclampsia has led to these biomarkers being validated for routine clinical use in some countries. However, extensive studies have demonstrated that these

biomarkers are more predictive of early onset preeclampsia and severe preeclampsia, and that the highest predictive values are demonstrated during second trimester screening (Table 2). Therefore, although maternal serum and urinary angiogenic proteins provide a strong predictive tool for a subset of preeclamptic women, they have a limited role in prediction for intermediate and late preeclampsia. Furthermore, for intervention to be successful, an early screening tool would be of more utility than a second trimester test.

Recently the potential use of VEGF family in the treatment of preeclampsia has also been explored. Li and colleagues describe a preeclampsia model in pregnant rats induced by adenoviral over-expression of sFLT-1 (Adv-sFLT-1). Infection with Adv-sFLT-1 in rats resulted in hypertension and proteinuria. Histologically, the kidneys from these rats showed glomerular endotheliosis, reminiscent of the renal lesions associated with preeclampsia in pregnant women. Administration of recombinant VEGF-A<sub>121</sub> resulted in a reduction in systolic blood pressure and proteinuria and an improvement in glomerular endotheliosis [152]. Similar findings have been reported in other animal models [153-155] suggesting that VEGF-A<sub>121</sub> may have a therapeutic potential in the management of preeclampsia. In a mouse model of preeclampsia, treatment with either VEGF-A<sub>164</sub> or PlGF-2 were shown to reduce the blood pressure but proteinuria was unaffected by either treatment [156]. These findings provide interesting insights into the potential role of VEGF-A and PlGF in the management of preeclampsia. Future studies focusing on the possible adverse effects of VEGF-A therapy on the fetus and the placenta will be beneficial.

**Table 2 Angiogenic biomarkers used for detection/prediction of preeclampsia**

Author	Sample size (n)	Predictive test	GA (weeks)	OR or RR	Sensitivity (%)	Specificity (%)	ROC
Tidwell et al 2001	39	Serum PIGF	5-15	95	90.9	90.5	0.72
Kusanovic et al 2009	1622	PIGF	6-15		62.9,PE; 77.8,EPE	60.4,PE; 69.7,EPE	0.65,PE; 0.74,EPE
		PIGF/sFLT-1	6-15		93.5,EPE	22.2,EPE	0.59,EPE
		PIGF/sEndoglin + sFLT-1	6-15		67.7,PE; 77.8,EPE	53.7,PE; 72.7,EPE	0.65,PE; 0.73,EPE
Thadani et al 2004	200	Serum PIGF	10	14.7			0.69
Poon et al 2009	7797	Maternal factors, MAP, Uterine PI, PAPP-A, PIGF	11-13		93.1,EPE		
Poon et al 2010	402	Maternal factors, MAP, Uterine PI, PIGF	11-13			88.5,EPE	
		Maternal factors, MAP, Uterine PI, PIGF, Activin-A, P-selectin	11-13			46.7,LPE	
Akolekar et al 2011	33602	Serum PIGF	11-13		53.5,EPE; 27,LPE		
		Maternal characteristics, MAP, Uterine RI serum PIGF, PAPP-A, PP13, sEndoglin, Inhibin-A, Activin-A, PTX3, P-Selectin	11-13		91,EPE; 60.9,LPE		
Akolekar et al 2008	824	Serum PIGF	11-14		52,EPE; 33, LPE		
		Maternal characteristics, Obstetric history Serum PIGF, Uterine PI	11-14		90,EPE; 49, LPE		
Levine et al 2004	240	Serum PIGF	13-20		7.4		

PE, Preeclampsia; SPE, Severe preeclampsia; EPE, Early preeclampsia; LPE, Late preeclampsia; MAP, mean arterial pressure; Uterine PI, uterine artery pulsatility index; Uterine RI,

uterine artery resistance index; UADV, uterine artery Doppler velocimetry; PAPP-A, pregnancy-associated plasma protein-A

**Table 2 Angiogenic biomarkers used for detection/prediction of preeclampsia - Continued**

Author	Sample size (n)	Predictive test	GA (weeks)	OR or RR	Sensitivity (%)	Specificity (%)	ROC
Yu et al 2010	124	Doppler PI at 22-24 wks, PIGF, Activin-A	12-16		0.9,PE	0.8,PE	0.91
		Doppler PI at 22-24 wks, PIGF, Activin-A, Inhibin-A	12-16		0.93,PE	0.8,PE	0.94
Su et al 2001	254	Serum PIGF	14-19	2.5	70	70	0.79
Lim et al 2008	140	sFLT-1	14-21	6.9,PE			0.76,PE
		sFLT-1/PIGF	14-21	38.8,PE			0.85,PE
Polliotti et al 2003	80	Serum PIGF	17	4.2			0.79
Stepan et al 2007	63	sFLT-1	19-24		0.62,PE; 0.67, EPE	0.7,PE; 0.89,EPE	
		PIGF	19-24		0.77,PE; 0.83,EPE	0.62,PE; 0.62,EPE	
		sFlt-1/PIGF	19-24		0.62,PE; 0.67, EPE	0.51,PE; 0.51,EPE	
		Doppler+sFLT-1	19-24		0.77,PE; 0.83,EPE	0.73,PE; 0.89,EPE	
		Doppler+PIGF	19-24		0.77,PE; 0.83,EPE	0.68,PE, 0.76,EPE	
Chapell et al 2001	65	Serum PIGF formula	20				
Kusanovic et al 2009		PIGF	20-25		51.6,PE; 100,EPE	76.4,PE; 95.8,EPE	0.65,PE; 0.99,EPE
		sFLT-1	20-25		66.7,EPE	93.2,EPE	0.86,EPE
		PIGF/sFLT-1	20-25		40.3,PE; 100,EPE	78.5,PE; 89.1, EPE	0.60,PE; 0.99,EPE
		PIGF/sEndoglin x sFLT-1	20-25		43.5,PE; 100,EPE	77.8,PE; 95.3,EPE	0.63,PE; 0.99,EPE
		PIGF/sEndoglin + sFLT-1	20-25		48.4,PE; 100,EPE	81.9,PE; 98,EPE	0.66,PE; 0.99,EPE

PE, Preeclampsia; SPE, Severe preeclampsia; EPE, Early preeclampsia; LPE, Late preeclampsia; MAP, mean arterial pressure; Uterine PI, uterine artery pulsatility index; Uterine RI,

uterine artery resistance index; UADV, uterine artery Doppler velocimetry; PAPP-A, pregnancy-associated plasma protein-A

**Table 2 Angiogenic biomarkers used for detection/prediction of preeclampsia - Continued**

Author	Sample size (n)	Predictive test	GA (weeks)	OR or RR	Sensitivity (%)	Specificity (%)	ROC
Madazli et al 2005	122	PIGF	21-26		92.9,PE; 100, SPE	94.4,PE; 88.1,SPE	0.99,PE
Espinoza et al 2007	3348	PIGF	22-26	2.6,PE; 5.5 , EPE			
		PIGF and abnormal UADV	22-26	8.6,PE; 43.8,EPE	27.3,PE; 64,EPE	96.4,PE; 96.5,EPE	
Abdalla et al 2008	108*	sFLT-1	23		96	87	0.94
		PIGF	23		88	81	0.89
		sFLT-1/PIGF	23		100	85	0.94
Chaiwaropongsa et al 2004	88	Serum sFLT-1	24-28		16.7	97.4	
De Vivo et al 2008	104	sFLT-1	24-28		73.1	80.8	0.87
		PIGF	24-28		92.3	80.8	0.83
		sFLT-1/PIGF	24-28		88.5	88.5	0.92
Hertig et al 2004	23	Serum sFLT-1	25-28		80	100	
Levine et al 2004	240	Serum sFLT-1	21-32	5.1			
Levine et al 2004	240	Urinary PIGF	21-32		22.5		
Moore Simas et al 2007	94	sFLT-1	22-36				0.90,EPE
		sFLT-1/PIGF	22-36				0.97, EPE

PE, Preeclampsia; SPE, Severe preeclampsia; EPE, Early preeclampsia; LPE, Late preeclampsia; MAP, mean arterial pressure; Uterine PI, uterine artery pulsatility index; Uterine RI,

uterine artery resistance index; UADV, uterine artery Doppler velocimetry; PAPP-A, pregnancy-associated plasma protein-A

## **VEGF family and Small for gestational age infants**

Being born small for gestational age (SGA) significantly increases the risk for neonatal complications and is a leading cause of perinatal death [157, 158]. SGA is also associated with impaired neural development and cognition demonstrated by lower intelligence, poor academic performance and behavioural abnormalities in childhood [159-161], as well as a lower academic achievement in adulthood [162]. A consistent association has also been demonstrated between SGA and adult onset diseases including increased risk for developing coronary artery disease, stroke, hypertension and type 2 diabetes mellitus [163-166]. Those born with low birth weight are known to develop endothelial dysfunction, which is an early event in atherosclerosis, that becomes apparent in childhood [167] and persists into adulthood when additional cardiovascular risk factors start to play a role in vascular pathology [168].

The majority of SGA babies are born to normotensive mothers (81.7%) and fewer are born to women with preeclampsia (10.7%) or gestational hypertension (7.6%) [169]. Pregnancies complicated by SGA caused by placental vasculopathy share many pathogenic abnormalities with preeclampsia. In addition, SGA pregnancies also demonstrate maternal endothelial cell dysfunction [170-172] and leukocyte activation [173].

Some studies report that maternal serum PIGF levels are lower in normotensive women with SGA infants as early as first trimester of pregnancy compared to gestational age matched controls [128, 174-177]. Others, however, report no significant difference between SGA and controls early in gestation but significant different levels later in gestation. A longitudinal study examining PIGF concentration in pregnant women at <14, 15-19, 21-25, 27-30, 35-38 weeks gestation has shown a lower level of PIGF approaching significance at 27-30 weeks in normotensive women delivering an SGA infant compared to normotensive controls, but did not observe any difference earlier in

gestation [123]. Another study reported that maternal serum PIGF levels in normotensive women with SGA infants were significantly lower than gestational age matched controls at 33 weeks but not at 17 or 25 weeks [124]. Urinary PIGF during mid pregnancy has not been shown to be affected by the presence of an SGA infant [132].

As with PIGF, several studies have investigated sFLT-1 in normotensive pregnancies with SGA infants compared to matched controls. Some report maternal serum sFLT-1 to be increased in normotensive women who deliver an SGA infant at term [118, 178] but others report no difference at term [122] in second [179] or first trimester [128]. A longitudinal study evaluating sFLT-1 at 4 week intervals from the first antenatal clinic visit until delivery has shown no difference in maternal serum sFLT-1 levels in normotensive women destined to deliver an SGA infant compared to controls [176].

A recent large study of 1,536 SGA and 31,314 non-SGA pregnancies reported that using a combination of maternal characteristics with mean arterial pressure, uterine artery pulsatility index, fetal nuchal translucency thickness, pregnancy-associated plasma protein-A (PAPP-A), free  $\beta$ -human chorionic gonadotrophin ( $\beta$ -hCG), PIGF, placental protein-13 (PP13) and A Disintegrin And Metalloprotease (ADAM12) can detect SGA in the absence of preeclampsia at 11-13 weeks. The detection rates in this study were 73% for SGA requiring delivery before 37 weeks and 46% for those delivering at term [177].

Drawing overall conclusions on the predictive value of VEGF family on SGA pregnancies is hampered by the widespread practice of using different definitions to diagnose SGA, as well as using the terms SGA and IUGR (intra uterine growth restriction) synonymously.

## **VEGF family and IUGR**

Intrauterine growth restriction (IUGR) remains a major challenge in pregnancy but lacks a precise definition. Intrauterine growth restriction results from many causes including congenital abnormalities, infections and substance abuse. However, most cases of IUGR are associated with placental insufficiency [180]. Umbilical and to a lesser extent uterine artery Doppler are used as surrogate markers to evaluate placental function and to diagnose IUGR.

Studies using uterine or umbilical artery Doppler in identifying IUGR in the absence of maternal hypertensive disease showed that maternal serum sFLT-1 is increased in these pregnancies compared to normotensive women delivering average for gestational age infants [139, 178, 181-184]. A recent study reported that there was no difference in mean maternal plasma sFLT-1 between normotensive women who delivered a SGA infant compared to matched controls but that a significant increase was only seen in a subset of women who had abnormal uterine artery Doppler and delivered a SGA infant [182]. This study also demonstrated that the magnitude of increase of sFLT-1 was related to the Doppler abnormalities in the maternal and fetal vasculatures suggesting that oxidative stress and shear stress may contribute to the increase in sFLT-1 in the maternal circulation [182, 185]. A study that measured maternal plasma PlGF and sFLT-1 in the second trimester in a subgroup of women with abnormal uterine perfusion, revealed that concurrent measurement of uterine perfusion with angiogenic proteins allows effective prediction of early onset IUGR. However, a strong predictive value was not detected for overall IUGR (Table 3) [139].

From the above findings, it appears that studies on IUGR pregnancies demonstrate more consistent results compared to those on SGA pregnancies. However, at present there are insufficient data to recommend VEGF family angiogenic growth factors as reliable biomarkers for the prediction of SGA or IUGR pregnancies.

**Table 3 Angiogenic biomarkers used for detection/prediction of SGA and IUGR**

Author	Sample size (n)	Predictive test	GA (weeks)	Sensitivity (%)	Specificity (%)
Stepan 2007	63 women with abnormal uterine perfusion	sFLT-1	19-24	0.64,IUGR; 0.8,early IUGR	0.54,IUGR; 0.94,early IUGR
		PIGF	19-24	0.36,IUGR; 0.4,early IUGR	0.84,IUGR; 0.84,early IUGR
		sFLT-1/PIGF	19-24	0.55,IUGR; 0.6,early IUGR	0.57,IUGR; 1.0,early IUGR
		sFLT-1 + PIGF	19-24	0.64,IUGR; 0.8,early IUGR	0.78,IUGR; 1.0,early IUGR
		Doppler + sFLT-1	19-24	0.64,IUGR; 0.8,early IUGR	0.70,IUGR; 1.0,early IUGR
		Doppler + PIGF	19-24	0.64,IUGR; 0.8,early IUGR	0.73,IUGR; 0.76,early IUGR
Karagiannis 2010	1536 SGA 31,314 Non SGA	Combination of maternal characteristics biophysical and biochemical markers including PIGF		0.73,SGA	

SGA, small for gestational age infant; IUGR, intrauterine growth restriction; GA, gestational age

### **Soluble KDR in preeclampsia and SGA**

The soluble form of the KDR receptor (sKDR) has been detected in human plasma [47] and the recombinant form of this protein is shown to have anti-angiogenic activity [186]. Maternal plasma sKDR is known to be reduced in preeclampsia [187] and in growth restricted pregnancies [178]. In a cross sectional study, plasma sKDR was analysed in 4 groups; non-pregnant women, women with uncomplicated pregnancy, preeclamptic women and non preeclamptic women who delivered an SGA infant. There was no significant difference in mean plasma sKDR between non pregnant women and women with uncomplicated pregnancies. Preeclamptic women, as well as non-preeclamptic women with SGA infants, were shown to have lower mean plasma sKDR compared to women with uncomplicated pregnancies. There was no significant difference in mean plasma sKDR between preeclamptic women and non-preeclamptic women with SGA infants [188]. Interestingly, no significant difference was observed in plasma sKDR concentration between SGA with and without abnormal uterine artery Doppler, indicating that utero-placental ischaemia may not be a major determinant of plasma sKDR [188]. In contrast to the above studies, one study reported that there was no difference in maternal plasma sKDR levels in preeclamptic women compared to women with uncomplicated pregnancies, however, the small sample size in this study may have yielded insufficient statistical power to detect a difference [189].

VEGF-A is known to mediate most of its actions via the membrane bound KDR receptor. The role of sKDR in VEGF-A signalling is yet to be determined. However, sKDR is known to have anti-angiogenic properties. The administration of adenovirus encoding murine sKDR to non-pregnant rats induces hypertension and proteinuria but this effect is not seen in pregnant rats. The high levels of unopposed PlGF produced by the placenta in the pregnant rat may explain these findings [12]. Reduced maternal plasma sKDR in preeclampsia and SGA compared to uncomplicated pregnancies is

unexpected given that preeclampsia and, to a lesser extent, SGA are considered anti-angiogenic conditions. While it is still not clear what biological factors contribute to the expression of sKDR, the expression of the membranous isoform of KDR is stimulated by VEGF-A [190]. Therefore, it is proposed that the low sKDR in preeclampsia and SGA may result from low availability of free VEGF-A to stimulate KDR on endothelial cells [12, 145, 188]. At present very few studies have evaluated the role of sKDR in normal and complicated pregnancies and future research is needed to validate the results.

### **VEGF family and Preterm birth**

Preterm deliveries are those that occur before the completion of 37 weeks of gestation. In developed countries the rate of preterm birth is 5-9% and as much as 12% in the USA [1]. Preterm birth contributes to 75% of perinatal mortality and increases long term morbidity [191]. In addition to the immediate complications during the neonatal period those born preterm are at increased risk for neuro-developmental and behavioural disorders in childhood and cardiovascular disorders and type 2 diabetes in adult life [192]. Preterm birth is clinically categorised as (1) *Spontaneous preterm birth*, which follows (a) preterm labour with intact membranes or (b) preterm premature rupture of membranes (PPROM) (2) *Indicated preterm birth*, in which labour is either induced or the infant is delivered by pre-labour caesarean section for maternal or fetal reasons that include, among other conditions, maternal preeclampsia, eclampsia, haemorrhage and fetal non-reassuring heart rate and intrauterine growth restriction [1]. Spontaneous preterm labour is known to be initiated by many factors including intrauterine infection, inflammation, uteroplacental ischaemia or haemorrhage, uterine overdistension, stress and other immunological processes [193, 194].

The most common types of placental pathology seen in spontaneous preterm birth include infection, inflammation and infarction/haemorrhage [195]. Recently a failure in maternal spiral artery remodelling was reported both in patients with preterm labour and intact membranes and those with preterm premature rupture of membranes [7, 8]. In the group with preterm labour with intact membranes, the mean percentage of spiral arteries with failure of physiological transformation in the myometrial and decidual segments was shown to be significantly higher than in normal pregnant women at term [7]. In the group with preterm premature rupture of membranes similar results were shown but the defect was confined to the myometrial segments of the spiral arteries [8]. The mechanisms responsible for this failure of physiological transformation are not yet determined. A possible mechanism is the inability of trophoblasts to down-regulate cell adhesion molecules characteristic of epithelial cells and up-regulate those characteristic of endothelial cells [86, 97].

At present data from clinical studies on the role of VEGF family in preterm birth are limited. One large study of 292 women with spontaneous preterm birth and 937 controls analysed PIGF and sFLT-1 in maternal serum collected between 10-14 weeks of gestation. This study did not find any association of early PIGF levels with subsequent preterm birth. However, women with higher circulating sFLT-1 at 10-14 weeks of gestation were at a lower risk of spontaneous preterm birth [196]. The biological basis of this finding is yet to be determined. It has been shown that VEGF-A is expressed in the myometrium of the pregnant human uterus [197]. In pregnant rats, VEGF-A, FLT-1 and Flk are expressed in the cervix and VEGF-A expression is known to peak in association with ripening of the cervix [198]. Therefore, it is proposed that low sFLT-1 resulting in increased free VEGF-A may promote preterm birth [196]. Hence it is plausible that high sFLT-1 could have a protective role in preterm birth. A recent study revealed that in the setting of inflammation (elevated high sensitivity C-Reactive

Protein), women experiencing spontaneous preterm labour had low serum PlGF levels [199]. These findings suggest an interaction between the angiogenic and inflammatory pathways that merits future research.

### **VEGF family and Recurrent Pregnancy Loss**

Spontaneous pregnancy loss affects 12-15% of couples [200] and 0.5 – 3% experience recurrent pregnancy loss defined as the occurrence of three or more consecutive pregnancy losses [2]. There is increasing evidence that there is no significant difference in the possible causes between those who experience two compared to three miscarriages [201] and it is recommended that couples should be investigated after two miscarriages [202, 203]. Parental chromosome abnormalities [204], uterine defects [204], thrombophilias [201, 205], antiphospholipid syndrome [201], endocrinological disorders, infections, nutritional and environmental factors [2] have all been documented as possible causes for recurrent miscarriage. However, in about 50% of recurrent pregnancy loss, the cause remains unknown [2, 201].

The majority of spontaneous miscarriage occurs during the first trimester of pregnancy. Blighted ovum, the most common type of first trimester miscarriage, is mostly associated with aneuploidy. With regards to defective placentation as a cause of early miscarriage, it should be noted that remodelling of spiral arteries in the decidual segments is well established by 8 weeks of gestation and is completed by the end of the first trimester [83]. However, failed trophoblast invasion and spiral artery remodelling are not demonstrated in early miscarriage [206]. In contrast to this, late sporadic miscarriage that occurs after 12 weeks of gestation and complicates approximately 1-2% of pregnancies [207] exhibits abnormalities in spiral artery transformation and trophoblast invasion [5].

VEGF-A, PlGF and the main receptors KDR and FLT-1 are known to regulate decidual vascularisation in early human pregnancy [208]. Decidual vascularisation is shown to be different in first trimester spontaneous miscarriages compared to matched controls. Decidual samples from miscarriage show fewer vessels with larger circumference in both decidua basalis and decidua parietalis compared to those obtained from first trimester terminations and these vascular differences correlate with increased expression of KDR and FLT-1 [209]. In contrast, a higher vessel density in the decidua parietalis [210] and decreased VEGF-A and FLT-1 are reported in missed abortions [211].

In addition to post implantation pregnancy loss, approximately 30% of spontaneously conceived embryos are lost prior to implantation and more than 50% of embryos conceived through *in-vitro* fertilization fail to implant [212]. In humans, the ability of the conceptus to implant in the endometrium is restricted to a few days in the menstrual cycle. This window of implantation minimises the risk of late implantation of compromised embryos [213]. Differentiation of endometrial stromal cells into specialized decidual cells, termed decidualization, is critical for the endometrium to achieve characteristics essential to recognize, respond to and eliminate implanting compromised embryos [213]. A recent paper by Dr. Jan Brosen's group demonstrates that impaired cyclic decidualization of the endometrium facilitates implantation yet predisposes to subsequent pregnancy loss by disabling natural embryo selection and by disrupting the maternal response to embryonic signals [214]. Intrauterine VEGF-A levels are known to be cycle dependant with increasing levels during the late secretory phase shown to be correlated with decidualization [215]. A positive correlation is also shown between intrauterine VEGF-A levels and the decidualization marker IGF binding protein (IGFBP-1) [215]. There is a sharp rise in intrauterine IGFBP-1 levels coinciding with the time of the closing of the implantation window suggesting that intrauterine IGFBP-1 may be involved in the mechanisms restricting endometrial receptivity [216].

Although correlations do not necessarily reflect causality, it can be hypothesized that low intrauterine VEGF-A levels may have a role in impaired decidualization and prolonged endometrial receptivity in recurrent pregnancy loss.

### **Role of other angiogenic and anti-angiogenic factors in pregnancy complications**

#### **Soluble endoglin**

Although not a member of the VEGF family, soluble endoglin, in combination with PlGF and sFLT-1, may be useful in prediction of preeclampsia. Endoglin (CD 105) is a transmembrane glycoprotein predominantly expressed on endothelial cells. It is a co-receptor for transforming growth factor (TGF)- $\beta_1$  and TGF- $\beta_3$  [217]. Endoglin modulates signalling of TGF- $\beta$  by interacting with TGF- $\beta$  receptors I and II [218]. Endoglin regulates nitric oxide-dependent vasodilatation [219] and its expression is up-regulated in tissues undergoing angiogenesis [218]. Endoglin gene mutations are associated with Hereditary Haemorrhagic Telangiectasia type 1 (HHT 1), a vascular disorder characterized by focal telangiectases and arteriovenous malformations [220]. These suggest the involvement of endoglin in angiogenesis, vascular development and in maintaining vessel wall integrity [218]. Proteolytic processing of the membrane bound endoglin results in soluble endoglin (sEng), an N-terminal cleavage product of the full-length endoglin [221]. *In vitro* studies have demonstrated that sEng inhibits TGF- $\beta$  signalling, blocks TGF- $\beta$  mediated vasodilatation and interferes with endothelial proliferation and capillary formation [221]. Endoglin deficiency does not affect vasculogenesis but results in poor vascular smooth muscle development and arrested endothelial remodelling and is shown to be essential for angiogenesis [222]. Soluble endoglin is also implicated in the pathophysiology of preeclampsia. Placental expression of sEng is up-regulated in preeclampsia. It is proposed that sEng enters the maternal circulation and inhibits TGF- $\beta$  signalling resulting in endothelial dysfunction

[221]. Over expression of sEng in rodents leads to increased vascular permeability and hypertension without proteinuria, and over-expression of both, sEng and sFLT-1 results in severe vascular damage, nephrotic-range proteinuria, severe hypertension, a syndrome similar to HELLP syndrome and IUGR [221]. Maternal serum sEng concentration increases during the last two months of normal pregnancy and this rise is higher and occurs earlier in women who develop preeclampsia. The increase in sEng is associated with an increase in the ratio of sFLT-1: PIGF and a composite measure incorporating all three molecules – the ratio of (sFLT-1+ sEng) : PIGF is considered a predictive biomarker for preeclampsia [223].

### **The angiopoietin family**

The angiopoietin family comprises angiopoietins 1-4 (ANG-1, ANG-2, ANG-3 and ANG-4) and the receptors (Tie-1 and Tie-2). Of these, ANG-1, ANG-2 and Tie-2 are the main molecules involved in the regulation of vascular integrity. ANG-1 and ANG-2 are both ligands for the Tie-2 receptor. ANG-1 activates Tie-2, whereas ANG-2 is known to antagonise the activation of the receptor [224]. ANG-1 acting through Tie-2 regulates vascular remodelling, maturation and stabilization of the vasculature [225]. Tie-2 knockout mice die by embryonic day 10.5 due to endocardial defects, haemorrhage and impaired vascular formation [225]. ANG-1 gene ablation results in similar vascular defects with embryonic lethality by day 12.5 demonstrating that ANG-1 plays a key role in embryonic angiogenesis [226]. ANG-1, ANG-2 and Tie-2 are expressed in the placenta throughout pregnancy and act in concert with the VEGF family to regulate placental angiogenesis [227]. In the first trimester placenta, ANG-1 and ANG-2 expression are mainly localized to the villous syncytiotrophoblasts. ANG-1 expression is also shown in endothelial cells of vessels within immature intermediate villi while ANG-2, Tie-1 and Tie-2 are also expressed by Hofbauer cells [228]. In first

trimester placenta, ANG-1, ANG-2 and Tie-2 immunostaining is also shown to be increased in intravascular extravillous trophoblasts suggesting that the angiopoietin family may regulate maternal spiral artery remodelling [66].

At present literature on maternal serum levels of the angiopoietin family in pregnancy complications is somewhat controversial. Some report that maternal serum ANG-1 is reduced [229] in preeclamptic women compared to normotensive women while others report increased levels [230] and no difference [231]. Similar results are reported for maternal serum ANG-2 concentrations with both increased [231] and decreased [230] levels reported. Differences in the gestational age at which sampling was performed may partially explain these discrepant findings. However, at present the literature on angiopoietin family in pregnancy complications is limited and future large studies are required to confirm the above findings.

### **The thrombospondin family**

The thrombospondin (TSP) family consists of TSP-1, TSP-2, TSP-3, TSP-4 and TSP-5. Within this family the molecules are grouped into two subfamilies (subgroup A, TSP-1 and TSP-2; subgroup B, TSP-3, TSP-4 and TSP-5) based on their secondary and tertiary structures [232]. Of these, TSP-1 and TSP-2 have been studied in relation to pregnancy complications.

Thrombospondin 1 (TSP-1) is a calcium binding glycoprotein expressed in many cells and is a major constituent of platelet  $\alpha$  granules. TSP-1 is released from platelets in response to activation by thrombin and stabilises platelet aggregation to injured endothelium through inhibition of ADAMTS13 degradation of ultra large von Willebrand factor multimers [233, 234]. In addition to its role in coagulation TSP-1 is well known for its strong anti-angiogenic properties [235]. During pregnancy, TSP-1 is expressed in the placenta. Placental expression of *TSP1* mRNA and protein are

increased in disorders of placental villous maturation suggesting that over-expression of *TSP1* may be implicated in the pathogenesis of pregnancies complicated by preeclampsia and SGA infants [236]. The angiogenic potential of cord blood endothelial colony forming cells (ECFC) is demonstrated to be impaired and the expression of both *TSP1* mRNA and protein are increased in low birthweight preterm infants [237]. Silencing *TSP1* is shown to restore the angiogenic properties of ECFC in LBW infants suggesting that TSP-1 may be implicated in the pathophysiology of impaired placental vascularisation demonstrated in growth restricted pregnancies. TSP-1 inhibits angiogenesis by interacting with vascular endothelial growth factor (VEGF-A) and indirectly by inhibiting the VEGF-A receptor KDR [238]. In addition, TSP-1 is a key molecule regulating inflammation and elevated TSP-1 levels are demonstrated in conditions associated with increased inflammation [239].

Thrombospondin-2 is a multifunctional molecule primarily described for its role in modulating the bioavailability and activity of proteases and growth factors in the extracellular matrix [240]. Thrombospondin-2 is expressed in endothelial cells but in contrast to TSP-1, detectable levels have not been demonstrated in platelets [241]. Similar to TSP-1, TSP-2 is demonstrated to have strong anti-angiogenic properties [242, 243]. Recently, it was reported that maternal serum TSP-2 level was increased in preeclamptic women compared to normotensive controls suggesting a potential role for TSP-2 in the pathogenesis of preeclampsia.

At present literature on the role of the TSP family in pregnancy complications is very limited. Considering the role of these molecules in angiogenesis and thrombosis, their contribution to pregnancy complications needs to be explored.

### **VEGF family genes and the genetic-conflict hypothesis**

The VEGF family provides a good example of the genetic conflict theory initially proposed by Haig [244]. According to the genetic conflict theory fetal genes are selected to increase the transfer of nutrients to the fetus and, maternal genes selected to restrict this transfer exceeding specific maternal limits [244]. In healthy pregnancy, the interaction between endovascular trophoblasts and decidual leukocytes, especially natural-killer cells, results in substantial VEGF-A and PlGF release that regulates endothelial cell integrity at the maternal-fetal interface. Placenta-derived sFLT-1 is up-regulated in preeclampsia, gains entry in to the maternal circulation and antagonises both VEGF-A and PlGF. In the first trimester, PlGF concentration is decreased in pregnancies with future preeclampsia and intrauterine growth restriction, whereas sFLT-1 concentration remains similar to that of healthy pregnancies suggesting that PlGF plays an essential role in early placentation. The rise in concentration of sFLT-1 in the third trimester of preeclamptic pregnancies is considered a fetal rescue mechanism in an attempt to restore the placental blood flow when the utero-placental blood supply is inadequate [4].

### **VEGF family gene polymorphisms and adverse pregnancy outcomes**

The VEGF family plays a pivotal role in normal pregnancy and consistent evidence exists for its causal role in pregnancy complications. Therefore, genetic variations in the genes encoding these angiogenic proteins are good candidates to study in pregnancy complications (Table 4).

### **VEGF family polymorphisms in preeclampsia**

Several groups have studied functional *VEGFA* polymorphisms in preeclampsia. The *VEGFA* +936C/T polymorphism is in the 3' untranslated region (3'UTR) of the *VEGFA*

gene and the T allele is associated with lower plasma VEGF-A compared to the C allele [245]. Two groups report the association of *VEGFA* +936C/T polymorphism with preeclampsia. A Korean study reports the association of the maternal *VEGF* +936 T allele with preeclampsia [246] while a Greek study reports no association of the maternal polymorphism with preeclampsia but found an association of the T allele with severe preeclampsia. However, their study sample was relatively small and the severe preeclamptic group comprised only 20 women [247].

The *VEGF* +405G/C is a promoter polymorphism and the GG genotype is associated with the highest plasma VEGF-A levels [248, 249]. The G allele of this polymorphism is shown to be associated with a reduced risk for severe preeclampsia [250] while the CC genotype is shown to be associated with an increased risk for HELLP syndrome [251]. The *VEGFA* -460C/T promoter polymorphism is in linkage disequilibrium with the +405G/C polymorphism and the TT genotype is shown to be associated with an increased risk for HELLP syndrome [251]

The *VEGFA* -2578C/A is a promoter polymorphism with the CC genotype having the highest transcriptional activity and VEGF-A protein production by leukocytes [252]. Four groups have studied this polymorphism and report no association with preeclampsia [247, 253], severe preeclampsia [250] or HELLP syndrome [251]. However, Banyasz reported that hypertension and proteinuria were detected earlier in carriers of the *VEGFA* -2578C/A A allele in their population of severe preeclamptic women [250]. Sandrim *et al.* report that when stratified by ethnicity, the AA genotype of the *VEGFA* -2578C/A polymorphism was less common in preeclamptic white women compared to healthy pregnant controls [253].

The *VEGFA* -634G/C and -1154G/A polymorphisms are located in the 5' untranslated region (5'UTR) of the *VEGFA* gene and the -634C and -1154G alleles are associated with higher VEGF-A production [252, 254]. Both polymorphisms have been studied in

preeclampsia and no association is reported [253, 255]. However, in the study by Sandrim *et al.* the GG genotype of the *VEGFA* -634G/C polymorphism is reported to be significantly less common in preeclamptic white women compared to controls.

At present genetic association studies on the above polymorphisms are limited by the sample size. A meta-analysis was deemed inappropriate due to the phenotypic differences in the preeclamptic study populations.

A recent large study evaluated 112 tagging polymorphisms in *VEGFA*, *VEGFB*, *VEGFC*, *FLT1* and *FLT4* in White and Black preeclamptic women with matched controls. This study reported the association of polymorphism in *VEGFC* and *FLT1* genes in both White and Black preeclamptic women but did not find the same polymorphism to be associated with preeclampsia in both ethnic groups. This may be partially explained by ethnic differences in the distribution of these polymorphisms and the relatively small group of White preeclamptic women in the study [256].

A dinucleotide repeat polymorphism in the 3' non-coding region of the *FLT1* gene has also been studied in preeclamptic Korean women with no association [257].

### **VEGF family polymorphisms in SGA infants**

At present literature on the role of VEGF family polymorphisms in SGA is limited. *VEGFA* +405G/C and -2578C/A polymorphisms have been studied in a group of low birthweight infants (LBW) compared to term healthy controls. The C allele of the +405G/C polymorphism is associated with an increased risk for low birthweight infants [258]. The *VEGF* -2578C/A polymorphism was not associated with LBW but the A allele was associated with increased risk for necrotizing enterocolitis and a decreased risk for acute renal failure during the perinatal period.

### **VEGF family polymorphisms in spontaneous preterm birth**

Only one study has so far looked at VEGF family polymorphisms in spontaneous preterm birth and reports an increased frequency of *VEGFA* +936C/T T allele in women who experience spontaneous preterm birth compared to controls [255].

### **VEGF family polymorphisms in recurrent pregnancy loss**

The *VEGFA* polymorphisms, -1154G/A, -2578C/A, +936C/T and -634G/C have been studied in women who have experienced recurrent pregnancy loss in a few ethnic groups. The *VEGFA* -1154G/A polymorphism has been investigated in six studies of which three report the association of the A allele with an increased risk for recurrent miscarriage [259-261] and the other three report no association [262-264].

The *VEGFA* -2578C/A, +936C/T and -634G/C polymorphisms have not been associated with recurrent pregnancy loss [260, 261, 265] except for a recent study that reported a decreased risk of the *VEGFA* -2578C/A A allele and an increased risk of the *VEGFA* -634G/C C allele for recurrent pregnancy loss [266].

Of these polymorphisms, a recent meta-analysis demonstrated that the *VEGFA* -1154G/A polymorphism was significantly associated with recurrent pregnancy loss [Odds ratio, 1.5; 95% confidence interval, 1.1-2.0] [267].

At present the candidate gene association studies on VEGF family have demonstrated consistent evidence only for an association of the *VEGFA*-1154G/A polymorphism with recurrent pregnancy loss.

**Table 4 Distribution of VEGF family polymorphisms in pregnancy complications**

<b>Pregnancy complication &amp; Author</b>	<b>Cases (n)</b>	<b>Controls (n)</b>	<b>Ethnicity</b>	<b>Polymorphism</b>	<b>rs number</b>	<b>Function</b>	<b>OR(95% CI)</b>
<b>Preeclampsia</b>							
Papagoglou 2004	42	73	Greek	<i>VEGFA -2578C/A</i>	rs699947	C allele - higher plasma VEGFA	No association
Banyasz 2006	84 <sup>a</sup>	96	Hungarian	<i>VEGFA -2578C/A</i>	rs699947	C allele - higher plasma VEGFA	No association
Nagy 2008	71 <sup>b</sup>	93	Hungarian	<i>VEGFA -2578C/A</i>	rs699947	C allele - higher plasma VEGFA	No association
Sandrim 2009	94	108	White and non white	<i>VEGFA -2578C/A</i>	rs699947	C allele - higher plasma VEGFA	No association
Papagoglou 2004	42	73	Greek	<i>VEGFA +936C/T</i>	rs3025039	T allele - lower plasma VEGFA	2.7 (1.1-6.6), SPE
Shim 2007	110	209	Korean	<i>VEGFA +936C/T</i>	rs3025039	T allele - lower plasma VEGFA	2.2 (1.5-3.4) for T allele
Banyasz 2006	84	96	Hungarian	<i>VEGFA +405G/C</i>	rs2010963	G allele - higher plasma VEGFA	0.3 (0.1-0.9) for G allele
Nagy 2008	71 <sup>b</sup>	93	Hungarian	<i>VEGFA +405G/C</i>	rs2010963	G allele - higher plasma VEGFA	3.7 (1.1-12.8) for CC genotype
	71 <sup>b</sup>	93	Hungarian	<i>VEGFA -460C/T</i>	rs833061	C allele - higher plasma VEGFA	3.0 (1.5-6.1) for TT genotype
Sandrim 2009	94	108	White and non white	<i>VEGFA -1154G/A</i>	rs1570360	G allele - higher plasma VEGFA	No association
	94	108	White and non white	<i>VEGFA -634G/C</i>	rs2010963	C allele - higher plasma VEGFA	No association
Papagoglou 2004	42	73	Greek	<i>VEGFA -634G/C</i>	rs2010963	C allele - higher plasma VEGFA	No association

<sup>a</sup> Severe preeclamptic women, SPE, severe preeclampsia; <sup>b</sup> HELLP syndrome, <sup>c</sup> infants with a birthweight  $\leq$  1500g

**Table 4 Distribution of VEGF family polymorphisms in pregnancy complications - continued**

<b>Pregnancy complication &amp; Author</b>	<b>Cases (n)</b>	<b>Controls (n)</b>	<b>Ethnicity</b>	<b>Polymorphism</b>	<b>rs number</b>	<b>Function</b>	<b>OR(95% CI)</b>
<b>Preeclampsia</b>							
Srinivas 2010	184	305	Black	<i>VEGFC -1830A/C</i>	rs1485766	tagSNP	1.6 (1.1-2.3)
	184	305	Black	<i>VEGFC -2698G/A</i>	rs6838834	tagSNP	1.6 (1.1-2.4)
	32	35	White	<i>VEGFC -33G/A</i>	rs7664413	tagSNP	2.0 (0.99-4.7)
	184	305	Black	<i>FLT1 -523G/C</i>	rs12584067	tagSNP	1.6 (1.0-2.4)
	184	305	Black	<i>FLT1 -4471C/T</i>	rs7335588	tagSNP	1.6 (1.1-2.4)
	32	35	White	<i>FLT1 +4244G/A</i>	rs722503	tagSNP	2.1 (1.1-4.2)
	32	35	White	<i>FLT4 +1480A/G</i>	rs307826	tagSNP	3.1 (1.2-7.9)
Kim 2008	170	201	Korean	Flt1 repeat		may affect signal transduction	No association
<b>SGA pregnancies</b>							
Banyasz 2006	128 <sup>c</sup>	200	Greek	<i>VEGFA +405G/C</i>	rs2010963	G allele - higher plasma VEGFA	1.3 (1.0-1.6) for C allele
	128 <sup>c</sup>	200	Greek	<i>VEGFA -2578C/A</i>	rs699947	C allele - higher plasma VEGFA	No association
<b>Spontaneous Preterm labour</b>							
Papazoglou 2004	54	79	Greek	<i>VEGFA -634G/C</i>	rs2010963	C allele - higher plasma VEGFA	No association

<sup>a</sup> Severe preeclamptic women, SPE, severe preeclampsia; <sup>b</sup> HELLP syndrome, <sup>c</sup> infants with a birthweight  $\leq 1500$ g

**Table 4 Distribution of VEGF family polymorphisms in pregnancy complications - continued**

<b>Pregnancy complication &amp; Author</b>	<b>Cases (n)</b>	<b>Controls (n)</b>	<b>Ethnicity</b>	<b>Polymorphism</b>	<b>rs number</b>	<b>Function</b>	<b>OR(95% CI)</b>
<b>Recurrent Miscarriage</b>							
Papazoglou 2005	52	82	Greek	<i>VEGFA -1154G/A</i>	rs1570360	G allele - higher plasma VEGFA	1.9 (1.2-3.2) for A allele
Coulam 2008	152	65	A variety of ethnics	<i>VEGFA -1154G/A</i>	rs1570360	G allele - higher plasma VEGFA	2.7 (1-7.4) for A allele
Lee 2010	215	113	Korean	<i>VEGFA -1154G/A</i>	rs1570360	G allele - higher plasma VEGFA	2 (1.2-3.5) for GA+AA
Eller 2011	99	181	Caucasian and Hispanic	<i>VEGFA -1154G/A</i>	rs1570360	G allele - higher plasma VEGFA	No association
Su 2011	115	170	Taiwanese	<i>VEGFA -1154G/A</i>	rs1570360	G allele - higher plasma VEGFA	No association
Xing 2011	339	291	Chinese Han	<i>VEGFA -1154G/A</i>	rs1570360	G allele - higher plasma VEGFA	No association
Papazoglou 2005	52	82	Greek	<i>VEGFA -2578C/A</i>	rs699947	C allele - higher plasma VEGFA	No association
Lee 2010	215	113	Korean	<i>VEGFA -2578C/A</i>	rs699947	C allele - higher plasma VEGFA	No association
Eller 2011	99	181	Caucasian and Hispanic	<i>VEGFA -2578C/A</i>	rs699947	C allele - higher plasma VEGFA	0.7 (0.5-0.9) for A allele
Papazoglou 2005	52	82	Greek	<i>VEGFA +936C/T</i>	rs3025039	T allele - lower plasma VEGFA	No association
Lee 2010	215	113	Korean	<i>VEGFA +936C/T</i>	rs3025039	T allele - lower plasma VEGFA	No association
Traina 2010	89	191	Brazilian	<i>VEGFA +936C/T</i>	rs3025039	T allele - lower plasma VEGFA	No association
Eller 2011	99	181	Caucasian and Hispanic	<i>VEGFA +936C/T</i>	rs3025039	T allele - lower plasma VEGFA	No association
Papazoglou 2005	52	82	Greek	<i>VEGFA -634G/C</i>	rs2010963	C allele - higher plasma VEGFA	No association
Lee 2010	215	113	Korean	<i>VEGFA -634G/C</i>	rs2010963	C allele - higher plasma VEGFA	No association
Traina 2010	89	191	Brazilian	<i>VEGFA -634G/C</i>	rs2010963	C allele - higher plasma VEGFA	No association
Eller 2011	99	181	Caucasian and Hispanic	<i>VEGFA -634G/C</i>	rs2010963	C allele - higher plasma VEGFA	1.5 (1.0-2.2) for C allele
Su 2011	115	170	Taiwanese	<i>KDR -168A/G</i>	rs6838752	tagSNP	0.4 (0.3-0.8)
				<i>KDR +116A/T</i>	rs1870377	tagSNP	0.5 (0.3-0.9)

## Discussion

Recent years have seen considerable advances in the role the VEGF family in pregnancy complications, mainly in preeclampsia. Extensive studies by Dr. Ananth Karumanchi's group have clearly demonstrated that the anti-angiogenic factors sFLT-1 and sEng play important roles in the pathophysiology of preeclampsia. These findings have been replicated in later years by other groups showing consistent evidence. Several prospective cohort studies have demonstrated that serum/plasma levels of PIGF, sFLT-1, sEng and the sFLT-1/PIGF ratio may be useful biomarkers in the prediction of preeclampsia although they may not be useful sufficiently early in gestation to permit intervention. The reviewed literature also demonstrates that these biomarkers in combination with other clinical and biochemical investigations are better at predicting preeclampsia than when used alone. The deviation of the plasma/serum levels of these biomarkers from normal reference ranges are more pronounced in women with earlier onset preeclampsia, while the deviation is minimal in those who develop late onset preeclampsia. Therefore, the clinical utility of these biomarkers is limited to a subset of women. Ohkuchi *et al.* recently proposed that these findings suggest the existence of thresholds for the onset of preeclampsia and set out to determine the thresholds for plasma concentrations of angiogenic (PIGF) and anti-angiogenic (sFLT-1 and sEng) factors at the onset of preeclampsia [268]. The authors report that these thresholds exist for sFLT-1 and sFLT-1/PIGF ratio, and the threshold for sFLT-1/PIGF ratio measured between 26-31 weeks of gestation may be useful for detecting preeclampsia with onset at <36 weeks. Consistent with previous studies, the authors also report that consideration of established maternal risk factors in the algorithm increases the likelihood ratios of the prediction model. The onset threshold levels of sFLT-1 and sFLT-1/PIGF ratio at earlier gestational age were very high and deviated markedly from

the reference ranges suggesting a possible reason for the lower incidence of early onset preeclampsia compared to late onset disease.

Endothelial dysfunction has long been recognized to play a key role in the pathophysiology of preeclampsia. However, most studies on prediction of preeclampsia have not incorporated measures of endothelial function into the predictive algorithms. Maternal endothelial function is shown to be markedly altered before the onset of clinical features of preeclampsia [269]. Therefore, inclusion of non-invasive measures of endothelial function may improve the predictive value of these models [270].

Maternal endothelial dysfunction in preeclampsia is mainly attributed to defective placentation. However, there is increasing evidence to suggest that abnormal placentation may not be the sole reason for altered endothelial function in preeclamptic women [271, 272]. A history of preeclampsia, as well as delivery of a growth restricted baby, are associated with increased long term risk for vascular diseases including coronary artery disease and stroke [273]. It is debatable as to whether endothelial dysfunction manifests as a consequence of these pregnancy complications and persists throughout life or whether women with risk factors for endothelial dysfunction manifest these vascular diseases at different stages. The shared risk factors for both preeclampsia and later life vascular diseases, as well as familial segregation of all these disorders suggest that these diseases share a common genetic predisposition that interact with the environment and may predispose individuals to vascular disorders that manifest at different time points throughout the life course.

Therefore, genetic variations in the angiogenic pathway need to be further evaluated. At present most candidate gene association studies in this pathway have evaluated polymorphisms in *VEGFA*. Although, PlGF protein is considered an important biomarker in the prediction of preeclampsia no studies have so far explored *PGF* polymorphisms in relation to pregnancy complications. The literature on *FLT1* and

*KDR* polymorphisms is also very limited. At present no literature exists on thrombospondin polymorphisms in pregnancy complications. One study has investigated a polymorphism in exon of the *ANGPT2* gene in recurrent pregnancy loss and reports no association [274], however no data exists on polymorphisms in *ANGPT1*. Although the placenta plays a critical role in all these pregnancy complications, at present no studies have evaluated the role of angiogenic gene polymorphisms in placental function. Another limitation at present is that there are no data on potential gene-environment interactions in the angiogenic pathway that may influence the risk for pregnancy complications.

Prospective cohort studies with the aim of collecting data on relevant clinical, environmental and lifestyle risk factors coupled with genotyping for functional variants in the angiogenic pathway will be beneficial in establishing interactions that predispose to pregnancy complications.

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# Hypotheses and Aims

## Hypotheses

The work described in this thesis is based on the following hypotheses:

1. A genetic predisposition to pregnancy complications exist via the angiogenic pathway.
2. Functional polymorphisms in angiogenesis regulating genes are implicated in abnormal placental function.
3. Functional polymorphisms in angiogenesis regulating genes are implicated in first trimester placental angiogenic gene expression.
4. Angiogenic gene expression is different between placentae from complicated pregnancy compared to uncomplicated pregnancy.

## Aims

1. To investigate whether functional polymorphisms in angiogenesis regulating genes are associated with pregnancy complications.
2. To investigate whether functional polymorphisms in angiogenesis regulating genes associate with abnormal uterine and umbilical artery Doppler as a surrogate marker of impaired placentation.
3. To investigate whether functional polymorphisms in angiogenesis regulating genes are associated with reduced first trimester placental angiogenic gene expression.
4. To investigate whether angiogenic gene expression is different in pregnancies complicated by preeclampsia, SGA infants and spontaneous preterm birth compared to uncomplicated pregnancy.

## Manuscript 2

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## Manuscript 3

### **Single nucleotide polymorphisms in the *KDR* gene in pregnancies complicated by gestational hypertensive disorders and small for gestational age infants**

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#### **Abstract**

**Introduction:** Pregnancies complicated by preeclampsia and small for gestational age infants (SGA) share placental vascular abnormalities and both disorders confer increased risk for later life coronary artery disease. *KDR* is the main receptor for VEGF-A, a potent angiogenic factor that regulates the development of the placental vasculature. Two polymorphisms in *KDR* (*-604T/C* and *Val297Ile*) are known to be associated with coronary artery disease. We investigated the association of these polymorphisms in pregnancies complicated by preeclampsia, gestational hypertension and SGA infants.

**Method:** Nulliparous pregnant women, their partners and infants were recruited to a prospective cohort study (n = 1169). Doppler ultrasound of the uterine and umbilical arteries was performed at 20 weeks gestation. Preeclampsia, gestational hypertension and SGA were defined according to international guidelines. DNA extracted from peripheral venous or cord blood was genotyped using the Sequenom MassARRAY system. Multivariable logistic regression was used to compare the odds for the

pregnancy complications between the genotype groups adjusting for potential confounders.

**Results:** Amongst 937 Caucasian pregnancies, 427 were uncomplicated (45.6%), 75 developed preeclampsia (8.0%), 102 developed gestational hypertension (10.9%) and 72 had SGA infants (7.7%) in the absence of maternal hypertensive disease. Paternal and neonatal *KDR -604T/C* was associated with preeclampsia (adjusted OR [aOR] 1.6, 95% CI 1.0-3.0 and aOR 2.2, 95% CI 1.0-4.4), SGA (aOR 1.9, 95% CI 1.1-3.3 and aOR 2.2, 95% CI 1.2-3.9) and SGA with abnormal Doppler (aOR 2.7, 95% CI 1.2-5.9 and aOR 3.2, 95% CI 1.2-5.9).

**Conclusion:** Paternal and neonatal carriage of the *KDR -604T/C* polymorphism is associated with the risk of preeclampsia and SGA infants.

## Introduction

Preeclampsia, gestational hypertension and small for gestational age infants (SGA) complicate approximately 15-20% of all nulliparous pregnancies and are leading causes of maternal and neonatal morbidity and mortality [1, 2]. Preeclampsia and SGA pregnancies are associated with increased long term risk for vascular disorders including coronary artery disease and stroke [3-7]. Early placentation defects including impaired trophoblast invasion and maternal spiral artery remodelling are demonstrated in many cases of preeclampsia and SGA pregnancies [8].

Placental vascular development is regulated by many growth factors, of which vascular endothelial growth factor A (VEGF-A) signalling represents a critical step in vessel growth and remodelling. VEGF-A exerts biologic effects through two high-affinity tyrosine kinase receptors: VEGF receptor-1 (VEGFR-1) also called fms-like tyrosine kinase-1 (Flt-1), and VEGF receptor-2 (VEGFR-2) also called kinase insert domain receptor (KDR) in humans and fetal liver kinase-1 (Flk-1) in mice. KDR is the major mediator of the mitogenic, angiogenic, permeability enhancing and endothelial survival effects of VEGF-A [9]. VEGF-A acting through KDR is proposed to regulate early placental vasculogenesis, branching angiogenesis and spiral artery remodelling [10, 11]. Homozygous gene mutations in Flk-1 in mice result in abnormalities in formation of mature endothelial cells with early embryonic death, suggesting that this receptor is crucial for the development of the embryonic vasculature [12].

Two single nucleotide polymorphisms in *KDR* (*-604T/C* and *Val297Ile*) are known have potential biological functions and are also associated with coronary artery disease and stroke [13, 14].

Considering the role of KDR in placental vascular remodelling, the association of *KDR-604T/C* and *Val297Ile* polymorphisms with coronary artery disease and stroke, and the data demonstrating that preeclamptic women and SGA infants are at increased risk of

vascular disorders, we aimed to investigate the association of *KDR -604T/C* and *Val297Ile* polymorphisms in pregnancies complicated by preeclampsia, gestational hypertension and SGA infants.

## **Materials and Methods**

This is a nested case control study where participants were recruited from the Screening for Pregnancy Endpoints (SCOPE) study. The SCOPE study is an international, multicenter, prospective cohort study with the aim of developing screening tests to predict preeclampsia, SGA infants and preterm birth across different populations [15]. The participants were recruited at the Lyell McEwin Hospital to the Adelaide cohort of the SCOPE study. Ethics approval was gained from the Central Northern Adelaide Health Service, Ethics of Human Research Committee and the Human Ethics Committee of the University of Adelaide (REC 1712/5/2008).

Nulliparous women with singleton pregnancies attending hospital antenatal clinics, obstetricians, general practitioners or community midwives before 15 weeks of gestation were invited to participate. Consenting women were recruited between September 2005 and September 2008. Exclusion criteria included women considered at high risk for preeclampsia, SGA infants or preterm birth because of underlying medical conditions (chronic hypertension requiring antihypertensive drugs, diabetes, renal disease, systemic lupus erythematosus, antiphospholipid syndrome, sickle cell disease), three or more miscarriages or terminations of pregnancy, previous cervical cone knife biopsy, interventions that could modify pregnancy outcome (such as aspirin, cervical suture) or known major fetal anomaly or abnormal karyotype.

Women were interviewed and examined by a research midwife at  $15 \pm 1$  and  $20 \pm 1$  weeks of gestation. Maternal data collected included demographic information, medical history, previous obstetric history, family history of obstetric complications and medical

disorders. The woman's birthweight and the gestational age at which she was born were obtained. Current pregnancy data included information on any complications during current pregnancy, diet, smoking [15], alcohol and the use of recreational drugs. Maternal physical measurements obtained at 15 weeks of gestation included height, weight and blood pressure. Two consecutive manual blood pressure measurements (using mercury or aneroid sphygmomanometer, with a large cuff size if the arm circumference was  $\geq 33$ cm and Korotkoff V for diastolic blood pressure) were recorded. Proteinuria in a mid stream urine specimen was measured in all women by dipstick or a protein:creatinine ratio.

If the woman was certain of the identity of the infant's father, the father was invited to participate in the SCOPE study. Male participants who agreed to participate provided written informed consent and were interviewed at either the  $15 \pm 1$  or  $20 \pm 1$  weeks' SCOPE visit. Paternal data collected included age, ethnicity, socioeconomic index, birthweight and history of medical disorders. Paternal height, weight, abdominal circumference and blood pressure were measured by the research midwife.

All women were followed prospectively and ultrasound and Doppler studies of the umbilical and uterine arteries were performed at 20 weeks gestation [16]. Mean uterine artery resistance index (RI) was calculated from the left and right uterine artery RI. If only a left or right uterine artery resistance index was available, this was used as 'mean resistance index'. Both umbilical artery RI and mean uterine artery RI  $>90^{\text{th}}$  percentile were considered abnormal.

Pregnancy outcome data and measurements of the infant were recorded by research midwives usually within 72 hours of birth.

## **Outcome measures**

The primary outcomes were *gestational hypertension* defined as systolic blood pressure  $\geq 140$  mmHg or diastolic blood pressure  $\geq 90$  mmHg (Korotkoff V), or both, on at least two occasions four hours apart after 20 weeks' gestation but before the onset of labour; *pre-eclampsia* defined as gestational hypertension or postpartum hypertension with proteinuria (24 hour urinary protein  $>300$ mg or spot urine protein:creatinine ratio  $\geq 30$ mg/mmol creatinine or urine dipstick protein  $\geq ++$ ) or any multisystem complication of preeclampsia [17]. Multisystem complications included any of acute renal insufficiency; effects on liver including raised aspartate transaminase and/or alanine transaminase concentration, and severe right upper quadrant or epigastric pain or liver rupture; neurological effects including eclampsia, imminent eclampsia or cerebral haemorrhage; and haematological effects including thrombocytopenia, disseminated intravascular coagulation or haemolysis; *SGA* defined as a birth weight  $< 10^{\text{th}}$  customised centile adjusted for maternal height, weight, parity and ethnicity, as well as gestational age at delivery and infant sex [18]; *SGA with abnormal Doppler* defined as SGA with uterine and /or umbilical artery RI  $>90^{\text{th}}$  centile. *Uncomplicated pregnancy* was defined as a pregnancy with no antenatal medical or obstetric complications and resulting in the delivery of an appropriately grown, healthy infant at  $\geq 37$  weeks of gestation.

## **Definitions of other pregnancy complications**

*Normotensive SGA* (NSGA) was defined as birth of an SGA infant where the mother did not have hypertension. *Hypertensive SGA* (HSGA) was defined as birth of an SGA infant where the mother had either gestational hypertension or preeclampsia.

### **Gene variants selection**

*KDR -604T/C* (rs2071559) and *Val297Ile* (rs2305948) single nucleotide polymorphisms (SNPs) were selected based on their potential biological functions. The *KDR -604T/C* promoter variant may affect transcriptional factor E2F binding to the region, that may alter *KDR* expression [14]. The variant allele of *KDR -604T/C* is also shown to be associated with lower *KDR* protein levels [13]. The *KDR* exonic variant (exon\_7) rs2305948 results in a nonsynonymous amino acid change at *Val297Ile*. The amino acid is located at the third extracellular Ig-like domain that is important for ligand-receptor binding [13, 14].

### **Genotyping**

Peripheral blood samples were collected from the women and partners and cord blood was collected at delivery. DNA was extracted from buffy coats separated from peripheral venous or cord blood. Genotyping for the *KDR -604T/C* (rs2071559) and *Val297Ile* (rs2305948) polymorphisms was performed at the Australian Genome Research Facility (AGRF, Brisbane, Australia) using the Sequenom MassARRAY system (Sequenom Inc, San Diego, California). As a quality control measure 300 independent samples that were genotyped in-house for the same polymorphisms using quantitative reverse transcription-polymerase chain reaction (qRT-PCR) were genotyped using the Sequenom MassARRAY system at AGRF. The concordance rate of the qRT-PCR results and MassARRAY results was 100%. The primer sequences and cycling conditions for qRT-PCR and the primer sequences for Sequenom MassARRAY system are shown in tables 1 and 2. Each sample was also genotyped for Amelogenin to ensure that the sex of the sample was correct [19]. Parental and neonatal genotype data were checked for a Mendelian pattern of inheritance and those found to be inconsistent were excluded from the analyses.

**Table 1 Sequence of primers used for qRT-PCR**

<b>Polymorphism</b>	<b>Sequence</b>	<b>PCR product length (base pair)</b>
<b><i>KDR-604T/C</i> rs2071559</b>		
Forward primer	5'-CGATGGACAAAAGCCTTCTTG -3'	107
Reverse primer	5'-GAAAACGCACTTGCCCAGTT-3'	
<b><i>KDR Val297Ile</i> rs2305948</b>		
Forward primer	5'-TGGATGCTGCACAGGTGTACA-3'	100
Reverse primer	5'-AAAACCCAGTCTGGGAGTGAGA-3'	

**qRT-PCR for genotyping**

qRT-PCR was performed on a Rotor-Gene™ 6000 real-time PCR machine (Corbett Research, Sydney, Australia). All reactions were set up using a Cass 1200™ liquid handling system (Corbett Robotics, Brisbane, Australia). Each reaction was performed in a total volume of 10µL, containing 1µL 10x PCR buffer Gold (Applied Biosystems, Foster City, CA, USA), 2µL MgCl<sub>2</sub> (Applied Biosystems, Foster City, CA, USA), 0.2µL of dNTP, 0.1 µL of Taq Gold (Applied Biosystems, Foster City, CA, USA), 0.5µL of 20X EvaGreen (Biotium, Inc., Hayward, California, USA), 0.25 µL each of forward and reverse primer, 3.7µL of sterile water for injection and 2µL of DNA. The thermal cycling conditions were 10 minutes at 95°C, then with 40 cycles at 95°C for 15 seconds, 60°C for 10 seconds and 72°C for 10 seconds. Genotypes were determined by High Resolution Melt (HRM) curve analysis.

**Table 2 Sequence of primers used for Sequenom MassARRAY system**

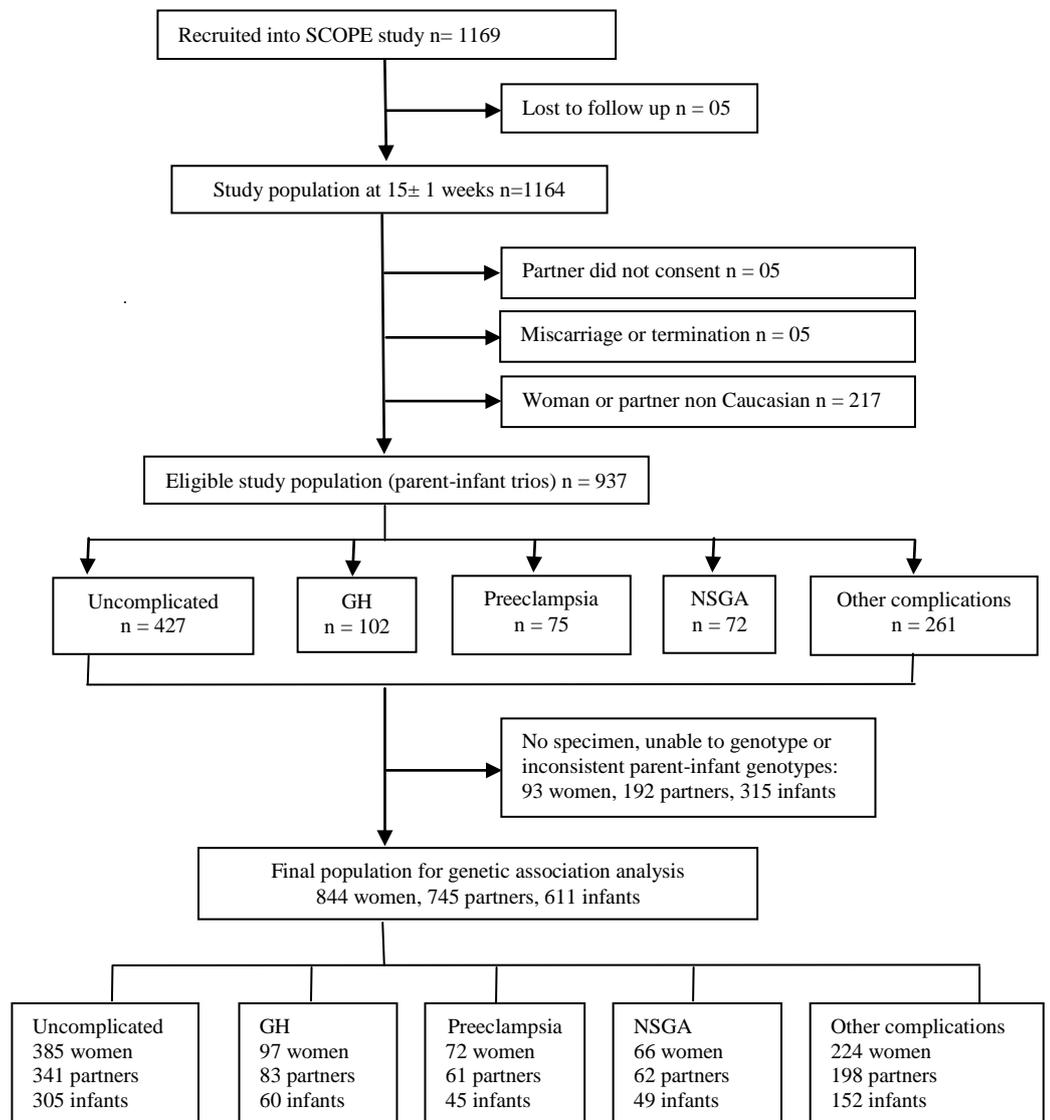
<b>Gene variant</b>	<b>1st PCR primer</b>	<b>2nd PCR primer</b>	<b>Extend primer</b>
<i>KDR</i> rs2071559	ACGTTGGATGTCACCTCAAACCTGGAGCCG	ACGTTGGATGATCAGAAAACGCACTTGCCC	GGGGAAATAGCGGGAATG
<i>KDR</i> rs2305948	ACGTTGGATGAGTCTGGGAGTGAGATGAAG	ACGTTGGATGTGTACAATCCTTGGTCACTC	GCACCTTAACTATAGATGGT

## **Statistics**

Women, partners and infants in the adverse pregnancy outcome groups were compared with women, partners and infants in the uncomplicated pregnancy group in a nested case control study design. Missing data were excluded from the analyses. The chi-square test was used to test the genotypes at each polymorphic locus for Hardy-Weinberg Equilibrium and to compare categorical variables. ANOVA was used to compare continuous variables. Adjusted and non-adjusted odds ratios were calculated for the genotype frequencies in adverse pregnancy outcome groups compared to controls using dominant and recessive genotype models by unconditional logistic regression analysis. The covariates for the logistic regression models for preeclampsia and gestational hypertension included maternal age, body mass index (BMI), mean arterial pressure at 15 weeks gestation, smoking at 15 weeks gestation and paternal age and BMI [20, 21]. The covariates for the logistic regression model for SGA included maternal age, BMI, birthweight, smoking, and paternal BMI and birthweight [22-24]. All data analyses were performed using PASW version 17.02 (SPSS, Inc, Cary, North Carolina). Results were reported as number and percent [n (%)] or mean  $\pm$  standard deviation (SD) where appropriate.  $P < 0.05$  was considered statistically significant.

## **Results**

Of those recruited, 620 women, 547 partners and 459 infants were included in the case control study. The exclusions are detailed in Figure 1.



**Figure 1** Study population

Amongst 937 eligible Caucasian pregnancies, 427 were uncomplicated (45.6%), 75 developed preeclampsia (8.0%), 102 developed gestational hypertension (10.9%), 72 had SGA infants (7.7%) in the absence of maternal hypertensive disease and the remaining 261 pregnancies developed other obstetric, medical or surgical complications during pregnancy (27.8%). Of the 75 preeclamptic women, 18 (24.0%) developed preterm preeclampsia requiring delivery before 37 weeks of gestation and 57 (76.0%) developed term disease. Sixty one percent (n = 11) of the women who had preterm preeclampsia, 12.3% (n = 7) of the women who had term preeclampsia and 18.6% (n = 19) of the women who developed gestational hypertension delivered a SGA infant. Maternal and paternal characteristics, Doppler results and pregnancy outcome data in relation to adverse pregnancy outcomes are detailed in table 3.

**Table 3 Characteristics of the study population**

	<b>Uncomplicated pregnancy (n = 427)</b>	<b>Preeclampsia (n = 75)</b>	<b>P</b>	<b>GH (n = 102)</b>	<b>P</b>	<b>SGA (n = 109)</b>	<b>P</b>
<b>Maternal characteristics</b>							
Age (years)	23.6 ± 4.9	24.1 ± 4.3	0.4	24.3 ± 5.0	0.1	24.3 ± 5.3	0.1
BMI (kg/m <sup>2</sup> )	25.9 ± 5.6	29.4 ± 8.0	<b>&lt;0.001</b>	30.5 ± 6.7	<b>&lt;0.001</b>	26.3 ± 5.7	<b>0.01</b>
Birthweight	3287 ± 549	3198 ± 562	0.2	3268 ± 611	0.7	3136 ± 544	<b>0.01</b>
Mean arterial pressure at 15 weeks	78.3 ± 6.9	82.8 ± 7.9	<b>&lt;0.001</b>	85.1 ± 8.1	<b>&lt;0.001</b>	81.2 ± 8.6	<b>&lt;0.001</b>
Smoking at 15 weeks	90 (21.1%)	11 (14.7%)	0.2	19 (18.6%)	0.6	48 (44.0%)	<b>&lt;0.001</b>
<b>Paternal characteristics</b>							
Age	26.4 ± 6.1	26.8 ± 5.1	0.5	28.3 ± 6.6	<b>0.004</b>	27.6 ± 6.3	0.057
BMI (kg/m <sup>2</sup> )	26.9 ± 4.9	28.7 ± 6.1	<b>0.005</b>	27.6 ± 5.1	0.2	27.3 ± 5.0	0.4
Birthweight (g)	3462 ± 593	3490 ± 522	0.7	3385 ± 700	0.3	3347 ± 558	0.08
<b>Uterine artery Doppler at 20 weeks</b>							
Abnormal mean uterine artery RI	37 (8.8%)	16 (21.3%)	<b>&lt;0.001</b>	11 (11.0%)	0.5	27 (25.0%)	<b>&lt;0.001</b>
Abnormal umbilical artery RI	54 (12.8%)	14 (20%)	0.2	14 (14.0%)	0.7	18 (16.7%)	0.3
<b>Pregnancy outcome</b>							
Neonatal birth weight (g)	3575 ± 390	3075 ± 754	<b>&lt;0.001</b>	3337 ± 520	<b>&lt;0.001</b>	2554 ± 536	<b>&lt;0.001</b>
Customised birthweight centile	54 ± 25	43 ± 32	<b>&lt;0.001</b>	39 ± 30	<b>&lt;0.001</b>	4 ± 3	<b>&lt;0.001</b>
Gestational age at delivery (weeks)	39.7 ± 1.2	37.7 ± 2.4	<b>&lt;0.001</b>	39.2 ± 1.4	<b>&lt;0.001</b>	38.4 ± 3.4	<b>&lt;0.001</b>

Data are either N (%) or Mean ± SD; comparisons using Pearson chi-square or ANOVA; GH, Gestational hypertension; SGA includes both normotensive

and hypertensive SGA; *P* values in bold are significant

Genotype data of 3 (4.0%) women, 14 (18.7%) partners and 20 (26.7%) infants in the preeclampsia group; 5 (4.9%) women, 19 (18.6%) partners and 42 (41.2%) infants in the gestational hypertension group; 2 (7.3%) women, 11 (10.9%) partners and 40 (36.7%) infants in the SGA group and 42 (4.9%) women, 83 (19.4%) partners and 122 (28.6%) infants in the uncomplicated pregnancy group could not be analysed due to non availability of samples, genotyping failure and Mendelian inconsistencies in parent-infant genotypes. Both *KDR -604T/C* and *Val297Ile* polymorphisms were in Hardy Weinberg Equilibrium.

### **Genotype distribution associated with preeclampsia and gestational hypertension**

Homozygosity for the variant allele of the *KDR -604T/C* polymorphism was increased in fathers and infants in the preeclampsia group compared to fathers and infants in the uncomplicated pregnancy group (Table 4). Maternal *KDR -604T/C* polymorphism approached significance for an association with preeclampsia ( $p = 0.07$ , table 4). Maternal, paternal and infant *KDR Val297Ile* polymorphisms were not associated with preeclampsia (Table 5). *KDR -604T/C* and *Val297Ile* polymorphisms in father, mother and the infant were not associated with gestational hypertension (Tables 4 and 5).

### **Genotype distribution associated with SGA**

Homozygosity for the variant allele of the *KDR -604T/C* polymorphism was increased in fathers and infants in the SGA group compared to fathers and infants in the uncomplicated pregnancy group (Table 4). Homozygosity for the variant allele of the *KDR -604T/C* polymorphism was increased in fathers and infants in the SGA group with abnormal uterine and/or umbilical artery Doppler, compared to fathers and infants in the uncomplicated pregnancy group (Table 4). Maternal *KDR -604T/C* was not associated with SGA and approached significance for an association with SGA with

abnormal uterine and/or umbilical artery Doppler ( $p = 0.07$ , table 4). Maternal, paternal and infant *KDR Val297Ile* was not associated with either SGA or SGA with abnormal Doppler (Table 5). Post-hoc analysis on the SGA sub groups demonstrated that the paternal and infant *KDR -604T/C* SNP was associated with hypertensive SGA but not with normotensive SGA (data not presented).

**Table 4 Distribution of maternal, paternal and neonatal *KDR -604T/C* polymorphism in adverse pregnancy outcomes and uncomplicated pregnancy**

<i>KDR -604T/C</i> variant	Genotype n (%)			Dominant model CT+CC vs TT		Recessive model CC vs TT+CT	
	TT	CT	CC	OR (95% CI)	aOR (95% CI)	OR (95% CI)	aOR (95% CI)
<i>Maternal KDR-604T/C</i>							
Uncomplicated pregnancy	102 (26.5)	189 (49.1)	94 (24.4)	1.0 (ref)	1.0 (ref)	1.0 (ref)	1.0 (ref)
Preeclampsia	14 (19.4)	34 (47.3)	24 (33.3)	1.5 (0.8-2.7)	1.5 (0.8-2.9)	1.1 (0.9-1.3)	1.6 (0.9-2.8)
Gestational hypertension	32 (33.0)	38 (39.2)	27 (27.8)	0.7 (0.5-1.2)	0.7 (0.4-1.2)	1.2 (0.7-1.9)	1.3 (0.7-2.4)
SGA	21 (20.8)	50 (49.5)	30 (29.7)	1.3 (0.8-1.9)	1.3 (0.9-1.9)	1.1 (0.9-1.2)	1.2 (0.9-1.7)
SGA with abnormal Doppler	6 (15)	20 (50)	14 (35)	2 (0.8-5.0)	1.9 (0.8-5.0)	1.7 (0.8-3.3)	1.7 (0.9-3.1)
<i>Paternal KDR-604T/C</i>							
Uncomplicated pregnancy	93 (27.3)	172 (50.4)	76 (22.3)	1.0 (ref)	1.0 (ref)	1.0 (ref)	1.0 (ref)
Preeclampsia	12 (19.7)	28 (45.9)	21 (34.4)	1.5 (0.8-3.0)	1.7 (0.8-3.4)	<b>1.8 (1.0-3.3)</b>	<b>1.6 (1.0-3.0)</b>
Gestational hypertension	17 (20.5)	40 (48.2)	21 (31.3)	1.3 (0.7-2.4)	1.5(0.8-2.8)	1.3 (0.7-2.3)	1.3 (0.7-2.3)
SGA	23(23.4)	38 (38.8)	37 (37.8)	1.2 (0.7-2.1)	1.2 (0.9-2.4)	<b>2.1 (1.3-3.4)</b>	<b>1.9 (1.1-3.3)</b>
SGA with abnormal Doppler	6 (15.4)	17 (43.6)	16 (41)	2.1 (0.8-5.1)	3.2 (0.9-11.1)	<b>2.4 (1.2-4.8)</b>	<b>2.7 (1.2-5.9)</b>

aOR (95% CI) for preeclampsia and gestational hypertension are adjusted for maternal age, BMI, mean arterial pressure at 15 weeks gestation, smoking at 15 weeks gestation, and paternal BMI; aOR (95% CI) for SGA and SGA with abnormal Doppler are adjusted for maternal age, BMI, birthweight, smoking at 15 weeks gestation, and paternal BMI and birthweight; ref, referent; OR (95% CI) values in bold are significant

**Table 4 Distribution of maternal, paternal and neonatal *KDR -604T/C* polymorphism in adverse pregnancy outcomes and uncomplicated pregnancy - Continued**

<i>KDR -604T/C</i> variant	Genotype n (%)			Dominant model CT+CC vs TT		Recessive model CC vs TT+CT	
	TT	CT	CC	OR (95% CI)	aOR (95% CI)	OR (95% CI)	aOR (95% CI)
Neonatal <i>KDR-604T/C</i>							
Uncomplicated pregnancy	87 (28.5)	154 (50.5)	64 (21.0)	1.0 (ref)	1.0 (ref)	1.0 (ref)	1.0 (ref)
Preeclampsia	12 (26.7)	17 (37.8)	16 (35.6)	1.1 (0.6-1.8)	1.2 (0.6-1.7)	<b>2.1 (1.1-4.1)</b>	<b>2.2 (1.0-4.4)</b>
Gestational hypertension	15 (25.0)	27 (45.0)	18 (30.0)	1.2 (0.6-2.3)	1.4 (0.7-2.8)	1.6 (0.9-3.0)	1.5 (0.7-3.0)
SGA	12 (17.4)	32 (46.4)	25 (36.2)	1.6 (0.9-2.8)	1.4 (0.9-2.9)	<b>2.1 (1.2-3.8)</b>	<b>2.2 (1.2-3.9)</b>
SGA with abnormal Doppler	2 (7.7)	12 (46.2)	12 (46.2)	<b>4.8 (1.1-20.7)</b>	<b>4.3 (1.0-18.9)</b>	<b>3.2 (1.4-7.3)</b>	<b>3.2 (1.2-5.9)</b>

aOR (95% CI) for preeclampsia and gestational hypertension are adjusted for maternal age, BMI, mean arterial pressure at 15 weeks gestation, smoking at 15 weeks gestation, and paternal BMI; aOR (95% CI) for SGA and SGA with abnormal Doppler are adjusted for maternal age, BMI, birthweight, smoking at 15 weeks gestation, and paternal BMI and birthweight; ref, referent; OR (95% CI) values in bold are significant

**Table 5 Distribution of maternal, paternal and neonatal *KDR Val297Ile* polymorphism in adverse pregnancy outcomes and uncomplicated pregnancy**

<i>KDR Val297Ile</i> variant	Genotype n (%)			Dominant model CT + TT vs CC		Recessive model TT vs CC + CT	
	CC	CT	TT	OR (95% CI)	aOR (95% CI)	OR (95% CI)	aOR (95% CI)
<i>Maternal KDR Val297Ile</i>							
Uncomplicated pregnancy	308 (82.6)	62 (16.6)	3 (0.8)	1.0 (ref)	1.0 (ref)	1.0 (ref)	1.0 (ref)
Preeclampsia	54 (78.3)	14 (20.3)	1 (1.4)	1.3 (0.7-2.5)	1.3 (0.9-2.5)	1.8 (0.2-17.7)	1.7 (0.2-18.2)
Gestational hypertension	77 (80.2)	18 (18.8)	1 (1.0)	1.2 (0.7-2.1)	1.4 (0.7-2.5)	1.3 (0.1-12.6)	1.3 (0.1-12.5)
SGA	82 (81.2)	19 (18.8)	0 (0.0)	1.1 (0.6-1.9)	1.1 (0.6-1.9)	0.5 (0.03-10.2)	0.6 (0.03-11.2)
SGA + abnormal Doppler	33 (82.5)	7 (17.5)	0 (0.0)	1.0 (0.4-2.4)	1.1 (0.4-2.4)	1.3 (0.07-25.7)	1.2 (0.07-26.3)
<i>Paternal KDR Val297Ile</i>							
Uncomplicated pregnancy	253 (77.4)	71 (21.7)	3 (0.9)	1.0 (ref)	1.0 (ref)	1.0 (ref)	1.0 (ref)
Preeclampsia	42 (72.4)	15 (25.9)	1 (1.7)	1.3 (0.7-2.4)	1.5 (0.8-2.4)	1.9 (0.2-18.6)	1.9 (0.2-18.8)
Gestational hypertension	57 (75.0)	18 (23.7)	1 (1.3)	1.1 (0.6-2.0)	1.1 (0.6-2.5)	1.4 (0.1-14.1)	1.2 (0.1-14.6)
SGA	70 (74.5)	23 (24.5)	1 (1.1)	1.2 (0.7-2.0)	1.1 (0.7-2.0)	1.2 (0.1-11.3)	1.2 (0.1-11.3)
SGA + abnormal Doppler	29 (78.4)	8 (21.6)	0 (0.0)	0.9 (0.4-2.2)	0.9 (0.5-2.4)	1.2 (0.06-24.4)	1.3 (0.08-26.2)

aOR (95% CI) for preeclampsia and gestational hypertension are adjusted for maternal age, BMI, mean arterial pressure at 15 weeks gestation, smoking at 15 weeks gestation, and paternal BMI; aOR (95% CI) for SGA and SGA with abnormal Doppler are adjusted for maternal age, BMI, birthweight, smoking at 15 weeks gestation, and paternal BMI and birthweight; ref, referent

**Table 5 Distribution of maternal, paternal and neonatal *KDR Val297Ile* polymorphism in adverse pregnancy outcomes and uncomplicated pregnancy – Continued**

<i>KDR Val297Ile</i> variant	Genotype n (%)			Dominant model CT + TT vs CC		Recessive model TT vs CC + CT	
	CC	CT	TT	OR (95% CI)	aOR (95% CI)	OR (95% CI)	aOR (95% CI)
Neonatal <i>KDR Val297Ile</i>							
Uncomplicated pregnancy	246 (83.1)	46 (15.5)	4 (1.4)	1.0 (ref)	1.0 (ref)	1.0 (ref)	1.0 (ref)
Preeclampsia	32 (72.7)	10 (22.7)	2 (4.5)	1.8 (0.9-3.8)	1.8 (0.8-3.7)	3.5 (0.6-19.6)	3.2 (0.7-18.4)
Gestational hypertension	44 (75.9)	14 (24.1)	0 (0.0)	1.6 (0.8-3.1)	1.7 (0.8-3.2)	0.6 (0.02-10.5)	0.6 (0.02-10.5)
SGA	50 (76.9)	14 (21.5)	1 (1.5)	1.5 (0.8-2.8)	1.5 (0.8-2.8)	1.1 (0.1-10.4)	1.2 (0.1-10.4)
SGA + abnormal Doppler	18 (69.2)	7 (26.9)	1 (3.8)	2.2 (0.9-5.3)	2.1 (0.9-5.5)	2.9 (0.3-27.1)	2.7 (0.3-26.5)

aOR (95% CI) for preeclampsia and gestational hypertension are adjusted for maternal age, BMI, mean arterial pressure at 15 weeks gestation, smoking at 15 weeks gestation, and paternal BMI; aOR (95% CI) for SGA and SGA with abnormal Doppler are adjusted for maternal age, BMI, birthweight, smoking at 15 weeks gestation, and paternal BMI and birthweight; ref, referent

### **Interaction between maternal *KDR -604T/C* polymorphism and smoking**

As a post hoc analysis, we stratified the cohort by maternal smoking at 15 weeks of gestation and investigated the association between the maternal *KDR -604T/C* polymorphism and adverse pregnancy outcomes. The prevalence of the maternal *KDR -604T/C* CC genotype was increased in preeclampsia (Table 6), SGA and SGA with abnormal Doppler (Table 6) in women who did not smoke at 15 weeks of gestation. Maternal *KDR -604T/C* polymorphism was not associated with any of the adverse pregnancy outcomes in women who continued to smoke at 15 weeks of gestation (data not presented). Maternal *KDR -604T/C* polymorphism was not associated with gestational hypertension in either smokers or non-smokers (Table 6). Associations between paternal and neonatal *KDR -604T/C* polymorphisms with adverse pregnancy outcomes were unaffected by maternal smoking (data not presented).

**Table 6 Distribution of maternal *KDR -604T/C* polymorphism in adverse pregnancy outcomes and uncomplicated pregnancy in non-smokers at 15 weeks of gestation**

Maternal <i>KDR -604T/C</i> variant	Genotype n (%)			Dominant model CC + CT vs TT	Recessive model CC vs TT + CT
	TT	CT	CC	OR (95% CI)	OR (95% CI)
Uncomplicated pregnancy	85 (28.1)	144 (47.7)	73 (24.2)	1.0 (ref)	1.0 (ref)
Preeclampsia	12 (19.7)	27 (44.2)	22 (36.1)	1.6 (0.8-3.2)	<b>2.1 (1.1-3.8)</b>
Gestational hypertension	25 (31.6)	31 (39.2)	23 (29.2)	0.8 (0.5-1.3)	1.3 (0.7-2.2)
SGA	8 (14.3)	26 (46.4)	22 (39.3)	<b>2.4 (1.1-5.2)</b>	<b>2.0 (1.1-3.7)</b>
SGA with abnormal Doppler	3 (13.0)	10 (43.5)	10 (43.5)	2.6 (0.8-9.0)	<b>2.4 (1.0-5.7)</b>

ref; referent; OR (95% CI) values in bold are significant

## Discussion

To our knowledge this is the first study to investigate the association of *KDR -604T/C* and *Val297Ile* polymorphisms in pregnancy complications. Our preliminary data demonstrate that homozygosity for the C allele of the *KDR -604T/C* polymorphism in both the father and the infant is associated with preeclampsia and small for gestational age pregnancies.

There is growing evidence that the father plays a significant role in the causation of both these pregnancy complications. The risk of fathering a preeclamptic pregnancy is increased among men who fathered a preeclamptic pregnancy with a different partner [25] and among men who were themselves the product of a preeclamptic pregnancy [26]. A paternal contribution to SGA has also been previously suggested by a positive correlation of paternal birthweight with infant birthweight [22, 27, 28] and that men who were SGA at birth are more likely than those with a normal birthweight to parent an SGA infant [22, 29]. In our study the paternal *KDR -604T/C* polymorphism remained associated with both preeclampsia and SGA after adjusting for established maternal and paternal risk factors for both disorders, demonstrating an independent association between the CC genotype of the *KDR -604T/C* polymorphism in the father with both pregnancy complications.

KDR is the main receptor for VEGF-A. Homozygous *KDR* gene (*Flk* in mice) ablation in mice results in failure to form mature endothelial cells leading to embryonic death demonstrating that this receptor is essential for embryonic and placental angiogenesis [12]. Placental expression of KDR is intense during early gestation and VEGF-A acting via KDR plays a key role in regulating angiogenesis and spiral artery remodelling [10]. Impaired spiral artery remodelling and angiogenesis are demonstrated in many cases of both preeclampsia and SGA pregnancies [8].

Our results demonstrate that the neonatal *KDR -604T/C CC* genotype is associated with both pregnancy complications and that the association is stronger for SGA with abnormal uterine and/or umbilical artery Doppler. As the infant's genotype is likely to represent the placental genotype, homozygosity for the C allele of the *KDR -604T/C* polymorphism may contribute to impaired placental vascular development.

In this study, the maternal *KDR -604T/C* polymorphism was not associated with preeclampsia or with SGA. This is an unexpected finding considering that *KDR* is not an imprinted gene. However, when the cohort was stratified by maternal smoking at 15 weeks of gestation we found a significant association between the maternal *KDR -604T/C CC* genotype and preeclampsia, SGA and SGA with abnormal Doppler in non-smokers. This may be due to the increase in expression of angiogenic growth factors in smokers [30].

We did not find a significant association of the *KDR -604T/C* polymorphism with gestational hypertension if not complicated by SGA or with SGA in the absence of maternal hypertensive disease. These are expected findings as the pathogenesis of these complications is different and placental insufficiency is not an established finding in these disorders.

Previous studies have shown an association between preeclampsia and SGA with later life vascular diseases including increased risk for developing coronary artery disease and stroke [3, 5-7]. These associations are thought to be the consequences of 'programming', whereby an insult during intrauterine life is predicted to have lifelong effects [31]. The placenta is considered a programming agent for future cardiovascular disease and animal models have demonstrated that abnormal endothelial development in the placenta is associated with increased vulnerability to heart disease [32]. Homozygosity for the variant allele of the *KDR -604T/C* polymorphism was previously shown to be associated with increased risk of coronary artery disease [13]. Here we

have shown that homozygosity for the variant allele of the *KDR* -604T/C is increased in fathers and infants in both preeclamptic and SGA pregnancies, providing further evidence for potential shared genetic factors between these pregnancy complications and cardiovascular disease.

The strengths of our study include a prospective cohort with excellent follow-up and collection of data on a large number of variables that enabled us to adjust for potential confounders. A limitation of our preliminary study is that our adverse pregnancy outcome groups were small and hence the study was relatively underpowered. We also excluded a number of cases and controls due to non availability of genotype results, and it is possible that this may have introduced bias into our results. A parent-infant trio analysis may provide substantial support for the apparent association seen in the case-control analysis. We were not able to perform a family based association analysis due to the small sample size in our preliminary cohort.

Previous studies have also demonstrated the association of other polymorphisms (Cys482Arg and Pro1147Ser) of the *KDR* gene with vascular diseases [33, 34] which suggests that a more comprehensive evaluation across the *KDR* gene investigating the possibility of linkage disequilibrium between polymorphisms may be beneficial. The novel association of a polymorphism in both the father and the infant with preeclampsia and SGA in this study is an interesting finding that needs to be replicated in a larger cohort.

In conclusion, the *KDR* -604T/C polymorphism which is a risk factor for coronary artery disease is associated with preeclampsia and SGA infants, suggesting that this polymorphism may associate with the risk of vascular disorders across the life course. Our data also demonstrate that there is a significant paternal genetic association with both preeclampsia and SGA pregnancies.

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## Manuscript 4

### **A functional variant in *ANGPT1* and the risk of pregnancies with hypertensive disorders and small for gestational age infants**

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#### **Abstract**

Pregnancies complicated by preeclampsia and small for gestational age (SGA) infants demonstrate impaired placental vascular remodelling. Angiopoietin-1 is an angiogenic growth factor that regulates vascular integrity and remodelling. The TT genotype of *angiopoietin 1 (ANGPT1) 1414T/A* polymorphism has been associated with increased plasma angiopoietin-1 levels compared to the AA genotype. We aimed to investigate the association between *ANGPT1 1414T/A* polymorphism and pregnancies complicated by gestational hypertensive disorders and SGA infants. We also aimed to investigate whether the polymorphism was associated with abnormal uterine artery Doppler as a surrogate marker of impaired placental vascular remodelling.

Genotyping data of 1361 nulliparous pregnant women, 1226 partners and 1190 infants were analysed. The prevalence of *ANGPT1 1414T/A* TT genotype was reduced in

women with preeclampsia (adjusted OR [aOR] 0.5, 95% CI 0.3-0.9,  $p = 0.01$ ), hypertensive SGA (aOR 0.5, 95% CI 0.2-0.9,  $p = 0.04$ ) and SGA with abnormal uterine artery Doppler findings (aOR 0.4, 95% CI 0.2-0.8,  $p = 0.009$ ) compared to women with uncomplicated pregnancy. The prevalence of maternal *ANGPT1 1414T/A* TT genotype was reduced in women with increased uterine artery resistance index (aOR 0.7, 95% CI 0.5-0.9,  $p = 0.03$ ) and bilateral notching of the uterine arteries (aOR 0.6, 95% CI 0.4-0.9,  $p = 0.004$ ).

Maternal *ANGPT1 1414T/A* TT genotype is associated with a reduced risk for preeclampsia, hypertensive SGA and abnormal uterine artery Doppler. These findings suggest that the TT genotype may protect against these pregnancy disorders by increasing angiopoietin-1 production at the maternal-fetal interface. The *ANGPT1 1414T/A* polymorphism may have a potential role in screening women to predict the risk of these pregnancy complications.

## Introduction

Preeclampsia, gestational hypertension and small for gestational age (SGA) infants complicate approximately 15-20% of all nulliparous pregnancies [1, 2]. Women who develop preeclampsia or deliver SGA infants are at increased risk for later life vascular disorders including coronary artery disease and stroke [3].

The cause of these pregnancy complications remains largely unknown but impaired maternal spiral artery remodelling is implicated in the pathogenesis of preeclampsia and SGA pregnancies [4, 5]. These abnormalities result in increased vascular impedance in the uterine circulation which is detected as an increased uterine artery resistance index on Doppler velocimetry [6, 7].

Many molecular pathways are involved in the regulation of maternal spiral artery remodelling, of which the angiotensin family mediated angiogenic pathway is recognized as playing a key role [8]. During pregnancy, angiotensin-1 (ANG-1) and its receptor Tie-2 are expressed in many cells at the maternal-fetal interface [9, 10]. *Angiotensin 1 (ANGPT1)* gene ablation in mice results in severe angiogenic defects and embryonic lethality further demonstrating that *ANGPT1* has a critical role in embryonic angiogenesis [11].

A single nucleotide polymorphism (SNP) located in the microRNA-211 (miRNA-211) target site in the 3' UTR of *ANGPT1* is found in approximately 58% of Caucasian populations (*ANGPT1 1414T/A* [rs2507800], NCBI db SNP database). The A allele is known to suppress angiotensin-1 translation by facilitating miRNA-211 binding while the T allele is resistant to miRNA -211 induced reduction in translation. The TT genotype is also associated with higher plasma angiotensin-1 levels compared to the AA genotype [12]. The TT genotype was recently shown to be associated with a reduced risk of stroke in two independent large cohorts [12].

Considering the role of angiotensin-1 in placental vascular remodelling, the consistent association of *ANGPT1 1414T/A* polymorphism with stroke and the data showing that women who develop preeclampsia or deliver SGA infants are at a higher risk of stroke, we aimed to investigate the association between the *ANGPT1 1414T/A* polymorphism and pregnancies complicated by preeclampsia, gestational hypertension and SGA infants. We also aimed to investigate the association of this polymorphism with abnormal uterine artery Doppler as a surrogate marker of impaired spiral artery remodelling.

## **Materials and Methods**

### **Study population**

We conducted a nested case control study where participants were recruited from the SCOPE (Screening for Pregnancy Endpoints) study. The SCOPE study is an international, multicenter, prospective cohort study with the aim of developing screening tests to predict preeclampsia, SGA infants and preterm birth across different populations. Ethics approval was gained from local ethics committees and all participants provided written informed consent.

Nulliparous women with singleton pregnancies were recruited between November 2004 and September 2008 in Adelaide, Australia and Auckland, New Zealand. Women considered at high risk of preeclampsia, SGA infants or preterm birth because of underlying medical, obstetric or gynaecological conditions were not eligible [13]. Women were interviewed and examined by a research midwife at  $15 \pm 1$  and  $20 \pm 1$  weeks of gestation. Maternal data on demographics, medical history, obstetric history, family history of obstetric and medical disorders as well as current pregnancy data on any complications during pregnancy, diet, smoking, alcohol and the use of recreational drugs were collected. Maternal height, weight and blood pressure were measured at 15

weeks gestation. Proteinuria in a mid stream urine specimen was measured by dipstick or a protein:creatinine ratio.

If the woman was certain of the identity of the infant's father, the father was invited to participate in the study. Consenting male participants were interviewed at either the 15  $\pm$  1 or 20  $\pm$  1 weeks' visit. Paternal data collected included age, ethnicity and birthweight. Paternal height and weight were also measured.

All women were followed prospectively and Doppler ultrasound of the uterine arteries was performed at 20 weeks gestation [14]. Mean uterine artery resistance index (RI) was calculated from the left and right uterine RI. Notching of each uterine artery was also recorded. A mean uterine artery RI  $>90^{\text{th}}$  percentile was considered abnormal. Pregnancy outcome data and measurements of the infant were recorded within 72 hours of birth.

The primary outcomes were *gestational hypertension* defined as systolic blood pressure  $\geq 140$ mmHg and/or diastolic blood pressure  $\geq 90$  mmHg on two or more measurements 6 hours apart after 20 weeks' gestation but before the onset of labour; *preeclampsia* defined as gestational hypertension or postpartum hypertension with proteinuria (24 hour urinary protein  $>300$ mg or spot urine protein:creatinine ratio  $\geq 30$ mg/mmol creatinine or urine dipstick protein  $\geq ++$ ) or any multisystem complication of preeclampsia [15]; *SGA* defined as a birth weight  $< 10^{\text{th}}$  customised percentile adjusted for maternal height, weight, parity and ethnicity, as well as gestational age at delivery and infant sex [16]; *normotensive SGA*, defined as birth of an SGA infant where the mother did not have hypertension; *hypertensive SGA*, defined as birth of an SGA infant where the mother had either gestational hypertension or preeclampsia, *SGA with abnormal Doppler* defined as SGA with mean uterine artery RI  $>90^{\text{th}}$  percentile. *Uncomplicated pregnancy* was defined as a pregnancy with no antenatal medical or

obstetric complications and resulting in the delivery of an appropriately grown, healthy infant at  $\geq 37$  weeks of gestation.

Peripheral blood samples were collected from the women. Peripheral blood, buccal swabs or saliva samples were collected from partners. Cord blood, buccal swabs or saliva samples were collected from infants. The buccal swabs were applied to Whatman FTA cards (Whatman Inc, Piscataway, NJ, USA) immediately following sample collection and saliva was collected using Oragene kits (DNA Genotek Inc, Kanata, Ontario, Canada).

### **Genotyping**

DNA was extracted from buffy coats from peripheral or cord blood, Whatman FTA cards or from saliva (Oragene<sup>®</sup> DNA kits) according to the manufacturers' instructions. Genotyping for the *ANGPT1 1414T/A* (rs2507800) polymorphism was performed at the Australian Genome Research Facility (AGRF, Brisbane, Australia) using the Sequenom MassARRAY system (Sequenom Inc, San Diego, California). The primer sequences were, 1<sup>st</sup> PCR primer 5'-ACGTTGGATGGGGAGAAAAATTGGCAAAC-3', 2<sup>nd</sup> PCR primer 5'-ACGTTGGATGTTCTTAGTCAGGTGACTATG-3' and extension primer 5'-GGCAAACACTATTATATGTAAGGGA-3'. As a quality control measure, each sample was also genotyped for Amelogenin to ensure that the sex of the sample was correct [17].

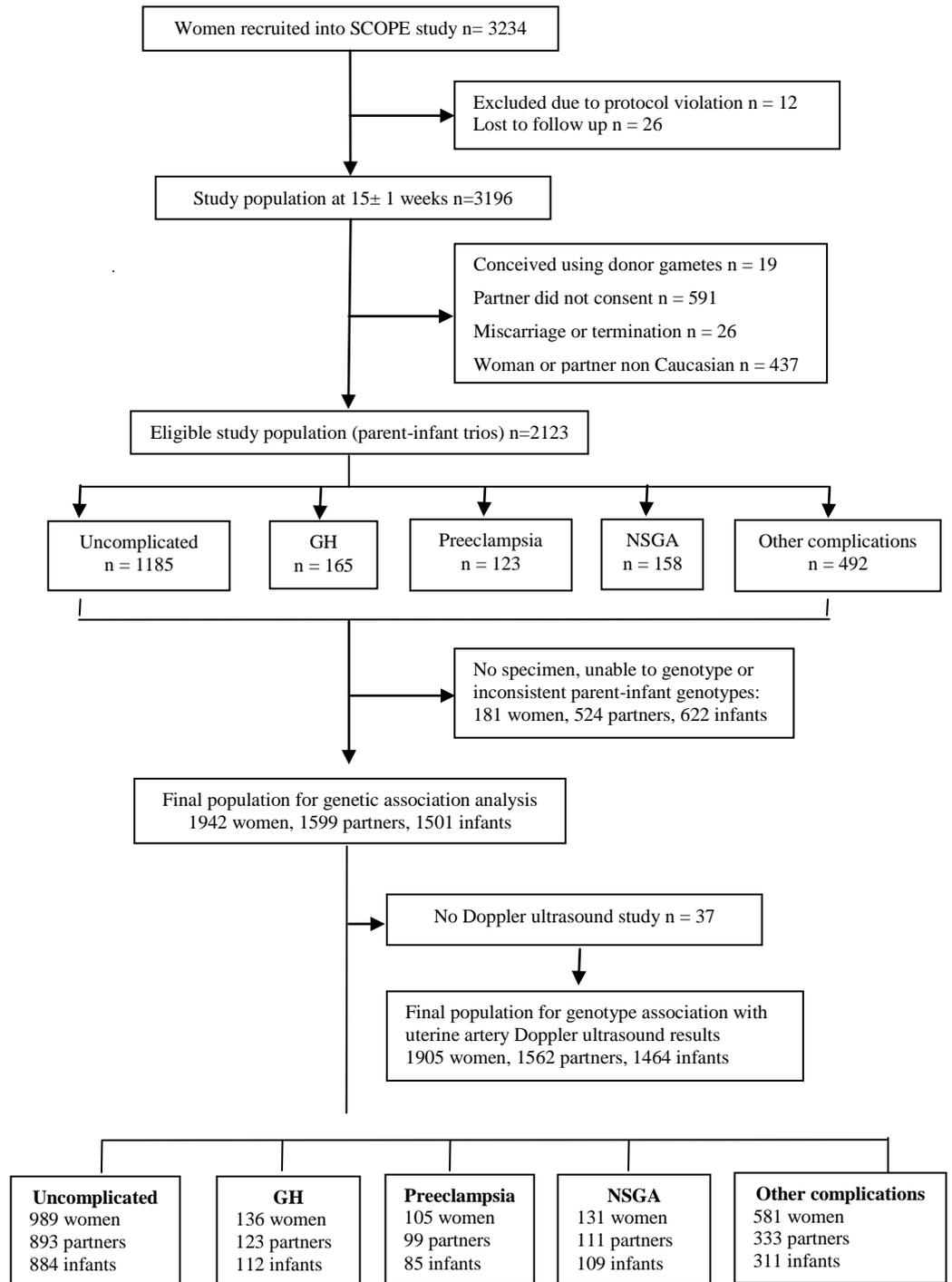
### **Statistics**

Women, partners and infants in the adverse pregnancy outcome groups (cases) were compared with women, partners and infants in the uncomplicated pregnancy group (control subjects) in a nested case control study design. Missing data were excluded from the analyses. The Chi-square test was used to test the genotypes at the

polymorphic locus for Hardy-Weinberg Equilibrium and to compare categorical variables. ANOVA or Student's *t*-test was used to compare continuous variables. Adjusted and non-adjusted odds ratios were calculated for the genotype frequencies in the adverse pregnancy outcome groups compared to the uncomplicated pregnancy group using additive, dominant and recessive genotype models by unconditional logistic regression analysis adjusting for previously established risk factors [15, 18, 19]. A false discovery rate (FDR) correction was performed to adjust for multiple comparisons controlling the FDR at 15% [20]. All data analyses were performed using PASW, version 17.02 (SPSS, Inc, Cary, North Carolina). Results were reported as number and percent [n (%)] or mean  $\pm$  standard deviation (SD) where appropriate.  $P < 0.05$  was considered statistically significant.

## **Results**

After the exclusion criteria detailed in figure 1, genotyping data of 1361 women, 1226 partners and 1190 infants were analysed.



**Figure 1** Study population. GH, Gestational hypertension; NSGA, Normotensive SGA

Maternal and paternal characteristics, Doppler results and pregnancy outcome data in relation to adverse pregnancy outcomes are detailed in table 1.

**Table 1 Characteristics of the study population**

Characteristic	Uncomplicated Pregnancy (n = 989)	Preeclampsia (n = 105)	<i>P</i> value	GH (n = 136)	<i>P</i> value	NSGA (n = 131)	<i>P</i> value
<b>Maternal characteristics</b>							
Age (years)	28.2 ± 5.6	27.3 ± 5.2	0.07	27.4 ± 6.4	<b>0.1</b>	28.9 ± 5.9	0.1
BMI (kg/m)	24.9 ± 4.5	28.4 ± 7.2	<b>&lt;0.001</b>	28.9 ± 6.1	<b>&lt;0.001</b>	25.3 ± 5.0	0.3
Birthweight (g)	3335 ± 530	3190 ± 551	<b>0.007</b>	3253 ± 605	<b>0.08</b>	3173 ± 533	<b>0.001</b>
Mean arterial pressure at 15 weeks	77.6 ± 7.4	83.6 ± 7.7	<b>&lt;0.001</b>	84.8 ± 7.3	<b>&lt;0.001</b>	78.3 ± 7.5	0.3
Smoking at 15 weeks gestation	91 (9.2)	9 (8.6)	0.9	20 (14.7)	<b>0.04</b>	27 (20.6)	<b>&lt;0.001</b>
Socio economic index	42.2 ± 16.5	37.7 ± 16.3	<b>0.006</b>	36.2 ± 16.7	<b>&lt;0.001</b>	39.9 ± 16.9	0.1
<b>Paternal characteristics</b>							
Age (years)	30.7 ± 6.3	29.3 ± 5.6	<b>0.01</b>	30.3 ± 6.5	0.4	31.4 ± 6.4	0.2
BMI (kg/m)	26.6 ± 4.0	28.4 ± 5.5	<b>&lt;0.001</b>	27.7 ± 4.4	<b>0.002</b>	27.0 ± 4.5	0.2
Birthweight (g)	3488 ± 569	3489 ± 561	0.9	3419 ± 638	0.2	3287 ± 520	<b>&lt;0.001</b>
<b>Doppler studies at 20 weeks</b>							
Abnormal mean uterine artery RI	63 (6.4)	19 (18.1)	<b>&lt;0.001</b>	15 (11.0)	<b>0.04</b>	22 (16.8)	<b>&lt;0.001</b>
Bilateral uterine artery notching	95 (9.6)	20 (19.1)	<b>0.001</b>	15 (11.0)	0.5	17 (12.9)	0.2
<b>Pregnancy outcome</b>							
Birthweight (g)	3596 ± 393	3076 ± 714	<b>&lt;0.001</b>	3342 ± 540	<b>&lt;0.001</b>	2688 ± 482	<b>&lt;0.001</b>
Customised birthweight centile	54 ± 25	44 ± 32	<b>&lt;0.001</b>	41 ± 30	<b>&lt;0.001</b>	5 ± 3	<b>&lt;0.001</b>
Gestational age at delivery (weeks)	39.7 ± 1.2	37.6 ± 2.3	<b>&lt;0.001</b>	38.9 ± 1.5	<b>&lt;0.001</b>	38.9 ± 3.1	<b>&lt;0.001</b>

GH, Gestational hypertension; NSGA, Normotensive SGA; Data are either N (%) or Mean ± SD, comparisons using Pearson chi-square or Student's *t*-test, *P* values are for comparison with uncomplicated pregnancies and *P* values in bold are significant

The *ANGPT1 1414T/A* polymorphism showed no deviation from Hardy Weinberg Equilibrium. The TT genotype of the maternal *ANGPT1 1414T/A* polymorphism was associated with a reduced risk for preeclampsia (adjusted OR, 0.5; 95% CI, 0.3-0.9,  $p = 0.01$ , recessive model, table 2), hypertensive SGA (adjusted OR, 0.5; 95% CI, 0.2-0.9,  $p = 0.04$ , recessive model, table 2), and SGA with abnormal Doppler (adjusted OR, 0.4; 95% CI, 0.2-0.8,  $p = 0.009$ , dominant model, table 2), Maternal *ANGPT1 1414T/A* was not associated with gestational hypertension or with normotensive SGA ( $p > 0.05$ , table 2). Paternal and neonatal *ANGPT1 1414T/A* were not associated with any of the adverse pregnancy outcomes (data not presented).

**Table 2 Associations between maternal *ANGPT1 1414T/A* variant and pregnancy complications**

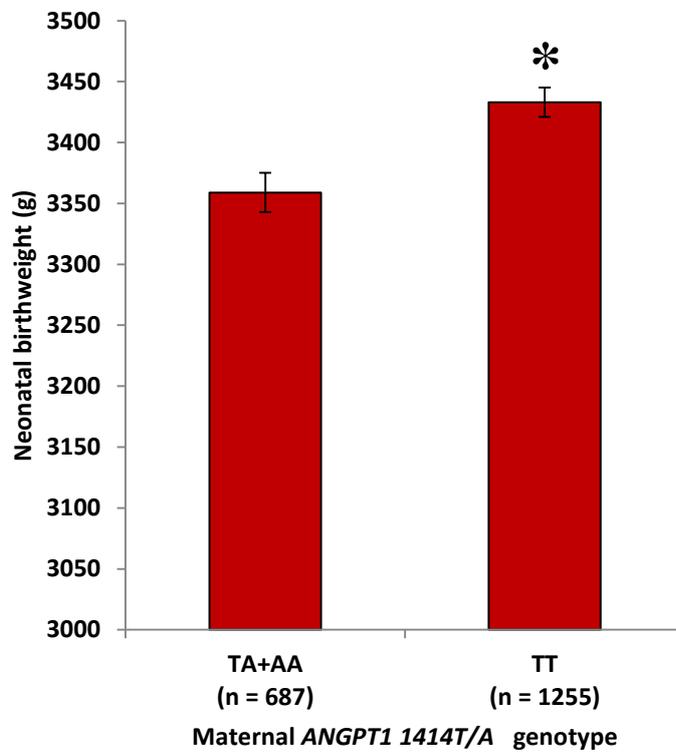
Pregnancy outcome	T allele		Genotype n (%)			Model	P value	OR (95%CI)	aP value	aOR(95%CI)
	%	OR (95% CI)	AA	AT	TT					
Uncomplicated pregnancy	61	ref (1.0)	147 (14.9)	477 (48.2)	365 (36.9)			1.0 (ref)		1.0 (ref)
Preeclampsia	54.3	0.8 (0.6-1.0)	19 (18.1)	58 (55.2)	28 (26.7)	Additive 1	0.8	0.9 (0.5-1.6)	0.7	0.9 (0.5-1.6)
						Additive 2	0.09	0.6 (0.3-1.1)	0.2	0.6 (0.3-1.3)
						Dominant	0.7	0.8 (0.5-1.3)	0.8	0.9 (0.5-1.6)
						Recessive	<b>0.04</b>	<b>0.6 (0.4-0.9)</b>	<b>0.01</b>	<b>0.5 (0.3-0.9)</b>
Gestational hypertension	61.8	1.0 (0.8-1.3)	21 (15.4)	62 (45.6)	53 (39.0)	Additive 1	0.7	0.9 (0.5-1.5)	0.8	0.9 (0.6-1.4)
						Additive 2	0.9	1.0 (0.6-1.7)	0.7	1.1 (0.6-1.8)
						Dominant	0.8	0.9 (0.6-1.6)	0.7	0.8 (0.4-1.8)
						Recessive	0.6	1.1 (0.8-1.6)	0.5	1.2 (0.7-1.5)
Normotensive SGA	60.7	0.9 (0.7-1.3)	22 (16.8)	59 (45.0)	50 (38.2)	Additive 1	0.5	0.8 (0.5-1.4)	0.4	0.8 (0.6-1.6)
						Additive 2	0.7	0.9 (0.5-1.6)	0.9	1.0 (0.4-1.9)
						Dominant	0.6	0.9 (0.5-1.4)	0.7	0.9 (0.5-1.8)
						Recessive	0.8	1.1 (0.7-1.5)	0.7	1.1 (0.7-2.1)
Hypertensive SGA	52.2	0.7 (0.5-1.1)	7 (15.2)	30 (65.2)	9 (19.6)	Additive 1	0.5	1.3 (0.6-3.1)	0.2	0.5 (0.2-1.3)
						Additive 2	0.2	0.5 (0.2-1.4)	0.7	0.2 (0.4-3.9)
						Dominant	0.9	0.9 (0.4-2.2)	0.5	0.7 (0.3-1.8)
						Recessive	<b>0.02</b>	<b>0.4 (0.2-0.9)</b>	<b>0.04</b>	<b>0.5 (0.2-0.9)</b>

**Table 2 Associations between maternal *ANGPT1 1414T/A* variant and pregnancy complications - Continued**

Pregnancy outcome	T allele		Genotype n (%)			Model	P value	OR(95% CI)	aP value	aOR(95% CI)
	%	OR (95% CI)	AA	AT	TT					
SGA + abnormal Doppler	47.7	<b>0.6 (0.4-0.9)</b>	13 (30.2)	19 (44.2)	11 (25.6)	Additive 1	<b>0.03</b>	<b>0.5 (0.2-0.9)</b>	<b>0.01</b>	<b>0.5 (0.2-0.9)</b>
						Additive 2	<b>0.008</b>	<b>0.3 (0.1-0.8)</b>	<b>0.009</b>	<b>0.3 (0.2-0.9)</b>
						Dominant	<b>0.003</b>	<b>0.4 (0.2-0.8)</b>	<b>0.009</b>	<b>0.4 (0.2-0.8)</b>
						Recessive	0.5	0.6 (0.3-1.2)	0.6	0.6 (0.4-1.6)

Abbreviations: SGA, small for gestational age infant; T allele %, T allele frequency; Additive model 1; AT compared to AA; Additive model 2; TT compared to AA, Dominant model AT+TT compared to AA; Recessive model TT compared to AA+AT; aP value and aOR (95%CI) for preeclampsia and gestational hypertension adjusted for maternal factors; maternal age, mean arterial pressure at 15 weeks of gestation, body mass index, family history of preeclampsia, family history of coronary artery disease, maternal birthweight, vaginal bleeding for at least five days during pregnancy, previous single miscarriage with the same partner, taking at least 12 months to conceive, low intake of fruit, cigarette smoking and alcohol use in the first trimester and paternal factors; age and body mass index; aP value and aOR(95%CI) for normotensive SGA, hypertensive SGA and SGA with abnormal Doppler adjusted for maternal factors; age, body mass index, birthweight, smoking, low fruit and vegetable intake, and paternal body mass index and birthweight; bold indicates significant values

The maternal *ANGPT1* 1414T/A TT genotype was also associated with a higher infant birthweight that was adjusted for gestational age at delivery and maternal smoking at 15 weeks gestation ( $p = 0.03$ , recessive model, figure 2).



**Figure 2** Distribution of maternal *ANGPT1* 1414T/A in neonatal birthweight. Neonatal birthweight is adjusted for gestational age at delivery and maternal smoking. Error bars represent SEM. \* $P = 0.03$

The maternal *ANGPT1* 1414T/A TT genotype was associated with a reduced risk for abnormal uterine artery Doppler (OR, 0.7; 95% CI, 0.5-0.9,  $p = 0.03$ , dominant model, table 3) and bilateral notching of the uterine artery waveform (OR, 0.6; 95% CI, 0.4-0.9,  $p = 0.004$ , dominant model, table 3). When adjusted for maternal smoking at 15 weeks gestation the results remained unchanged (Table 3). All results remained significant after correcting for multiple testing.

**Table 3 Associations of the maternal *ANGPT1 1414T/A* variant with abnormal uterine artery Doppler findings**

Doppler finding	T allele		Genotype n(%)			Model	P value	OR(95% CI)	aP value	aOR(95% CI)
	%	OR (95% CI)	AA	AT	TT					
Normal Doppler	60.1	ref (1.0)	275 (16.0)	823 (47.8)	623 (36.2)			1.0 (ref)		1.0 (ref)
Increased Uterine artery RI	52.9	<b>0.7 (0.6-0.9)</b>	41 (22.3)	91 (49.5)	52 (28.3)	Additive 1	0.1	0.7 (0.5-1.1)	0.1	0.7 (0.5-1.1)
						Additive 2	<b>0.008</b>	<b>0.6 (0.4-0.9)</b>	<b>0.008</b>	<b>0.6 (0.4-0.9)</b>
						Dominant	<b>0.03</b>	<b>0.7 (0.5-0.9)</b>	<b>0.03</b>	<b>0.7 (0.5-0.9)</b>
						Recessive	<b>0.03</b>	<b>0.7 (0.5-0.9)</b>	<b>0.03</b>	<b>0.7 (0.5-0.9)</b>
B/L notching	53.9	<b>0.8 (0.6-0.9)</b>	49 (23.7)	93 (44.9)	65 (31.4)	Additive 1	<b>0.01</b>	<b>0.6 (0.4-0.9)</b>	<b>0.01</b>	<b>0.6 (0.4-0.9)</b>
						Additive 2	<b>0.008</b>	<b>0.6 (0.4-0.9)</b>	<b>0.008</b>	<b>0.6 (0.4-0.9)</b>
						Dominant	<b>0.004</b>	<b>0.6 (0.4-0.9)</b>	<b>0.004</b>	<b>0.6 (0.4-0.9)</b>
						Recessive	0.2	0.8 (0.6-1.1)	0.2	0.8 (0.6-1.1)

Abbreviations: T allele %, T allele frequency; Additive model 1; AT compared to AA; Additive model 2; TT compared to AA, Dominant model AT+TT compared to AA; Recessive model TT compared to AA+AT; aP value and aOR(95%CI), adjusted for maternal smoking at 15 weeks gestation; bold indicates significant values

## Discussion

To our knowledge this is the first study to investigate the *ANGPT1 1414T/A* polymorphism in pregnancy complications. Our study demonstrates that the maternal *ANGPT1 1414T/A* TT genotype is associated with a reduced risk for preeclampsia, hypertensive SGA and SGA with abnormal uterine artery Doppler. The maternal *ANGPT1 1414T/A* TT genotype is also associated with a reduced risk for abnormal uterine artery Doppler.

Impaired maternal spiral artery remodelling is implicated in the pathogenesis of the aforementioned pregnancy complications [4]. Angiopoietin-1 and its receptor Tie-2 are expressed at the maternal-fetal interface and are of critical importance in placental vascular remodelling [8-10, 21]. The A allele of the *ANGPT1 1414T/A* polymorphism is known to suppress angiopoietin-1 translation by facilitating miR-211 binding. The T allele is resistant to miR-211 induced reduction in translation, and subjects carrying the TT genotype have higher plasma angiopoietin-1 levels than those with the AA or AT genotypes [12]. We observed that the frequency of the TT genotype was reduced in women who developed preeclampsia, hypertensive SGA and SGA with abnormal uterine artery Doppler Findings. We hypothesise that the TT genotype may contribute to increased angiopoietin-1 production at the maternal-fetal interface.

Consistent with this, we found that the frequency of the TT genotype was reduced in women who had abnormal mean uterine artery RI as well as in those with bilateral notching of the uterine artery waveform at the 20 weeks Doppler scan. As smoking is known to influence the expression of angiogenic growth factors and also impacts on uterine artery Doppler indices possibly through this mechanism, we included maternal smoking at 15 weeks of gestation in our logistic regression model [22]. The protective effect of the *ANGPT1 1414T/A* TT genotype was independent of maternal smoking.

We did not find a significant association of the polymorphism and either gestational hypertension if not complicated by SGA or normotensive SGA. This is consistent with the theory that utero-placental vascular remodelling is unaffected in gestational hypertension not associated with SGA [23].

A history of preeclampsia is associated with increased long term risk for vascular diseases including coronary artery disease and stroke [3]. The relationship between preeclampsia and later life vascular disease involves shared risk factors for both, including chronic endothelial dysfunction [24, 25]. Furthermore, familial segregation of preeclampsia, as well as cardiovascular and cerebro-vascular disease, suggest that these diseases share a common genetic predisposition that interact with the environment and may predispose individuals to vascular disorders that manifest at different time points throughout the life course. The TT genotype of *ANGPT1 1414T/A* polymorphism which is associated with higher plasma angiotensin-1 was recently shown to be associated with a significant reduced risk for both haemorrhagic and ischaemic stroke in two large independent cohorts [12]. Angiotensin-1 acting through Tie-2 receptor plays a critical role in maintaining the integrity of the vascular endothelium and in suppressing endothelial permeability [26]. The protective effect of the TT genotype of *ANGPT1 1414T/A* polymorphism on preeclampsia and haemorrhagic stroke suggests that the associations may be due to its contribution in reducing endothelial dysfunction.

The strengths of our study include a large prospective cohort and collection of data on a large number of clinical variables. The clinical variables used in our multivariable logistic regression models were previously shown to be associated with preeclampsia and SGA pregnancies in our study cohort [15, 18, 19]. Our data show that the association of the maternal *ANGPT1 1414T/A* polymorphism with preeclampsia, hypertensive SGA and SGA with abnormal Doppler is independent of these clinical risk factors. The availability of uterine artery Doppler ultrasound prior to the development of

pregnancy complications also enables us to comment on the potential mechanistic role of the polymorphism in the pathogenesis of these pregnancy disorders.

We acknowledge the following limitations in our study. Given the sample size of our cohort and a prevalence of *ANGPT1 1414T/A* TT genotype in 36.4% of women in the study cohort with complete genotyping information (n = 1361), the preeclampsia, gestational hypertension and normotensive SGA groups had 80% power to detect an OR of 0.5 ( $\beta = 80\%$ ,  $\alpha = 0.05$ ) but our groups of hypertensive SGA and SGA with abnormal uterine artery Doppler were relatively small. Hence, replication in other independent cohorts as well as investigating the effects of genotype on plasma angiotensin-1 during pregnancy will be beneficial.

At present literature on the predictive role of plasma angiotensin-1 on pregnancy complications is limited, but it has been shown that the ratio of plasma angiotensin-1 to angiotensin-2 is reduced in preeclampsia [27]. However, angiotensin-1 production is regulated by many factors and also the changes are detected only during the latter part of the pregnancy [27, 28] making it an unsuitable marker for screening for pregnancy complications.

Considering the functionality of the polymorphism and the known association with stroke, our results are consistent with previous evidence. If similar genotype associations are demonstrated in a replication cohort, this polymorphism in combination with other clinical risk factors may have a potential role in early screening to predict the risk of these pregnancy complications.

In conclusion, our study demonstrates that the maternal *ANGPT1 1414T/A* polymorphism is associated with a reduced risk for preeclampsia, hypertensive SGA and SGA with abnormal uterine artery Doppler and that it may have a protective effect on the pathogenesis of these pregnancy complications.

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## Manuscript 5

### **A functional variant in the thrombospondin-1 gene and the risk of small for gestational age infants**

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#### **Abstract**

**Introduction:** Thrombospondin 1 (TSP-1) is a pro-thrombotic and anti-angiogenic glycoprotein expressed in the placenta. A functional single nucleotide polymorphism in the *TSP1* gene (*TSP1 2210A/G*) is a risk factor for familial premature myocardial infarction. Small for gestational age (SGA) infants are at increased risk of coronary artery disease in adult life and common genetic factors may underlie both conditions. We investigated the association of *TSP1 2210A/G* polymorphism in SGA infants and their parents.

**Method:** 3234 nulliparous pregnant women, their partners and babies were recruited in Adelaide, Australia and Auckland, New Zealand to a prospective multicenter cohort study. Amongst 2123 Caucasian women, 216 (10.2%) delivered an SGA infant defined as birthweight <10<sup>th</sup> customised percentile adjusted for maternal height, weight, parity

and ethnicity, as well as gestational age at delivery and infant sex. Uncomplicated pregnancies served as controls (n=1185). DNA extracted from peripheral/cord blood or buccal swabs was genotyped using the Sequenom MassARRAY system. Multivariable logistic regression was used to compare the odds of SGA between the genotype groups adjusting for potential confounders.

**Results:** Paternal (adjusted OR [aOR] 1.4, 95% CI 1.0-2.0) and neonatal (aOR 1.8, 95% CI 1.1-2.) *TSP1 2210A/G* associates with SGA. The maternal polymorphism approaches significance for an association with SGA (aOR 1.3, 95% CI 0.9-1.9). Maternal *TSP1 2210A/G* associates with a reduced maternal birthweight adjusted for gestational age at delivery ( $p = 0.03$ ).

**Conclusion:** The *TSP1 2210A/G* polymorphism which is a risk factor for myocardial infarction is associated with SGA pregnancies, suggesting that this polymorphism may associate with the risk of vascular disorders across the life course.

## Introduction

Small for gestational age infants (SGA) are at increased risk of later life vascular disorders including hypertension, coronary artery disease and stroke [1, 2]. An anti-angiogenic state is increasingly being recognised to be implicated in the pathophysiology of SGA pregnancies [3]. It is proposed that an angiogenic defect originating during the antenatal period may contribute to the risk of SGA and later life vascular disorders. A recent study demonstrated that the angiogenic potential of cord blood endothelial colony forming cells was impaired and that the expression of thrombospondin 1 (TSP-1) was increased in low birthweight preterm infants [4].

Thrombospondin 1 (TSP-1) is a calcium binding glycoprotein expressed in many cells and is a major constituent of platelet  $\alpha$  granules. TSP-1 is released from platelets in response to activation by thrombin and stabilises platelet aggregation to injured endothelium through inhibition of ADAMTS13 degradation of ultra large von Willebrand factor multimers [5, 6]. In addition to its role in coagulation TSP-1 is well known for its strong anti-angiogenic properties [7]. During pregnancy, TSP-1 is expressed in the placenta. Placental expression of *TSP1* mRNA and protein are increased in disorders of placental villous maturation suggesting that over-expression of *TSP1* may be implicated in the pathogenesis of SGA pregnancies [8].

A single nucleotide polymorphism in the *TSP1* gene (*TSP1* 2210A/G [rs2228262]) which results in the substitution at residue 700 of a serine (Ser-700) for an asparagine (Asn-700) is found in 8-10% of European populations. The Ser-700 variant is associated with lower  $\text{Ca}^{2+}$  binding capacity and conformational stability, enhanced interaction with fibrinogen on platelet surfaces and a higher rate and extent of platelet aggregation [9-11]. The expression of TSP-1 on the surface of platelets from carriers of the Ser-700 variant is also known to be increased [11]. The *TSP1* 2210A/G polymorphism is

reported to be a significant risk factor for familial premature myocardial infarction in both homozygous and heterozygous carriers of the variant allele [12, 13].

The association of an angiogenic imbalance in the pathophysiology of both small for gestational age pregnancies and coronary artery disease suggests that common genetic factors may underlie both these conditions [14]. A recent large study demonstrated that offspring birthweight (adjusted for gestational age at delivery) is inversely associated with cardiovascular mortality in parents, implying that shared genetic factors may contribute to these findings [15]. Familial correlations in birthweight have mostly been explained by fetal and maternal genetic factors [16]. However, there is growing evidence of associations of paternal characteristics with small for gestational age infants which suggest a significant contribution by paternal genes that merits further investigation [17-20].

Considering the anti-angiogenic potential of TSP-1, the strong association of *TSP1* 2210A/G polymorphism with familial premature myocardial infarction and the data showing that infants born small for gestational age are at increased risk of coronary artery disease, we investigated the association of *TSP1* 2210A/G polymorphism in small for gestational age infants (SGA) and their parents in a Caucasian cohort.

## **Materials and Methods**

We conducted a nested case control study where participants were recruited from the Screening for Pregnancy Endpoints (SCOPE) study. The SCOPE study is an international, multicenter, prospective cohort study with the aim of developing screening tests to predict preeclampsia, SGA infants and preterm birth across different populations. Ethics approval was gained from local ethics committees (Australia REC 1712/5/2008 and New Zealand AKX/02/00/364) and the SCOPE study is registered with the Australian and New Zealand Clinical Trial Registry-ACTRN12607000551493.

Nulliparous women with singleton pregnancies attending hospital antenatal clinics, obstetricians, general practitioners or community midwives before 15 weeks' of gestation were invited to participate. Consenting women were recruited between November 2004 and September 2008 in Adelaide, Australia and Auckland, New Zealand. Those considered at high risk of preeclampsia, SGA or preterm birth because of underlying medical conditions (including known pre-existing chronic hypertension on hypertensive medication or with a blood pressure  $>160/100$  mmHg at 15 weeks of gestation), gynaecological history, three or more miscarriages or terminations of pregnancy or couples who received medical or surgical interventions that could modify pregnancy outcome were not eligible. If the woman was certain of the identity of the infant's father, the father was invited to participate in the SCOPE study. Male participants who agreed to participate provided written informed consent.

Recruited couples were excluded for the following reasons: protocol violation, lost to follow up, conceived with donor sperms or oocytes, miscarriage or termination and woman or partner not of Caucasian ethnicity.

Couples who agreed to participate were interviewed and examined by a research midwife at  $15 \pm 1$  and  $20 \pm 1$  weeks of gestation. Data were collected at each time point and included demographic information, medical history, previous obstetric history, family history of obstetric complications and medical disorders. The woman's birthweight and the gestational age at which she was born as well as the partner's birthweight were also recorded. Current pregnancy data included information on any complications during current pregnancy, diet, smoking, alcohol and the use of recreational drugs. A low fruit intake was defined as less than one portion per week. A low intake of green leafy vegetables was defined as less than two portions per week. Maternal and paternal physical measurements obtained at 15 weeks of gestation included height, weight and blood pressure.

All women were followed prospectively and pregnancy outcome data and infant measurements were recorded by research midwives usually within 72 hours of birth.

### **Specimen collection**

Peripheral blood samples were collected from the women and partners. All women provided blood samples. Buccal swabs or saliva samples were collected from partners who were unwilling to undergo venepuncture. The buccal swabs were applied to Whatman FTA cards (Whatman Inc, Piscataway, NJ) immediately following sample collection and saliva was collected using Oragene kits (DNA Genotek Inc, Kanata, Ontario, Canada). Cord blood was collected at delivery. If cord blood was not obtained at delivery, a buccal swab or saliva sample was collected from the baby.

### **Outcome measure**

The primary outcome was *SGA (small for gestational age)*, defined as a birth weight below the 10<sup>th</sup> customised percentile adjusted for maternal height, weight, parity and ethnicity, as well as gestational age at delivery and infant sex [21].

### **Definitions of pregnancy outcome**

*Gestational hypertension* was defined as systolic blood pressure of  $\geq 140$  mmHg, and/or diastolic blood pressure  $\geq 90$  mmHg, on at least two occasions, four hours apart, after 20 weeks of gestation, but before the onset of labour. *Preeclampsia* was defined as gestational hypertension or post-partum hypertension with proteinuria (24 hour urinary protein level of  $>300$ mg or a spot urine protein: creatinine ratio of  $\geq 30$ mg/mmol creatinine or urine dipstick protein level of  $\geq ++$ ) or any multisystem complication of preeclampsia [22]. *Normotensive SGA* was defined as birth of an SGA infant where the mother did not have hypertension and *hypertensive SGA* was defined as birth of an SGA

infant where the mother had either gestational hypertension or preeclampsia. *Severe SGA* was defined as birthweight below the 5<sup>th</sup> customised percentile. *Uncomplicated pregnancy* was defined as a pregnancy with no antenatal medical or obstetric complications and resulting in the delivery of an appropriately grown, healthy baby at  $\geq$  37 weeks of gestation.

### **Genotyping**

DNA was extracted from buffy coats from peripheral or cord blood, Whatman FTA cards or from saliva (Oragene<sup>®</sup>DNA kits) according to the manufacturers' instructions. Genotyping for *TSP1 2210A/G* (rs2228262) single nucleotide polymorphism was performed at the Australian Genome Research Facility (AGRF, Brisbane, Australia) using the Sequenom MassARRAY system. For quality control, each sample was also genotyped for Amelogenin to ensure that the sex of the sample was correct [23]. Parental and neonatal genotyping data were checked for a Mendelian pattern of inheritance and those found to be inconsistent were excluded from the analyses.

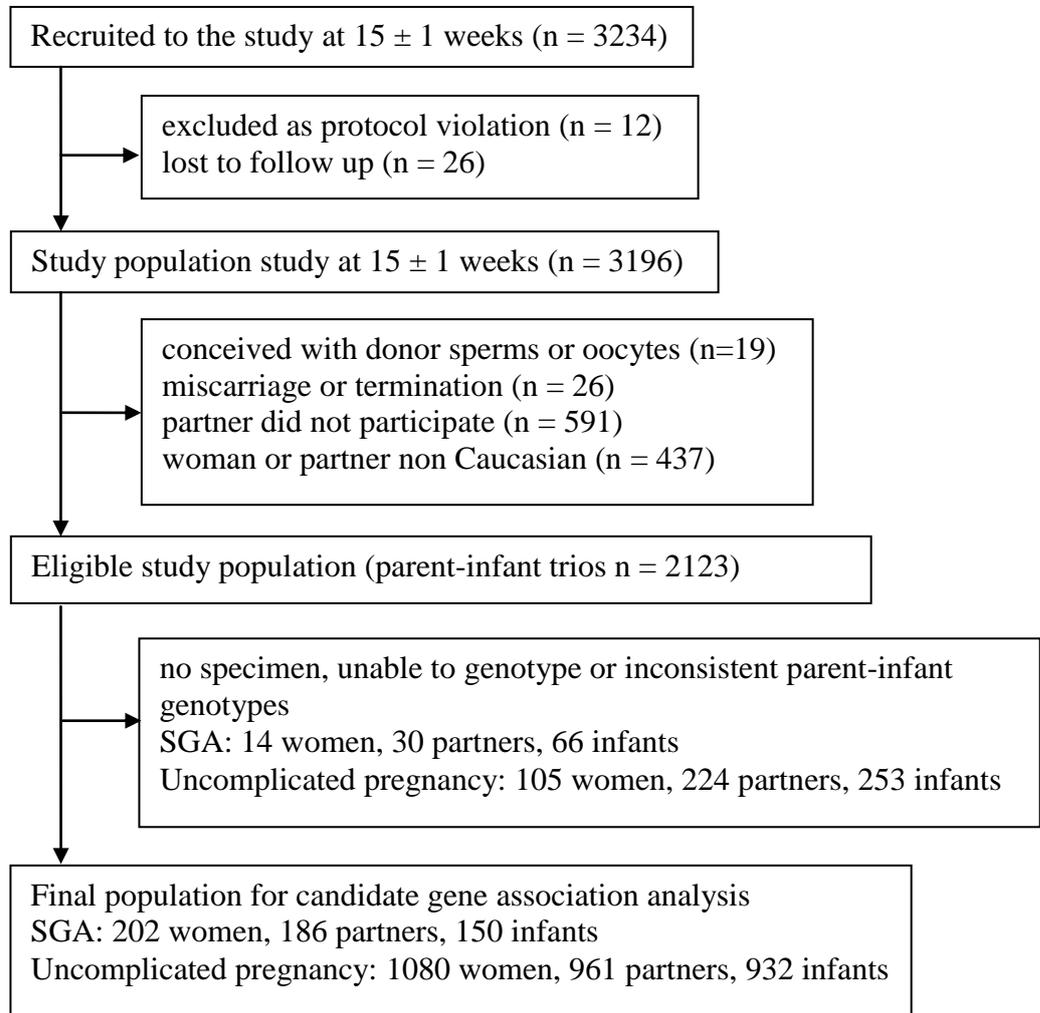
### **Statistical methods**

SGA infants and their parents were compared with parent-infant trios from uncomplicated pregnancies in a nested case control study design. Missing data were excluded from the analyses. The chi-square test was used to test the genotypes at the polymorphic locus for Hardy-Weinberg Equilibrium and to compare categorical variables. ANOVA was used to compare continuous variables between the three genotype groups with posthoc Sidak test for pairwise comparisons. Multivariable logistic regression was used to compare the odds of SGA between carriers of the variant allele and the additive model (GA+GG) compared to the reference common genotype (AA) adjusting for previously established risk factors for SGA. The covariates for the

logistic regression model included maternal age, BMI, birthweight, smoking at 15 weeks of gestation, pre-pregnancy low fruit intake, pre-pregnancy low green leafy vegetable intake, and paternal BMI and birthweight [20, 24]. Adjusted odds ratios and 95% CIs were also calculated for heterozygous and GG homozygous infants <50<sup>th</sup>, 40<sup>th</sup>, 30<sup>th</sup>, 20<sup>th</sup>, 10<sup>th</sup>, 9<sup>th</sup>, 8<sup>th</sup>, 7<sup>th</sup>, 6<sup>th</sup> and 5<sup>th</sup> customised centiles compared to those >50<sup>th</sup> customised centile to identify any genotype correlation with the birthweight centile. All data analyses were performed using PASW version 17.02 (SPSS, Inc, Cary, North Carolina). Results were reported as number and percent [n (%)] or mean  $\pm$  standard deviation (SD) where appropriate.  $P < 0.05$  was considered statistically significant. On the basis of prevalence of *TSP-1 2210A/G* polymorphism in 10% of Caucasians in the general population and a ratio of 6 control subjects to 1 case, 150 SGA infants and 932 control subjects has 80% power to detect an odds ratio of 2.0 ( $\beta = 80\%$ ,  $\alpha = 0.05$ ).

## **Results**

Of the 3234 eligible parent-infant trios, 2123 trios were included in this study. The exclusions are detailed in Figure 1.



**Figure 1** Study Population

Amongst 2123 Caucasian pregnancies, 1185 (55.8%) were uncomplicated, 216 (10.2%) had SGA infants and the remaining 722 (34.0%) developed other obstetric, medical or surgical complications during pregnancy. One hundred and eleven infants (5.2%) had a birthweight < 5<sup>th</sup> customised percentile. Of the 216 SGA infants, 158 (73.2%) were born to normotensive mothers and 58 (26.8%) were born to hypertensive mothers [preeclampsia (n=28), gestational hypertension (n=30)]. The characteristics of the participants are shown in table 1. In the hypertensive SGA group both parents had a higher body mass index (BMI) compared to the uncomplicated pregnancy group. Both parents had lower birthweights in the normotensive, as well as in the hypertensive, SGA group compared to the uncomplicated pregnancy group. Smoking was more prevalent among normotensive and hypertensive women who delivered an SGA infant compared to women who had an uncomplicated pregnancy.

**Table1 Characteristics of the study population**

<b>Characteristic</b>	<b>Control (n=1185)</b>	<b>NSGA (n=158)</b>	<b>P</b>	<b>HSGA (n=58)</b>	<b>P</b>
<b>Maternal characteristics</b>					
Age (years)	28.2 ± 5.6	28.6 ± 6.0	0.4	28.1 ± 6.6	0.9
BMI (kg/m)	24.9 ± 4.5	25.5 ± 5.1	0.1	27.9 ± 6.2	<b>&lt;0.001</b>
Birthweight (g)*	3331 ± 530	3171 ± 553	<b>0.001</b>	3154 ± 455	<b>0.014</b>
Smoking at 15 weeks gestation	105 (9%)	36 (22.8%)	<b>&lt;0.001</b>	11 (18.9%)	<b>0.014</b>
<b>Paternal characteristics</b>					
Age (years)	30.7 ± 6.3	31.4 ± 6.6	0.2	30.2 ± 6.8	0.5
BMI (kg/m)‡	26.6 ± 4.0	26.9 ± 4.5	0.2	27.7 ± 4.7	<b>0.041</b>
Birthweight (g)¶	3492 ± 571	3305 ± 528	<b>&lt;0.001</b>	3336 ± 504	<b>0.05</b>
<b>Pregnancy outcome</b>					
Neonatal birthweight (g)	3590 ± 394	2640 ± 559	<b>&lt;0.001</b>	2442 ± 539	<b>&lt;0.001</b>
Customised birthweight centile	54 ± 25	5 ± 3	<b>&lt;0.001</b>	4 ± 3	<b>&lt;0.001</b>
Gestational age at delivery (weeks)	39.7 ± 1.2	38.6 ± 3.7	<b>&lt;0.001</b>	37.7 ± 3.0	<b>&lt;0.001</b>

NSGA, Normotensive SGA; HSGA, Hypertensive SGA; Data are either N (%) or Mean ± SD, Comparisons with controls using Pearson chi-square or Student's *t*-test; \*Controls n = 1144, NSGA n = 156, HSGA n=56; ‡Controls n = 1148, NSGA n=152; ¶Controls n = 1099, NSGA n=144, HSGA n=53; *P* values in bold are significant

Genotype data of 14 (6.5%) women, 30 (13.9%) partners and 66 (30.6%) infants in the SGA group and 105 (8.9%) women, 224 (18.9%) partners and 253 (21.3%) infants in the uncomplicated pregnancy group could not be analysed due to non availability of samples, genotyping failure and Mendelian inconsistencies in parent-infant genotypes. Genotype data were available for 202 women, 186 partners and 150 infants in the SGA group and 1080 women, 961 partners and 932 infants in the uncomplicated pregnancy group.

### **Genotype distribution associated with SGA**

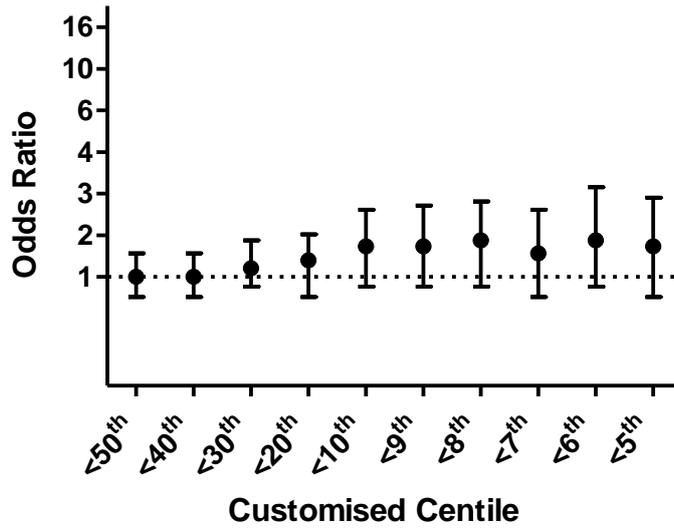
The *TSP1 2210A/G* polymorphism was in Hardy-Weinberg Equilibrium. There were no sporadic mutations detected in the genotyped infants in this study cohort. The prevalence of paternal and neonatal *TSP1 2210A/G* polymorphism was increased in the SGA group compared to the uncomplicated pregnancy group ( $p = 0.03$  for the additive model for the paternal SNP and  $p = 0.02$  for the additive model for the neonatal SNP, table 2). The prevalence of paternal and neonatal *TSP1 2210A/G* polymorphism was increased in the severe SGA group compared to the uncomplicated pregnancy group ( $p = 0.01$  for the additive model for the paternal SNP and  $p = 0.02$  for GG homozygosity for the neonatal SNP, table 2). The maternal SNP approached a significant association with SGA ( $p = 0.06$  for the additive model, table 2).

**Table 2 Distribution of maternal, paternal and neonatal *TSP1 2210A/G* polymorphism in SGA and in uncomplicated pregnancy**

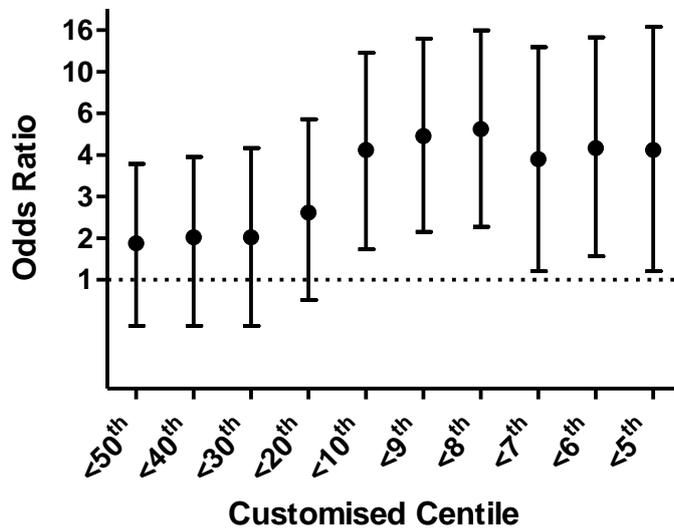
<i>TSP1 2210A/G</i>	Uncomplicated pregnancy (n%)	SGA <10 <sup>th</sup> centile n (%)	OR(95% CI)	aOR(95%CI)	SGA <5 <sup>th</sup> centile n (%)	OR(95% CI)	aOR(95%CI)
Maternal	n=1080	n=202			n=104		
AA	835 (77.3)	145 (71.8)	1.0 (ref)	1.0 (ref)	75 (72.1)	1.0 (ref)	1.0 (ref)
GA	231 (21.4)	53 (26.2)	1.3 (0.9-1.9)	1.3(0.9-1.9)	28 (26.9)	1.3 (0.9-2.1)	1.4 (0.8-2.3)
GG	14 (1.3)	4 (2.0)	1.7 (0.6-5.5)	1.5 (0.4-5.5)	1 (1.0)	0.8 (0.1-6.1)	0.8 (0.1-6.7)
GA+GG	245 (22.7)	57 (28.2)	1.3 (0.9-1.2)	1.3(0.9-1.9)	29 (27.9)	1.3 (0.8-2.1)	1.3 (0.8-2.2)
Paternal	n=961	n=186			n=97		
AA	761 (79.2)	135 (72.6)	1.0 (ref)	1.0 (ref)	66 (68.0)	1.0 (ref)	1.0 (ref)
GA	190 (19.8)	46 (24.7)	1.4 (0.9-1.9)	1.3(0.9-2.1)	29 (29.9)	<b>1.8 (1.1-2.8)</b>	<b>1.6 (1.0-3.1)</b>
GG	10 (1.0)	5 (2.7)	2.8 (0.9-8.3)	<b>4.2 (1.2-14.7)</b>	2 (2.1)	2.3 (0.5-10.8)	1.9 (0.8-14.5)
GA+GG	200 (20.8)	51 (27.4)	<b>1.4 (1.0-2.1)</b>	<b>1.4(1.0-2.0)</b>	31 (32.0)	<b>1.8 (1.1-2.8)</b>	<b>1.5 (1.0-2.6)</b>
Neonatal	n=932	n=150			n=74		
AA	728 (78.1)	105 (70.0)	1.0 (ref)	1.0 (ref)	52 (70.2)	1.0 (ref)	1.0 (ref)
GA	194 (20.8)	39 (26.0)	1.4 (0.9-2.1)	<b>1.6 (1.0-2.4)</b>	19 (25.7)	1.4 (0.8-2.4)	1.4 (0.8-2.5)
GG	10 (1.1)	6 (4.0)	<b>4.2 (1.5-11.7)</b>	<b>5.9 (1.9-17.8)</b>	3 (4.1)	<b>4.2 (1.1-15.8)</b>	<b>7.0 (1.7-28.5)</b>
GA+GG	204 (21.9)	45 (30.0)	<b>1.5 (1.0-2.2)</b>	<b>1.8 (1.1-2.7)</b>	22 (29.8)	1.5 (0.9-2.5)	1.6 (0.9-2.7)

aOR(95% CI) adjusted for maternal factors: maternal age, BMI, birthweight, smoking at 15 weeks gestation, pre-pregnancy low fruit intake, pre-pregnancy low green leafy vegetable intake; paternal factors: paternal, BMI and birthweight; GA+GG, Additive model is compared with the reference AA genotype; ref, referent, OR(95% CI) values in bold are significant

Forest plots of the association of the neonatal *TSP1 2210A/G* polymorphism with infants <50<sup>th</sup>, 40<sup>th</sup>, 30<sup>th</sup>, 20<sup>th</sup>, 10<sup>th</sup>, 9<sup>th</sup>, 8<sup>th</sup>, 7<sup>th</sup>, 6<sup>th</sup> and 5<sup>th</sup> customised centiles compared to infants >50<sup>th</sup> customised centile are shown in figures 2 and 3. A significant association was evident from below the 10<sup>th</sup> customised centile for infants homozygous for the G allele (Figure 3).



**Figure 2** Forest plots show the association between neonatal heterozygosity for *TSP1* 2210A/G polymorphism and infants <50<sup>th</sup> centile, data are presented as aOR (95% CI)



**Figure 3** Forest plots show the association between neonatal homozygosity for the G allele of the *TSP1* 2210A/G polymorphism and infants <50<sup>th</sup> centile, data are presented as aOR (95% CI)

As a post-hoc analysis we subcategorised SGA in to normotensive and hypertensive groups. SGA subgroup analysis demonstrated that the prevalence of the neonatal *TSP1 2210A/G* SNP was increased in SGA infants born to normotensive women compared to neonates born to women with uncomplicated pregnancies ( $p = 0.02$  for the additive model, table 3). The prevalence of the GA and GG genotypes of the maternal and paternal *TSP1 2210A/G* was increased in the normotensive SGA group compared to the uncomplicated group but the results were not significant ( $p = 0.08$  for the additive model for the maternal SNP and  $p = 0.08$  for the additive model for the paternal SNP, table 3). Maternal, paternal and neonatal *TSP1 2210A/G* polymorphisms were not associated with hypertensive SGA although the frequency of GA and GG genotypes were increased in the hypertensive SGA group compared to uncomplicated pregnancy (table 3).

**Table 3 Distribution of maternal, paternal and neonatal *TSP1 2210A/G* polymorphism in normotensive and hypertensive SGA and in uncomplicated pregnancy**

<i>TSP1 2210A/G</i> genotype	Uncomplicated pregnancy	NSGA n (%)	OR (95% CI)	aOR (95% CI)	HSGA n (%)	OR (95% CI)	aOR (95% CI)
Maternal	n = 1080	n = 148			n = 54		
AA	835 (77.3)	106 (71.6)	1.0 (ref)	1.0 (ref)	39 (72.2)	1.0 (ref)	1.0 (ref)
GA	231 (21.4)	40 (27)	1.4 (0.9-2.0)	1.8 (0.8-2.0)	13 (24.1)	1.2 (0.6-2.3)	1.2 (0.6-2.4)
GG	14 (1.3)	2 (1.4)	1.1 (0.3-5.0)	1.2 (0.4-5.0)	2 (3.7)	3.1 (0.7-13.9)	3.5 (0.7-17.6)
GA+GG	245 (22.7)	42 (28.4)	1.3 (0.9-1.9)	1.8 (0.8-1.9)	15 (27.8)	1.3 (0.7-2.4)	1.3 (0.7-2.6)
Paternal	n = 961	n = 131			n = 55		
AA	761 (79.2)	96 (73.3)	1.0 (ref)	1.0 (ref)	39 (70.9)	1.0 (ref)	1.0 (ref)
GA	190 (19.8)	31 (23.7)	1.3 (0.8-1.9)	1.7 (0.7-1.9)	15 (27.3)	1.5 (0.8-2.9)	1.6 (0.8-3.3)
GG	10 (1.0)	4 (3.1)	3.2 (0.9-10.3)	4.6 (0.9-19.4)	1 (1.8)	1.9 (0.2-15.6)	1.9 (0.2-21.2)
GA+GG	200 (20.8)	35 (26.7)	1.4 (0.9-2.1)	1.3 (0.9-2.1)	16 (29.1)	1.6 (0.9-2.9)	1.7 (0.9-3.3)

NSGA, Normotensive SGA; HSGA, Hypertensive SGA; aOR(95% CI) adjusted for maternal factors: maternal age, BMI, birthweight, smoking at 15 weeks gestation, pre-pregnancy low fruit intake, pre-pregnancy low green leafy vegetable intake; paternal factors: paternal, BMI and birthweight GA+GG, Additive genetic model is compared with the reference AA genotype; ref, referent; OR (95% CI) values in bold are significant

**Table 3 Distribution of maternal, paternal and neonatal *TSP1 2210A/G* polymorphism in normotensive and hypertensive SGA and in uncomplicated pregnancy - Continued**

<i>TSP1 2210A/G</i> genotype	Uncomplicated pregnancy	NSGA n (%)	OR (95% CI)	aOR (95% CI)	HSGA n (%)	OR (95% CI)	aOR (95% CI)
Neonatal	n = 932	n = 113			n = 37		
AA	728 (78.1)	78 (69.0)	1.0 (ref)	1.0 (ref)	27 (73.0)	1.0 (ref)	1.0 (ref)
GA	194 (20.8)	30 (26.5)	1.4 (0.9-2.3)	1.6 (0.9-2.6)	9 (24.3)	1.3 (0.6-2.7)	1.4 (0.6-3.0)
GG	10 (1.1)	5 (4.4)	<b>4.7 (1.6-14)</b>	<b>7.8 (2.4-25.9)</b>	1 (2.7)	2.7 (0.3-21.8)	2.9 (0.4-32.7)
GA+GG	204 (21.9)	35 (31)	<b>1.6 (1.0-2.5)</b>	<b>1.8 (1.1-2.8)</b>	10 (27.0)	1.3 (0.6-2.8)	1.4 (0.3-3.1)

NSGA, Normotensive SGA; HSGA, Hypertensive SGA; aOR(95% CI) adjusted for maternal factors: maternal age, BMI, birthweight, smoking at 15 weeks gestation, pre-pregnancy low fruit intake, pre-pregnancy low green leafy vegetable intake; paternal factors: paternal, BMI and birthweight GA+GG, Additive genetic model is compared with the reference AA genotype; ref, referent; OR (95% CI) values in bold are significant

### **Genotype distribution associated with maternal birthweight**

The mother's own birthweight adjusted for gestational age at birth was on average 232g lower in women with *TSP1* 2210A/G GG genotype compared to those with the AA genotype ( $p = 0.03$ , Table 4). We were not able to investigate the association between paternal *TSP1* 2210A/G SNP and paternal birthweight adjusted for gestational age at birth as we did not collect paternal data relating to gestational age at delivery.

**Table 4 Distribution of maternal *TSP1 2210A/G* polymorphism in maternal birthweight**

<i>TSP1 2210A/G</i> genotype	Maternal birthweight* (n = 1875) <sup>‡</sup>	<i>P</i>
AA	3291 ± 538	ref
GA	3269 ± 568	0.07
GG	3059 ± 503	0.03

Data are presented as mean ± SD; \* ANOVA with posthoc Sidak test and adjusted for gestational age at delivery; <sup>‡</sup>After exclusion of 83 women with missing birthweight; ref, referent

### **Genotype distribution in parent-infant trios**

The prevalence of the *TSP1 2210A/G* polymorphism in both the infant and either of the parents was increased in SGA pregnancy trios compared to trios from uncomplicated pregnancies. The effect was similar (OR 1.9) in maternal, as well as in paternal, carriage of the variant allele if the infant was a carrier of the polymorphism (Table 5).

**Table 5 Distribution of the *TSP1 2210A/G* polymorphism in parent-infant trios**

	<b>Control trios n = 690</b>	<b>SGA trios n = 119</b>	<b>OR (95% CI)</b>
Mother-father-infant polymorphism absent	413 (59.9%)	59 (49.6%)	1
Father-infant polymorphism absent Mother polymorphism present	63 (9.1%)	5 (4.2%)	0.6 (0.2-1.4)
Mother-infant polymorphism absent Father polymorphism present	55 (8.0%)	10 (8.4%)	1.3 (0.6-2.6)
Father polymorphism absent Mother-infant polymorphism present	67 (9.7%)	19 (16.0%)	<b>1.9 (1.1-3.5)</b>
Mother polymorphism absent Father-infant polymorphism present	59 (8.6%)	16 (13.4%)	<b>1.9 (1.0-3.5)</b>
Infant polymorphism absent Mother-father polymorphism present	8 (1.2%)	3 (2.5%)	1.8 (0.4-1.4)
Mother-father-infant polymorphism present	25 (3.6%)	7 (5.9%)	1.9 (0.8-4.7)

Data are N (%) using Pearson chi-square or Fisher's exact test;

Polymorphism absent, AA genotype;

Polymorphism present, GA or GG genotype

## Discussion

To our knowledge this is the first study to investigate the prevalence of *TSP1* 2210A/G polymorphism in small for gestational age infants and their parents. Our data demonstrate that the neonatal and paternal polymorphisms are associated with SGA. Our data also show that the association with SGA is stronger for neonatal homozygosity for the polymorphism and that the effect is similar in maternal as well as in paternal carriage of the variant allele. As the infant's genotype is likely to represent the placental genotype our data suggest that the polymorphism may have an effect through the placenta.

A successful pregnancy requires the development of an adequate utero-placental circulation and impaired placental villous vascularisation is demonstrated in the pathophysiology of small for gestational age pregnancies [3, 25]. The angiogenic potential of cord blood endothelial colony forming cells (ECFC) is impaired and the expression of both *TSP1* mRNA and protein is increased in low birthweight preterm infants [4]. Silencing TSP-1 is shown to restore the angiogenic properties of ECFC in LBW infants suggesting that TSP-1 may be implicated in the pathophysiology of impaired placental vascularisation demonstrated in growth restricted pregnancies. TSP-1 inhibits angiogenesis by interacting with vascular endothelial growth factor (VEGF-A) and indirectly by inhibiting the VEGF-A receptor KDR [26].

In addition to its role in inhibiting angiogenesis, TSP-1 has pro-thrombotic properties. TSP-1 is released from activated platelets, binds to the platelet surface in a  $\text{Ca}^{2+}$  dependant manner and increases platelet aggregation [27]. The Ser-700 variant of the *TSP1* 2210A/G SNP exhibits higher affinities for platelets and fibrinogen and shows increased platelet surface expression compared to the Asn-700 variant [11]. Enhanced platelet aggregation and increased TSP-1 surface expression are both consistent with a pro-thrombotic phenotype.

Placentae from pregnancies affected by fetal growth restriction demonstrate thrombotic lesions within fetal and chorionic vessels suggesting that placental thrombosis may contribute to fetal growth restriction [28]. Similar pathological changes underlie pregnancies complicated by SGA fetuses [29]. Our data show that heterozygosity for the *TSP1 2210A/G* SNP in infants is associated with an adjusted OR of 1.6 and homozygosity with an adjusted OR of 5.9 with SGA. As a potential pathophysiological mechanism, we hypothesise that the SNP may contribute to a pro-thrombotic and anti-angiogenic phenotype in the placenta.

Our data also demonstrate a borderline significant association between the paternal *TSP1 2210A/G* polymorphism and SGA. A paternal contribution to SGA has been previously suggested by a positive correlation of paternal birthweight with infant birthweight [18-20] and that men who were SGA at birth are more likely than those with a normal birthweight to parent an SGA infant [17]. In our study the paternal *TSP1 2210A/G* SNP remained associated with SGA after adjusting for established maternal and paternal risk factors for SGA, demonstrating an independent association between the GG genotype of the *TSP1 2210A/G* SNP in the father with birth of an SGA infant.

We did not find a significant association between the maternal polymorphism and SGA but found that homozygosity for the G allele in the mother was associated with a reduced maternal birthweight adjusted for gestational age at delivery. Considering the consistent association of maternal birthweight with neonatal birthweight, the *TSP1 2210A/G* polymorphism may contribute to SGA through influences on the mother's own birthweight.

Although thrombophilias and angiogenic growth factor imbalances have been much studied in pregnancy complications, there is a paucity of literature on the potential role of TSP-1 in normal and complicated pregnancies. Topol and co-workers reported that the *TSP1 2210A/G* SNP was strongly associated with familial premature myocardial

infarction in Caucasians homozygous for the G allele [12]. This was later confirmed in a large study which reported that the *TSP1 2210A/G* SNP was a risk factor for myocardial infarction in both homozygous and heterozygous carriers of the G allele [13].

A consistent association has been demonstrated between SGA and adult onset diseases including increased risk for developing hypertension, insulin resistance and coronary artery disease [1, 2, 30]. It is proposed that these associations result from fetal programming in response to the intrauterine environment with long-term consequences for metabolic and cardiovascular function [31]. An alternative explanation is that common genetic factors may underlie both restricted fetal growth and adult onset diseases [14]. In support of this theory, a few previous studies have identified polymorphisms in candidate genes that are associated with either reduced birthweight or SGA and adult onset disorders. Hattersley *et al.* reported that a mutation in the glucokinase gene that results in adult onset impaired glucose tolerance was associated with a reduction in birthweight [32]. Polymorphisms in genes associated with adult onset diabetes are also associated with being born small for gestational age [33]. In agreement with this theory Infante-Rivard *et.al* demonstrated that a cardio protective polymorphism in the Apo-lipoprotein gene was less prevalent among SGA infants [34]. Here we have shown that the prevalence of the *TSP1 2210A/G* polymorphism, which was previously shown to be associated with myocardial infarction in two independent studies, was increased in infants and fathers of SGA pregnancies, providing further evidence for potential shared genetic factors between SGA and cardiovascular disease. The strengths of our study include a large prospective cohort with excellent follow-up and rich metadata which enabled us to adjust for potential confounders. Our study has a few limitations which should be acknowledged. Although our prospective cohort was large our group of SGA pregnancies was relatively small, therefore the borderline significance of the paternal polymorphism may be due to the sample size. We also

excluded a number of cases and controls due to no genotype-available, and it is possible that this has introduced bias into our results. Since this is a novel finding, our results need to be replicated in other independent cohorts.

### **Conclusion**

This study demonstrates that the prevalence of the *TSP1 2210A/G* polymorphism that was previously shown to be a risk factor for familial premature myocardial infarction is increased in infants and fathers of SGA pregnancies suggesting that this polymorphism may associate with the risk of vascular disorders across the life course. Our data also show that there is a paternal genetic association with SGA.

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## Manuscript 6

### Interaction between maternal BMI and angiogenic gene polymorphisms associates with the risk of spontaneous preterm birth

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#### Abstract

Obesity is associated with an increased level of inflammation. Interactions between inflammatory and angiogenic pathways are implicated in the major pregnancy disorders. The aim of this study was to investigate whether functional polymorphisms in angiogenesis regulating genes (*VEGFA* -2578C/A, *VEGFA* +936C/T, *KDR* -604T/C and *ANGPT1* 1414A/T) interact with maternal BMI to modify the risk of spontaneous preterm birth (sPTB).

We conducted a nested case control study of 1190 nulliparous Caucasian women (107 sPTB and 1083 controls). Spontaneous PTB was defined as spontaneous preterm labour or preterm premature rupture of membranes resulting in preterm birth at <37 weeks of

gestation. DNA was extracted from peripheral blood and genotyped using Sequenom MassARRAY system.

Among overweight or obese women ( $BMI \geq 25$ ), *VEGFA* -2578C/A AA genotype was associated with a higher risk of sPTB (OR = 2.4, 95% CI: 1.4-4.6,  $p = 0.001$ ) and a significant interaction between BMI and the polymorphism was detected (OR = 4.2, 95% CI: 1.7-10.9,  $p = 0.003$ ). Among women with a  $BMI < 25$ , *ANGPT1* 1414A/T AA genotype was associated with a higher risk of sPTB (OR = 2.3, 95% CI: 1.2-4.4,  $p = 0.02$ ) and a significant interaction between BMI and the polymorphism was detected (OR = 3.3, 95% CI: 1.1-9.3,  $p = 0.02$ ). All results remained significant after adjusting for potential confounding factors.

Maternal BMI interacts with angiogenesis regulating gene polymorphisms to modify the risk of sPTB.

## **Introduction**

Preterm birth (delivered before 37 weeks' gestation) accounts for approximately 75% of neonatal deaths [1]. In addition to the immediate complications during the neonatal period those born preterm are at increased risk for neuro-developmental and behavioural disorders in childhood and cardiovascular disorders and type 2 diabetes in adult life [2]. Preterm birth may result from iatrogenic delivery for maternal or fetal concerns or following spontaneous onset of preterm labour. Spontaneous preterm birth (sPTB) is known to be initiated by many factors including intrauterine infection, inflammation, utero-placental ischaemia or haemorrhage, uterine over-distension, stress and other immunological processes [3, 4]. Impaired placentation is evident in approximately 15% of spontaneous preterm births. In a proportion of spontaneous preterm deliveries, the cause remains unknown and a genetic contribution is suggested with heritability in the range of 27-36% [5]. There is growing evidence that polymorphisms in disease-susceptibility genes and gene-environment interactions may account for differences in the prevalence of preterm birth across populations [6-8].

The vascular endothelial growth factor family consisting of VEGF-A, placental growth factor (PlGF) and the receptors fms-like tyrosine kinase-1 (Flt-1) and kinase-insert domain (KDR) along with the angiopoietins (ANG-1, ANG-2 and Tie-2 receptor) are the major angiogenic growth factors expressed at the maternal-fetal interface. As regulators of spiral artery remodelling, these molecules have a key role in placentation. Polymorphisms in these angiogenic genes regulate the transcriptional activity of the genes as well as protein production. We recently reported that functional polymorphisms in *VEGFA*, *KDR* and *ANGPT1* were associated with pregnancies complicated by preeclampsia and small for gestational age infants [9-11]. As in preeclampsia and fetal growth restriction, a failure in maternal spiral artery remodelling has been reported in sPTB following both preterm labour with intact membranes and

preterm premature rupture of membranes [12, 13]. Therefore, polymorphisms in angiogenesis regulating genes may also contribute to the risk of sPTB.

Recently, there has been a significant increase in the number of women of reproductive age who are either overweight or obese with resultant increased rates of adverse pregnancy outcomes including preeclampsia and gestational diabetes mellitus. The literature regarding the association of maternal obesity with preterm birth is controversial with reports of increased [14], decreased [15] and no risk [16]. However, a recent review incorporating data from developed as well as developing countries reported that overweight and obese women were at increased risk of sPTB [17]. Further, obesity is known to be associated with alteration in angiogenic factors including vascular endothelial growth factor A (VEGF-A) and angiopoietin-1 [18-20].

The aim of this study was to investigate whether functional single nucleotide polymorphisms in angiogenesis regulating genes (*VEGFA* -2578C/A, *VEGFA* +936C/T, *KDR* -604T/C and *ANGPT1* 1414T/A) interact with maternal BMI to modify the risk of spontaneous preterm birth (sPTB).

## **Materials and Methods**

### **Study population**

The SCOPE study is an international, multicenter, prospective cohort study with the aim of developing screening tests to predict preeclampsia, SGA infants and preterm birth across different populations [21]. Ethics approval was gained from local ethics committees and all participants provided written informed consent.

Nulliparous women with singleton pregnancies attending hospital antenatal clinics, obstetricians, general practitioners or community midwives before 15 weeks of gestation were invited to participate. Consenting women were recruited between November 2004 and September 2008 in Adelaide, Australia and Auckland, New

Zealand. Women considered at high risk of preeclampsia, SGA infants or preterm birth because of underlying medical conditions (chronic hypertension requiring antihypertensive drugs, diabetes, renal disease, systemic lupus erythematosus, antiphospholipid syndrome, sickle cell disease), three or more miscarriages or terminations of pregnancy, previous cervical cone knife biopsy, interventions that could modify pregnancy outcome (such as aspirin, cervical suture) or known major fetal anomaly or abnormal karyotype were not eligible. Recruited women were excluded for the following reasons: protocol violation, lost to follow up, conceived with donor sperms or oocytes, miscarriage or termination and woman or partner not of Caucasian ethnicity. The final study population comprised 1190 nulliparous Caucasian women (107 sPTB and 1083 controls).

Women were interviewed and examined by a research midwife at  $15 \pm 1$  and  $20 \pm 1$  weeks of gestation. Maternal data on demographic information, medical history, previous obstetric history, family history of obstetric complications and medical disorders were collected [22]. Current pregnancy data included information on any complications during current pregnancy, diet, smoking, alcohol and the use of recreational drugs. Maternal physical measurements obtained at 15 weeks of gestation included height, weight and blood pressure. Body mass index (BMI) was calculated as weight (kg)/ height ( $m^2$ ). Obesity was defined as  $BMI \geq 30$  and overweight was defined as  $BMI \geq 25$  and  $< 30$ .

If the woman was certain of the last menstrual period (LMP), the expected date of delivery (EDD) was calculated from the LMP. The EDD was adjusted only if either a scan at  $< 16$  weeks' gestation found a difference of more than seven days or a scan at 20 weeks' gestation found a difference of 10 or more days between the scan gestation and that calculated by LMP. If the LMP was not known the scan dates were used to estimate the EDD. All women were followed prospectively and pregnancy outcome data and

measurements of the infant were recorded by research midwives usually within 72 hours of birth.

The primary outcome was spontaneous preterm birth (sPTB) defined as spontaneous preterm labour or preterm premature rupture of membranes resulting in preterm birth at <37 weeks. Preterm labour (PTL) was defined as spontaneous uterine contractions occurring > 6 per hour (at least every 10 minutes) for more than 1 hour, with or without ruptured membranes at <37weeks. Preterm premature rupture of membranes (PPROM) was defined as confirmed rupture of membranes in the absence of labour and the time between the rupture of membranes to delivery is at least six hours greater than the combined time for established labour (URL: <https://www.anzctr.org.au>). *Uncomplicated pregnancy* was defined as a pregnancy with no antenatal medical or obstetric complications and resulting in the delivery of an appropriately grown, healthy infant at  $\geq 37$  weeks of gestation.

### **Genotyping**

Peripheral blood samples were collected from the women and DNA was extracted from buffy coats according to the manufacturers' instructions. Genotyping for the *VEGFA* -2578C/A (rs699947), *VEGFA* +936C/T (rs3025039), *KDR* -60C/T (rs2071559) and *ANGPT 1414* (rs2507800) polymorphisms was performed at the Australian Genome Research Facility (AGRF, Brisbane, Australia) using the Sequenom MassARRAY system (Sequenom Inc, San Diego, California). As a quality control measure, each sample was also genotyped for Amelogenin to ensure that the sex of the sample was correct [23]. The primers used for genotyping are detailed in table 1.

**Table 1 Primer sequences for Sequenom MassARRAY system**

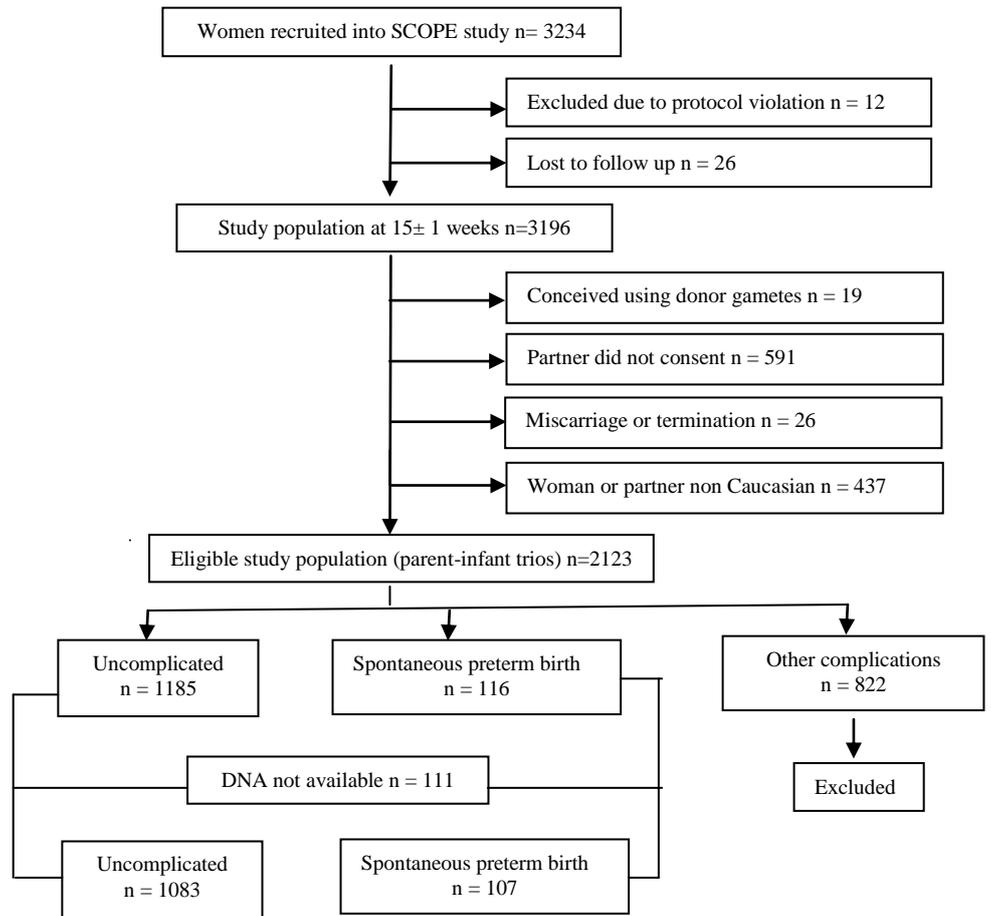
<b>Gene variant</b>	<b>1st PCR primer</b>	<b>2nd PCR primer</b>	<b>Extend primer</b>
<i>VEGFA</i> -2578C/A ( rs699947)	ACGTTGGATGAGTCAGTCTGATTATCCACC	ACGTTGGATGTTCTCAGTCCATGCCTCCAC	TAATTCTGATTATCCACCCAGATC
<i>VEGFA</i> +936C/T ( rs3025039)	ACGTTGGATGATGGCGAATCCAATTCCAAG	ACGTTGGATGAGACTCCGGCGGAAGCATT	CTAAGCCAATTCCAAGAGGGACC
<i>KDR</i> -604C/T (rs2071559)	ACGTTGGATGTCACTTCAAACCTTGGAGCCG	ACGTTGGATGATCAGAAAACGCACTTGCCC	GGGGAAATAGCGGGAATG
<i>ANGPT1</i> 1414T/A ( rs2507800)	ACGTTGGATGGGGAGAAAAATTGGCAAAAC	ACGTTGGATGTTCTTAGTCAGGTGACTATG	GGCAAAACTATTATATGTAAGGGA

## **Statistics**

Women in the sPTB group (cases) were compared with women in the uncomplicated pregnancy group (control subjects) in a nested case control study design. Missing data were excluded from the analyses. Chi-square test was used to test the genotypes at each polymorphic locus for Hardy-Weinberg Equilibrium and to compare categorical variables. Analysis of variance or Student's *t*-test was used to compare continuous variables. We used multivariable logistic regression models to estimate the individual and combined associations of maternal BMI and the genotypes of the polymorphisms in relation to sPTB with and without adjustments for the following maternal variables: age, smoking and alcohol consumption at 15 weeks gestation, birthweight, mean arterial blood pressure at 15 weeks gestation, born preterm and family history of PTB. In our preliminary analysis we evaluated 3 possible genetic models: dominant, recessive and additive. BMI was analysed as a continuous variable as well as a dichotomous variable grouping as BMI <25 and BMI  $\geq$  25. We grouped the overweight and obese women together as the sample size in each category was small to detect clinically significant results. A false discovery rate (FDR) correction was performed to adjust for multiple comparisons controlling the FDR at 15% [24]. All data analyses were performed using PASW version 17.02 (SPSS, Inc, Cary, North Carolina). Results were reported as number and percent [n (%)] or mean  $\pm$  standard deviation (SD) where appropriate.  $P < 0.05$  was considered statistically significant.

## **Results**

Of those recruited, a total of 1190 Caucasian women (107 in the preterm birth group and 1083 uncomplicated pregnancies) were included in the study. The exclusions are detailed in figure 1.



**Figure 1** Study population

Of the women in the sPTB birth group, 44 (41.1%) had preterm premature rupture of membranes and 63 (58.9%) had spontaneous onset of labour with intact membranes.

The characteristics of the participants are shown in table 2.

**Table 2 Characteristics of the study population**

<b>Characteristic</b>	<b>Uncomplicated Pregnancy (n = 1083)</b>	<b>sPTB (n = 107)</b>	<b>P value</b>
<b>Maternal characteristics</b>			
Age (years)	28.2 ± 5.6	27.9 ± 6.2	0.7
BMI (kg/m <sup>2</sup> )	24.9 ± 4.5	25.9 ± 5.4	0.02
Smoking at 15 weeks gestation	111 (10.2)	20 (18.7)	0.007
Participant born preterm	110 (10.2)	19 (17.8)	0.015
Family history of preterm birth	139 (12.8)	25 (23.4)	0.002
<b>Pregnancy outcome</b>			
Birthweight (g)	3591 ± 394	2394 ± 743	<0.001
Customised birthweight centile	54 ± 25	50 ± 31	<0.001
SGA infants	0	13 (12.1)	<0.001
Gestational age at delivery (weeks)	39.7 ± 1.2	33.6 ± 3.4	<0.001

Data are either N (%) or Mean ± SD, comparisons using Pearson chi-square or Student's *t*-test

Smoking at 15 weeks gestation ( $p = 0.007$ ), being born preterm ( $p = 0.015$ ) and a positive family history of preterm birth ( $p = 0.002$ ) were more prevalent among women who had sPTB compared to women with uncomplicated pregnancy (table 2). A higher maternal body mass index (BMI) was also demonstrated in the sPTB group ( $p = 0.02$ , table 2) compared to the uncomplicated pregnancy group. The distribution of maternal BMI groups in the study population is shown in table 3.

**Table 3 Distribution of maternal BMI groups in the study population**

<b>BMI category (kg/m<sup>2</sup>)</b>	<b>Uncomplicated pregnancy</b>	<b>Spontaneous preterm birth</b>
Underweight (< 18.5 )	16 (1.5)	1 (0.9)
Normal weight (≥ 18.5 and < 25)	635 (58.6)	51 (47.7)
Overweight (≥ 25 and < 30)	300 (27.7)	33 (30.8)
Obese (≥ 30)	132 (12.2)	22 (20.6)

Data are N (%)

All polymorphisms were in Hardy Weinberg equilibrium. No significant individual association was detected between the polymorphisms and sPTB ( $p > 0.05$ , table 4).

**Table 4 Genotype distribution of angiogenic gene polymorphisms in spontaneous preterm birth and uncomplicated pregnancy**

<b>Maternal variant</b>	<b>Uncomplicated pregnancy n (%)</b>	<b>sPTB n (%)</b>	<b>P</b>
<sup>1</sup> <i>VEGFA -2578C/A</i>			
CC	270 (25.1)	33 (31.4)	0.3
CA	537 (50)	46 (43.8)	
AA	267 (24.9)	26 (24.8)	
<sup>2</sup> <i>VEGFA +936C/T</i>			
CC	793 (73.9)	75 (70.1)	0.7
CT	257 (24)	29 (27.1)	
TT	23 (2.1)	3 (2.8)	
<sup>3</sup> <i>KDR -604T/C</i>			
TT	274 (25.6)	25 (23.6)	0.6
CT	522 (48.7)	57 (53.8)	
CC	276 (25.7)	24 (22.6)	
<sup>4</sup> <i>ANGPT1 1414A/T</i>			
AA	170 (15.7)	22 (20.8)	0.3
AT	514 (47.5)	49 (46.2)	
TT	399 (36.8)	35 (33)	

Abbreviations; sPTB, spontaneous preterm birth, genotyping failure <sup>1</sup>n = 11, <sup>2</sup>n = 10, <sup>3</sup>n = 12, <sup>4</sup>n = 01; results are expressed as N (%); comparisons are with uncomplicated pregnancy

After adjusting for confounding factors, maternal BMI  $\geq 25$  was not associated with the risk of sPTB compared to BMI  $< 25$  (aOR = 1.3, 95% CI: 0.9-1.9,  $p = 0.1$ , table 5).

When *VEGFA* -2578C/A polymorphism was considered, the association between maternal BMI and sPTB differed by the phenotype. Among women with a BMI  $< 25$  the *VEGFA* -2578C/A polymorphism had no significant association (Table 5). Among women with a BMI  $\geq 25$ , *VEGFA* -2578C/A AA genotype was associated with a higher risk of preterm birth (OR = 2.4, 95% CI: 1.4-4.6,  $p = 0.001$ , table 5). Maternal BMI had a significant interaction on the association between *VEGFA* -2578C/A polymorphism and the risk of sPTB (OR = 4.2, 95% CI: 1.7-10.9,  $p = 0.003$ , table 5). The interaction was also significant when maternal BMI was considered as a continuous variable and when adjusted for confounding factors (Table 5).

**Table 5 Association of VEGFA -2578C/A polymorphism with spontaneous preterm birth according to maternal BMI status**

BMI group	Genotype	No	PTB %	<i>P</i> value	OR (95% CI)	<i>aP</i> value	aOR(95%CI)
BMI < 25		695	7.5		1.0		1.0
BMI ≥ 25		484	11.2	0.04	1.5 (1.0-2.2)	0.1	1.3 (0.9-1.9)
BMI < 25	CC/CA	518	8.1		1.0		1.0
	AA	177	5.1	0.2	0.6 (0.3-1.3)	0.9	0.9 (0.6-1.6)
BMI ≥ 25	CC/CA	358	8.4		1.0		1.0
	AA	126	19	0.001	2.4 (1.4-4.6)	0.005	2.3 (1.3-4.3)
Interaction <sup>1</sup>				0.003	4.2 (1.7-10.9)	0.008	3.8 (1.4-10.2)
Interaction <sup>2</sup>				<0.001	1.2 (1.1-1.3)	<0.001	1.2 (1.1-1.3)

*aP* value and aOR(95% CI), adjusted for maternal age, smoking and alcohol consumption at 15 weeks gestation, birthweight, mean arterial blood pressure at 15 weeks gestation, born preterm and family history of PTB, Interaction<sup>1</sup>, BMI as a categorical variable, Interaction<sup>2</sup>, BMI as a continuous variable

A significant interaction effect was also found between maternal BMI and maternal *ANGPT1 1414A/T* polymorphism in risk for sPTB. Among women with a BMI < 25, *ANGPT1 1414A/T* AA genotype was associated with a higher risk of sPTB (aOR = 2.3, 95% CI: 1.2-4.4,  $p = 0.02$ , table 6). Maternal BMI had a significant interaction on the association between *ANGPT1 1414A/T* polymorphism and the risk of sPTB (OR = 3.3, 95% CI: 1.1-9.3,  $p = 0.02$ , table 6). The interaction was also significant when maternal BMI was considered as a continuous variable and when adjusted for confounding factors (Table 6).

Significant interactions were not demonstrated between maternal BMI and *VEGFA +936C/T* and *KDR -604T/C* polymorphisms (data not presented). All results remained significant after correcting for multiple testing.

**Table 6 Association of *ANGPT 1414T/A* polymorphism with spontaneous preterm birth according to maternal BMI status**

BMI group	Genotype	No	PTB %	P	OR (95% CI)	aP value	aOR(95%CI)
BMI < 25	TT/TA	592	6.3		1.0		1.0
	AA	111	13.5	0.02	2.3 (1.2-4.4)	0.04	2.0 (1.0-3.9)
BMI ≥ 25	TT/TA	405	11.6		1.0		1.0
	AA	81	8.6	0.4	0.7 (0.3-1.7)	0.2	0.6 (0.2-1.4)
Interaction <sup>1</sup>				0.02	3.3 (1.1-9.3)	0.04	3.0 (1.1-8.8)
Interaction <sup>2</sup>				0.04	1.1 (1.0-1.2)	0.04	1.1 (1.0-1.5)

aP value and aOR(95% CI), adjusted for maternal age, smoking and alcohol consumption at 15 weeks gestation, birthweight, mean arterial blood pressure at 15 weeks gestation, born preterm and family history of PTB, Interaction<sup>2</sup>, BMI as a continuous variable

## Discussion

This is the first study to report gene-environment interactions in angiogenesis regulating genes associated with sPTB. We report that BMI has a significant interaction on the association between *VEGFA* -2578C/A and *ANGPT1* 1414T/A polymorphisms and the risk of sPTB.

In overweight and obese women, the *VEGFA* -2578C/A AA genotype was associated with an increased risk of sPTB, whereas this polymorphism was not associated with sPTB in women of normal weight. Although the direct mechanism leading to this increased risk is not clear at present, previous studies suggest a plausible explanation. As individuals become obese, their adipocytes enlarge and the adipose tissue undergoes molecular changes leading to increased production of pro inflammatory cytokines [25]. Therefore obesity is proposed to be associated with a chronic low grade inflammation. Interaction between inflammatory and angiogenic pathways have been recognized in the pathogenesis of many human diseases. A recent study revealed that in the setting of inflammation, women experiencing spontaneous preterm birth had low serum placental growth factor levels [26]. *VEGFA* -2578C/A AA genotype is associated with lower transcriptional activity and plasma VEGF-A levels [27]. Our findings demonstrate that overweight or obese women with genotypes associated with low VEGF-A production may be at increased risk of sPTB.

We also found that in women with a BMI < 25 the *ANGPT1* 1414T/A AA genotype had an increased risk of sPTB and that an interaction exists between BMI and the polymorphism. Angiopoietin 1 1414T/A single nucleotide polymorphism is located in the microRNA-211 (miRNA-211) target site in the 3' UTR of *ANGPT1* and results in an A to T base change. The A allele is known to suppress angiopoietin-1 translation by facilitating miRNA-211 binding while the T allele is resistant to miRNA -211 induced

reduction in translation. The AA genotype is also associated with lower plasma angiopoietin-1 levels compared to the TT genotype [28]. Adipocytes express angiopoietin-1 and obesity is known to be associated with up-regulation of *ANGPT1* [19, 20]. As non overweight/obese women are unlikely to have the physiological up-regulation of *ANGPT1* expression, we can hypothesize that the AA genotype may increase the risk of spontaneous preterm birth in these women. We earlier reported that the *ANGPT1 1414T/A* polymorphism was associated with preeclampsia, SGA in the presence of maternal hypertensive disease and SGA with abnormal uterine artery Doppler findings [11].

The increased risk in sPTB of the *VEGFA -2578C/A* AA genotype when BMI was  $\geq 25$  and the *ANGPT1 1414T/A* AA genotype when the BMI was  $< 25$  group suggests that modification in the risk of sPTB with these polymorphisms is influenced by BMI status. The controversial literature regarding the association of maternal obesity with sPTB may partially be due to potential gene-environment interactions.

The strengths of our study include a large prospective cohort and collection of data on a large number of clinical variables. The associations of these polymorphisms with preeclampsia and SGA infants, as well as with abnormal uterine artery Doppler in this cohort have been published before. It has previously been proposed that the pregnancy complications preeclampsia, SGA infants and preterm birth constitute a continuum of disorders with similar pathogenic mechanisms. Consistent with this theory, we found that the *ANGPT1 1414T/A* polymorphism which was previously shown to be associated with other pregnancy complications was associated with sPTB. Given the sample size of our cohort, the prevalence of a polymorphism in 25% of women and a ratio of 10 control subjects to 1 case, the spontaneous preterm birth group had 80% power to detect an OR of 2.0 ( $\beta = 80\%$ ,  $\alpha = 0.05$ ).

In summary, the findings of our preliminary study demonstrate that maternal BMI interacts with angiogenesis regulating gene polymorphisms to modify the risk of spontaneous preterm birth. The findings of this preliminary study need to be replicated in a larger cohort.

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## Manuscript 7

### Vascular endothelial growth factor family gene polymorphisms in preeclampsia in Sinhalese women in Sri Lanka

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#### Abstract

**Objective:** To investigate the association of polymorphisms in the VEGF family genes (*VEGFA* -2578C/A, *VEGFA* +936C/T, *PGF* 642C/A, *KDR* -604T/C and *KDR* Val297Ile) with preeclampsia in Sinhalese women in Sri-Lanka.

**Method:** We conducted a case-control study where 175 nulliparous Sinhalese women with preeclampsia and 171 normotensive women matched for age, ethnicity, parity and BMI were recruited in tertiary care maternity hospitals in Sri Lanka. Preeclampsia was diagnosed using international guidelines. DNA extracted from peripheral venous blood was genotyped using the Sequenom MassARRAY system. The chi square test was used to compare the distribution of allele and genotype frequencies between the cases and the control subjects.

**Results:** The frequency of *PGF* 642C/A variant allele (OR 1.5, 95% CI 1.1-2.1) and dominant genotype model (aOR 1.6, 95% CI 1.0-2.4) were increased in preeclamptic

women compared to controls. *VEGFA* -2578C/A, *VEGFA* +936C/T, *KDR* -604T/C and *KDR Val297Ile* polymorphisms were not associated with preeclampsia.

**Conclusion:** Maternal *PGF* 642C/A polymorphism is associated with preeclampsia in Sinhalese women in Sri Lanka.

## Introduction

Preeclampsia is a multisystem disorder complicating 4-6% of all nulliparous pregnancies and is a leading cause of maternal and neonatal morbidity and mortality [1, 2]. Women who develop preeclampsia during their first pregnancy are at increased risk for recurrence of preeclampsia in the second pregnancy and for later life vascular disorders including hypertension, ischemic heart disease, stroke and venous thromboembolism [2-4].

The cause of preeclampsia remains largely unknown but there is growing evidence that an imbalance between the VEGF family of angiogenic growth factors including vascular endothelial growth factor (VEGF-A), placental growth factor (PlGF) and the anti-angiogenic molecule soluble FLT-1 (sFLT-1) is closely related to the pathogenesis of the disease [5]. During pregnancy, these angiogenic peptides are expressed in many cells at the maternal-fetal interface and regulate placental angiogenesis and spiral artery remodelling [6-8]. VEGF-A exerts its effects principally via two receptors, fms-like-tyrosine kinase-1 (FLT-1) and kinase-insert domain receptor (KDR) [9]. PlGF exerts its effects only via FLT-1 [9]. Soluble FLT-1 which is endogenously produced by alternative splicing, inhibits both VEGF-A and PlGF. In women who subsequently develop preeclampsia, serum VEGF-A and PlGF are reduced and sFLT-1 is increased before the clinical onset of the disease [5, 10].

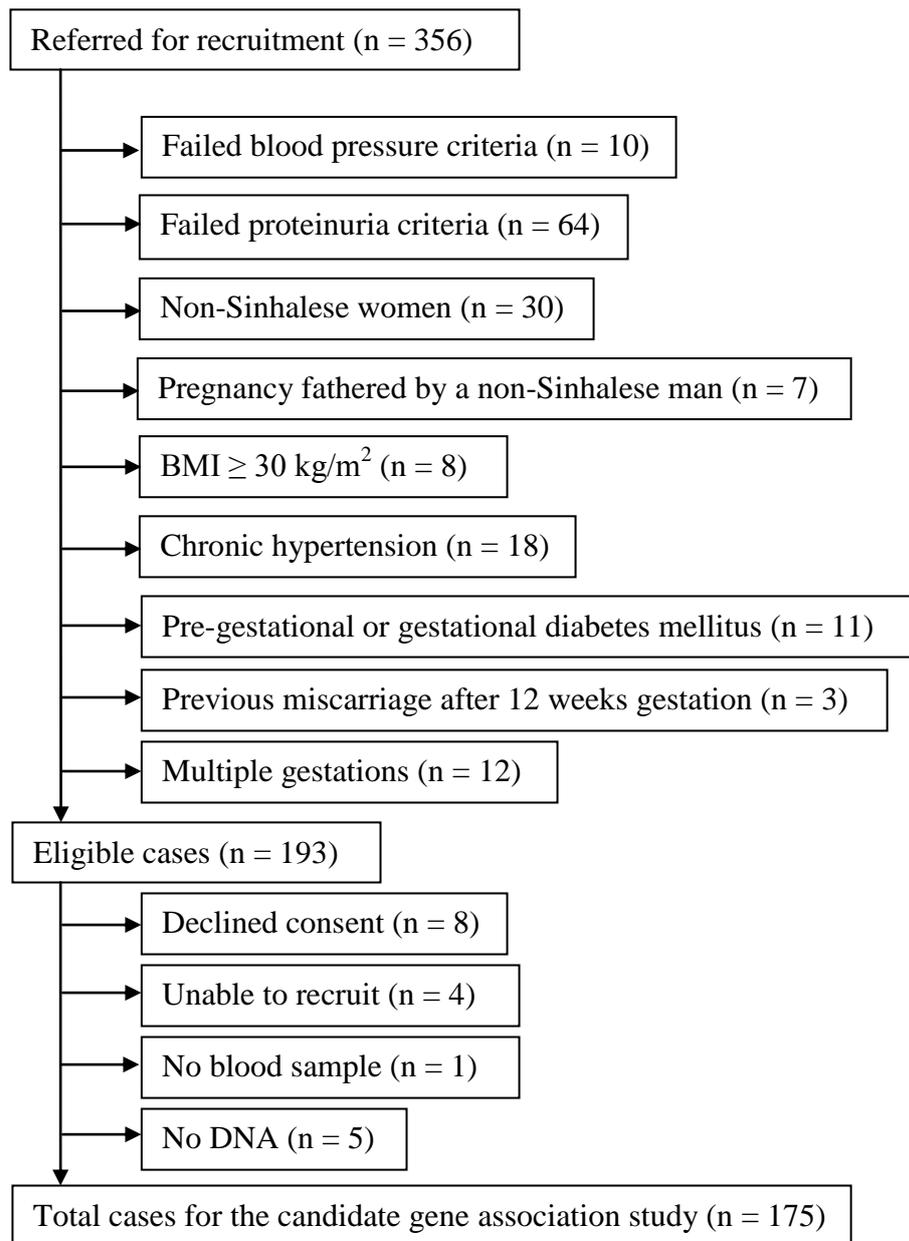
Several single nucleotide polymorphisms (SNPs) have been described in the VEGF family genes and some are known to alter gene expression and protein production. The aim of this study was to investigate the association of *VEGFA* -2578C/A, *VEGFA* +936C/T, *PlGF* 642C/A, *KDR* -604T/C and *KDR* Val297Ile polymorphisms with preeclampsia in Sinhalese women in Sri-Lanka.

## Materials and Methods

We conducted a case-control study where nulliparous Sinhalese women with preeclampsia and matched controls were recruited at two tertiary care maternity hospitals in Colombo between August 2001 and January 2003. Ethics approval was obtained from the Ethics Review Committee of the Faculty of Medicine, University of Colombo, Sri-Lanka (EC/08/133) and the Human Ethics Committee of the University of Adelaide, Australia (H-147-2008). Only women providing written informed consent were recruited. Preeclampsia was defined as systolic blood pressure  $\geq 140$  mmHg or diastolic blood pressure  $\geq 90$  mmHg, or both, on at least two occasions six hours apart after 20 weeks' gestation with proteinuria of  $\geq 1+$  on the urine protein heat coagulation test (HCT) not associated with urinary tract infection or ruptured membranes. The HCT has been validated for use in these maternity hospitals. A proteinuria of  $\geq 1+$  on the HCT is as sensitive and specific as  $\geq 2+$  on the dipstick test in detecting proteinuria of  $\geq 500$ mg/day on a 24 hour urine collection [11]. Small for gestational age (SGA) was defined as a birthweight  $\leq 10^{\text{th}}$  customised birthweight percentile adjusted for maternal ethnicity, weight, height, parity, gestational age at delivery and infant sex.

Exclusion criteria included non Sinhalese women or women of a mixed ethnicity, current pregnancy fathered by a non Sinhalese man, renal disease, chronic hypertension, persistent proteinuria (defined as  $\geq 1+$  on the HCT in the first three urine samples tested in pregnancy with or without urinary tract infection), ischemic heart disease, cerebro-vascular accidents, type 1 or 2 diabetes mellitus, body mass index (BMI)  $\geq 30$ kg/m<sup>2</sup> based on height and weight measured at the antenatal booking visit, a previous miscarriage after 12 weeks of gestation, hydatidiform mole, multiple gestations and gestational diabetes mellitus in the current pregnancy [12].

As cases, 356 nulliparous pregnant women were referred and 175 were included in the study. The exclusions are detailed in figure 1.



**Figure 1** Selection of cases for the candidate gene association study

As controls, 180 pregnant women, who were normotensive and non-proteinuric throughout the pregnancy delivering at term and matched for age, parity, ethnicity and body mass index (BMI) were recruited within 24 hours of delivery. Of the control women, nine delivered infants with a birthweight  $\leq 5^{\text{th}}$  customised birthweight percentile. As these infants were likely to have intra-uterine growth restriction (IUGR) which may have shared patho-physiological abnormalities with preeclampsia, these nine control women were excluded from the study.

Maternal data collected included demographic information, medical history and previous obstetric history. Current pregnancy data included information on any complications during current pregnancy and smoking. Maternal physical measurements obtained at the booking visit included height, weight and blood pressure. Two consecutive manual blood pressure measurements using a mercury sphygmomanometer with a cuff of the appropriate size and Korotkoff V for diastolic blood pressure were recorded. Proteinuria in a mid stream urine specimen was measured in all women by the heat coagulation test. Infants' measurements including the birthweight were obtained from the medical records.

### **Selection of the gene variants**

The gene variants were selected based on the HapMap genotype frequency data and potential biological functions. The *VEGFA* gene (*VEGFA*, OMIM 192240) is located on chromosome 6p12. Several transcription factor- binding sites are found in the *VEGFA* 5'-untranslated region (5'UTR) and variation within the region increases transcriptional activity [13]. *VEGFA* -2578C/A (rs699947) polymorphism located in the 5'UTR is associated with higher VEGF-A production by peripheral blood mononuclear cells [14] and the *VEGFA* +936C/T (rs3025039) polymorphism located in the 3'-untranslated region (3'UTR) is associated with reduced plasma VEGF-A levels [15]. The *PGF* gene

(*PGF*, OMIM 601121) is located on chromosome 14q24.3. At present there is paucity of literature on *PGF* polymorphisms. We searched for putative transcription factor binding sites and found that the single base substitution at rs1042886 (*PGF* 642C/A, Genbank Refseq X54936) may affect binding of several transcription factors. Carriers of the C allele have five additional transcription factor binding sites, including Glial cell missing-1 (GCM-1) which are not found in carriers with the A allele (<http://www.genomatix.de>). In addition, carriers of the A allele have the recognition sequence for myeloid zinc finger-1 (MZF-1) which is not found in carriers of the C allele (<http://www.cbrc.jp>). KDR is the major mediator of the mitogenic, angiogenic, permeability enhancing and endothelial survival effects of VEGF-A [9] and the *KDR* gene (OMIM 191306) is located on chromosome 4q11-q12. The *KDR* -60T/C (rs2071559) promoter variant may affect transcriptional factor E2F binding to the region, which may alter *KDR* expression [16]. The variant allele of *KDR* -60T/C is also shown to be associated with lower KDR protein levels [17]. The *KDR* exonic variant (exon\_7) rs2305948 results in a non-synonymous amino acid change at *Val297Ile*. The amino acid is located at the third extracellular Ig-like domain that is important for ligand-receptor binding [16, 17].

### **Genotyping**

Peripheral blood samples were collected from the women and DNA was extracted using QiaAmp blood midi DNA extraction kits (Qiagen). Genotyping was performed at the Australian Genome Research Facility (AGRF, Brisbane, Australia) using the Sequenom MassARRAY system (Sequenom Inc, San Diego, California). As a quality control measure 300 independent samples that were genotyped for the same SNPs using quantitative reverse transcription-polymerase chain reaction (qRT-PCR) were genotyped using the Sequenom MassARRAY system at AGRF. The concordance rate

of the qRT-PCR results and MassARRAY results was 100%. Each sample was also genotyped for Amelogenin to ensure that the sex of the sample was correct [18]. The primers used for genotyping are detailed in table 1.

**Table 1 Primer sequences for Sequenom MassARRAY system**

Gene variant	1st PCR primer	2nd PCR primer	Extend primer
<i>VEGFA</i> -2578C/A (rs699947)	ACGTTGGATGAGTCAGTCTGATTATCCACC	ACGTTGGATGTTCTCAGTCCATGCCTCCAC	TAATTCTGATTATCCACCCAGATC
<i>VEGFA</i> +936C/T ( rs3025039)	ACGTTGGATGATGGCGAATCCAATTCCAAG	ACGTTGGATGAGACTCCGGCGGAAGCATT	CTAAGCCAATTCCAAGAGGGACC
<i>PGF</i> 642C/A (rs1042886)	ACGTTGGATGGGCCCACTCTGTATGTGTC	ACGTTGGATGCTGGGACATTGTTCTTTCCG	CCCCACACTCTGTATGTGTCTCTTAG
<i>KDR</i> -604T/C (rs2071559)	ACGTTGGATGTCACTTCAAACCTGGAGCCG	ACGTTGGATGATCAGAAAACGCACTTGCCC	GGGAAATAGCGGGAATG
<i>KDR</i> Val297Ile (rs2305948)	ACGTTGGATGAGTCTGGGAGTGAGATGAAG	ACGTTGGATGTGTACAATCCTTGGTCACTC	GCACCTTAACTATAGATGGT

## **Statistical analyses**

The Chi-square test was used to test the genotypes at each polymorphic locus for Hardy-Weinberg Equilibrium. The Chi-square test or Fisher's exact test was used to compare the distribution of allele and genotype frequencies between the cases and the control subjects. In the genotype analyses, we evaluated dominant and recessive genotype models. All data analyses were performed using PASW version 17.02 (SPSS, Inc, Cary, North Carolina). Results were reported as number and percent [n (%)] or mean  $\pm$  standard deviation (SD) where appropriate.  $P < 0.05$  was considered statistically significant.

We collected peripheral blood from randomly selected Sinhalese men and women (n=80) from the general population and genotyped DNA extracted from buffy coats to determine the prevalence of the polymorphisms in the general population. On the basis of prevalence of a polymorphism in 20% of the general population, a ratio of 1 control subject to 1 case, 171 preeclamptic women and 171 control subjects has 80% power to detect an odds ratio of 2.0 ( $\beta = 80\%$ ,  $\alpha = 0.05$ ).

## **Results**

All polymorphisms were in Hardy-Weinberg Equilibrium. Of the 175 preeclamptic women, 73 (41.7%) developed preterm preeclampsia requiring delivery before 37 weeks of gestation and 102 (58.3%) developed term disease. Sixty one percent (n = 51) of the women who had preterm preeclampsia and 39% (n=33) of the women who had term preeclampsia delivered an SGA infant. In the preterm preeclamptic group mean gestational age at delivery was 30.6 weeks ( $30.6 \pm 4.5$ ) and the mean birthweight corrected for gestational age was 1150g ( $1150 \pm 492$ ). In the term preeclamptic group mean gestational age at delivery was 37.4 weeks ( $37.4 \pm 1.8$ ) and the mean birthweight corrected for gestational age was 2331g ( $2331 \pm 577$ ). All cases and control subjects

were non smokers. Maternal characteristics and pregnancy outcome data in relation to preeclampsia are detailed in table 2.

**Table 2 Characteristics of the study population**

<b>Characteristic</b>	<b>Control (n = 171)</b>	<b>Preeclampsia (n = 175)</b>	<b>P</b>
<b>Maternal characteristics</b>			
Age (years)	27.1 ± 5.1	26.9 ± 5.3	0.8
BMI (kg/m)	20.7 ± 3.2	21.0 ± 3.1	0.4
Systolic blood pressure	108 ± 9	112 ± 10	0.002
Diastolic blood pressure	69 ± 7	71 ± 7	0.002
Mean arterial pressure	81.9 ± 6.9	83.1 ± 13.2	0.3
<b>Pregnancy outcome</b>			
Neonatal birthweight (g)	3016 ± 412	1842 ± 797	<0.001
Customised birthweight centile	50 ± 30	21 ± 28	<0.001
Gestational age at delivery (weeks)	39.4 ± 1.1	34.5 ± 4.6	<0.001

Systolic and diastolic blood pressure measurements were taken at the antenatal booking visit, Data are either N (%) or Mean ± SD, using Pearson Chi-square or Student's *t*-test

The prevalence of the A allele and the dominant genotype model (CA+AA) of the *PGF 642C/A* polymorphism were increased in preeclamptic women compared to control subjects (table 3). *VEGFA -2578C/A*, *VEGFA +936C/T*, *KDR -604T/C* and *KDR Val297Ile* polymorphisms were not associated with preeclampsia (table 3).

**Table 3 Genotype distribution in cases and control subjects**

Gene variant	Allele		Genotype n (%)			Dominant model	Recessive model
	MAF%	OR (95% CI)	MM	Mm	mm	OR (95% CI)	OR (95% CI)
<i>VEGFA -2578C/A</i>							
Control subjects	49.7	1	40 (23.8)	89 (53.0)	39 (23.2)	1	1
Cases	50.9	1.0 (0.8-1.4)	42 (24.1)	87 (50.0)	45 (25.9)	1.0 (0.6-1.6)	1.1 (0.7-1.9)
<i>VEGFA +936C/T</i>							
Control subjects	8.9	1	139 (82.7)	28 (16.7)	1 (0.6)	1	1
Cases	11.2	1.3 (0.8-2.1)	140 (80.5)	29 (16.6)	5 (2.9)	1.2 (0.7-2.0)	4.9 (0.6-42.8)
<i>PGF642C/A</i>							
Control subjects	23.5	1	101 (59.4)	58 (34.1)	11 (6.5)	1	1
Cases	31.3	<b>1.5 (1.1-2.1)</b>	83 (48.5)	69 (40.4)	19 (11.1)	<b>1.6 (1.0-2.4)</b>	1.8 (0.8-3.9)
<i>KDR -604T/C</i>							
Control subjects	57.2	1	27 (16.2)	90 (53.9)	50 (29.9)	1	1
Cases	51.1	0.8 (0.6-1.1)	41 (23.5)	88 (50.6)	45 (25.9)	0.6 (0.4-1.1)	0.8 (0.5-1.3)
<i>KDR Val297Ile</i>							
Control subjects	12.5	1	128 (76.2)	38 (22.6)	2 (1.2)	1	1
Cases	11.5	0.9 (0.6-1.4)	137 (78.7)	34 (19.6)	3 (1.7)	0.9 (0.5-1.4)	1.5 (0.2-8.8)

MAF, minor allele frequency; M, major allele; m, minor allele; Dominant model, Mm + mm compared to MM, Recessive model, mm compared to MM + Mm; OR (95% CI) values in bold indicate significant results

## Discussion

Our study demonstrates the association of *PGF 642C/A* A allele with preeclampsia in Sinhalese women. *PGF 642C/A* polymorphism is located in the 3'UTR of the *PGF* gene. We searched for putative transcription factor binding sites in the 3'UTR of the *PGF* gene and found that carriers of the A allele have the recognition sequence for the transcription factor MZF-1 and lack the recognition sequence for the transcription factor GCM-1. In a recent study investigating the association of early pregnancy maternal peripheral blood gene expression, Enquobahrie *et al.* identified MZF-1 as one of the transcription factors responsible for differential gene expression in preeclampsia [19]. GCM-1 is a transcription factor that regulates the differentiation of villous and extra-villous human placental trophoblast cells [20]. Down-regulation of GCM-1 expression in placental villi and reduced trophoblast differentiation are demonstrated in preeclamptic placentae [21]. These findings suggest that altered transcription factor binding to the region may underlie the association of the *PGF 642C/A* polymorphism with preeclampsia.

In this study, we did not observe a significant association between the *VEGFA* polymorphisms and preeclampsia. Our results are consistent with the results of two other studies that investigated the *VEGFA-2578C/A* polymorphism in preeclampsia and found no association between the polymorphism and the disease [22, 23]. Banyasz *et al.* reported that hypertension and proteinuria were diagnosed earlier in carriers of the *VEGFA -2578C/A* A allele after adjusting for risk factors for preeclampsia but did not find an association of the polymorphism with preeclampsia [24].

Two previous studies report an association between *VEGFA +936C/T* polymorphism and preeclampsia. In a Korean population, Shim *et al.* demonstrated that the T allele of the *VEGFA +936C/T* polymorphism was more prevalent among preeclamptic women compared to controls [25]. In a Greek population, Papazoglou *et al.* demonstrated that

the T allele of the *VEGFA* +936C/T polymorphism was more prevalent among women with severe preeclampsia compared to controls [23]. The inconsistent results observed between our study and the aforementioned studies may partly be explained by genotype frequency differences between different ethnic groups as well as phenotypic differences between the study populations. In addition, the Greek study found no association of the polymorphism with preeclampsia and the only association was with severe preeclampsia which comprised only 20 women. We did not perform subgroup analysis within our preeclamptic population as the sample size in each category was not sufficient for adequate power to detect clinically relevant differences. We recently reported that the *VEGFA* +936C/T polymorphism was associated with small for gestational age infants in a large Caucasian study population and also found that the association was independent of maternal hypertensive disease [26]. The Korean study did not report the effects of growth restriction in their preeclamptic women and their preeclamptic population comprised infants with significantly lower birthweight compared to the controls. The Greek study also does not report the presence of growth restriction which is likely in their severe preeclamptic population. Therefore, the associations demonstrated in these studies may be due to the effects of growth restriction in their preeclamptic study populations.

We recently reported that the prevalence of the *KDR* -604T/C variant was increased in men who fathered a preeclamptic pregnancy as well as in infants born to preeclamptic women in a Caucasian cohort. We also found that the polymorphism was associated with preeclampsia in women who were non-smokers at 15 weeks of gestation [27]. However, we did not find a significant association of the polymorphism with preeclampsia in the Sri Lankan cohort. This may be due to genotype frequency differences between the two study groups.

The strengths of our study include the selection of cases based on a strictly defined disease phenotype and recruiting an appropriately matched control group. As with most candidate gene association studies of this nature, our study is limited by sample size. However, considering the consistent association of maternal serum PIGF levels with preeclampsia and the potential changes in transcription factor binding implicated by the polymorphism, the novel association of the polymorphism with preeclampsia may have potential clinical implications. Therefore, replication in other study populations as well as investigating the association of the polymorphism with the expression of GCM-1 and MZF-1 transcription factors may be beneficial.

### **Conclusion**

Our study demonstrates the association of *PGF 642C/A* single nucleotide polymorphism with preeclampsia in Sinhalese women in Sri Lanka.

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## Manuscript 8

### Placental expression of VEGF family mRNA in adverse pregnancy outcomes

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#### Abstract

**Introduction:** The pregnancy complications preeclampsia, gestational hypertension, small for gestational age infants (SGA) and preterm birth (PTB) affect approximately 21% of all pregnancies. The Vascular Endothelial Growth Factor family (VEGF) is implicated in the pathogenesis of these complications. We aimed to evaluate the placental mRNA expression of *VEGFA*, *PGF*, *FLT1* and *KDR* in pregnancies complicated by preeclampsia, gestational hypertension, SGA infants and preterm birth.

**Method:** Placentae were collected at delivery from women with pregnancies complicated by preeclampsia (n = 18), gestational hypertension (n = 15), normotensive SGA infants (n = 13), late spontaneous preterm birth (n = 10) and uncomplicated pregnancy (n = 30). RNA was extracted and *VEGFA*, *PGF*, *FLT1* and *KDR* expression were quantified using qRT-PCR. Kruskal Wallis test was used to compare placental mRNA expression in the adverse pregnancy outcome groups compared to uncomplicated term pregnancy.

**Results:** Compared to placental mRNA from uncomplicated pregnancies, *VEGFA* ( $p = 0.006$ ), *PGF* ( $p < 0.001$ ), *KDR* ( $p < 0.001$ ) and *FLT1* ( $p = 0.02$ ) mRNA were reduced in preeclamptic placentae; *VEGFA* ( $p < 0.001$ ), *PGF* ( $p = 0.01$ ) and *KDR* ( $p = 0.008$ ) mRNA were reduced in placentae from pregnancies complicated by gestational hypertension; *VEGFA* ( $p = 0.03$ ) mRNA was reduced in normotensive SGA pregnancies; *VEGFA* ( $p = 0.008$ ), *PGF* ( $p = 0.01$ ), *KDR* ( $p = 0.04$ ) and *FLT1* ( $p = 0.02$ ) mRNA were reduced in placentae from late spontaneous PTB.

**Conclusion:** VEGF family of angiogenic growth factor mRNA expression in the placenta is reduced in gestational hypertensive disorders, SGA and in spontaneous preterm birth.

## Introduction

The pregnancy complications preeclampsia, gestational hypertension, small for gestational age infants (SGA) and spontaneous preterm birth affect approximately 21% of all pregnancies [1, 2]. Early placentation defects, including impaired maternal spiral artery remodelling and placental villous vascularisation have been demonstrated in pregnancies complicated by preeclampsia, SGA infants and preterm birth [3-6]. Many molecular pathways are involved in the pathogenesis of these placentation defects, of which the vascular endothelial growth factor (VEGF) family mediated angiogenic pathway is recognized as playing an important role [7].

The VEGF family consists of VEGF-A, VEGF-B, VEGF-C, VEGF-D, placental growth factor (PlGF) and their receptors VEGFR-1 (FLT-1, fms-like-tyrosine-kinase receptor 1), VEGFR-2 (KDR, kinase insert domain receptor; Flk in mice), VEGFR-3 and the co-receptors Neuropilin-1 and Neuropilin-2. Of these, VEGF-A and PlGF acting through KDR and FLT-1 are known to regulate early placental vascular development.

Gene ablation studies in mice demonstrate that homozygous gene mutations in *VEGFA*, *FLT1* and *Flk* result in early embryonic death demonstrating that these molecules are critical for embryonic angiogenesis [8-12]. During pregnancy, VEGF-A, PlGF, FLT-1 and KDR are expressed in villous and extravillous trophoblasts, villous vascular endothelium, as well as in decidual natural killer cells, and the level of expression is known to be altered in adverse pregnancy outcomes [13-18].

To date, most studies on placental expression of the VEGF family of angiogenic growth factors in adverse pregnancy outcomes have focused mainly on the expression of *VEGFA* in preeclampsia. Some studies have shown that placental expression of *VEGFA* mRNA at term is reduced [19, 20] in preeclamptic placentae compared to normal placentae while others have demonstrated an increase [21] or no difference [22, 23]. The aim of this study was to evaluate the expression of *VEGFA*, *PlGF*, *FLT1* and *KDR*

mRNA in pregnancies complicated by preeclampsia, gestational hypertension, SGA infants or spontaneous preterm birth.

## **Materials and Methods**

This is a nested case control study where participants were recruited from the Screening for Pregnancy Endpoints (SCOPE) study. The SCOPE study is an international, multicenter, prospective cohort study with the aim of developing screening tests to predict preeclampsia, SGA infants and preterm birth across different populations [24]. The participants were recruited to the Adelaide cohort of the SCOPE study and ethics approval was gained from the local ethics committees. All women provided written informed consent for collection of placentae at delivery.

Nulliparous women with singleton pregnancies were recruited between September 2005 and September 2008. Women considered at high risk for preeclampsia, SGA infants or preterm birth were not eligible to participate [25].

Maternal data collected included demographic information, medical history, obstetric history, family history of obstetric complications and medical disorders. Current pregnancy data included information on any complications during current pregnancy, diet, smoking, alcohol and the use of recreational drugs [24]. Maternal physical measurements obtained at 15 weeks of gestation included height, weight and blood pressure.

*Gestational hypertension* was defined as systolic blood pressure  $\geq 140$  mmHg or diastolic blood pressure  $\geq 90$  mmHg, or both, on at least two occasions four hours apart after 20 weeks' gestation. *Preeclampsia* was defined as gestational hypertension with significant proteinuria and/or any multisystem complication of the disease [26]. *Severe hypertension* was defined as systolic blood pressure  $\geq 170$  mmHg or diastolic blood pressure  $\geq 110$  mmHg. *Small for gestational age* was defined as a birth weight  $< 10^{\text{th}}$

customised percentile adjusted for maternal height, weight, parity and ethnicity, as well as gestational age at delivery and infant sex [27]. *Normotensive SGA* was defined as birth of an SGA infant where the mother did not have hypertension. *Spontaneous preterm birth* was defined as spontaneous preterm labour or preterm premature rupture of membranes resulting in preterm birth at <37 weeks. *Uncomplicated pregnancy* was defined as a pregnancy with no antenatal medical or obstetric complications and resulting in the delivery of an appropriately grown, healthy infant at  $\geq 37$  weeks of gestation.

### **Placental tissue**

The study groups considered were: (1) preeclampsia (n = 18); (2) gestational hypertension (n = 15); (3) normotensive SGA (n = 13); (4) spontaneous preterm birth (n = 10) and (5) uncomplicated pregnancy (n = 30). The placental samples were randomly collected from a small subset of SCOPE women by labour ward midwives usually within 30 minutes of delivery. As far as we are aware there was no selection bias although if the baby or woman were very sick their placentae would not have been sampled. The placentae were weighed and a 1cm<sup>2</sup> full thickness block of tissue was immersed in RNAlater (Ambion, Austin, Texas) for at least 24 hours and frozen at -80 °C for subsequent RNA extraction. Total RNA was isolated from 100mg of each placental villous tissue using the TRIzol method according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). Potential RNA degrading contaminants were removed by treatment with the DNA-free™ DNase Treatment Kit (Ambion, Austin, Texas) and RNA integrity was determined by gel electrophoresis.

## Quantitative RT-PCR

For each placental sample, 2 µg of RNA was reverse transcribed to cDNA using random hexamer primers (GeneWorks, Adelaide, SA, Australia), and Superscript III (Invitrogen, Carlsbad, CA, USA) according to the manufacturers' instructions. Quantitative RT-PCR was performed on a Rotor-Gene™ 6000 real-time PCR machine (Corbett Research, Sydney, Australia). All reactions were set up using a Cass 1200™ liquid handling system (Corbett Robotics, Brisbane, Australia). The endogenous control, *18s rRNA* was used for normalization of the raw data. The primer sequences for *VEGFA*, *PGF*, *FLT1*, *KDR* and *18s rRNA* are detailed in table 1. All reactions were carried out in 10 µL of mixture containing, 5µL of SYBR Green PCR Master Mix (2X) (Applied Biosystems, Warrington, UK), 0.5µL each of forward and reverse primer, 2 µL cDNA and 2µL of sterile water for injection. The thermal cycling conditions were 10 minutes at 95°C, then with 40 cycles at 95°C for 15 seconds, 60°C for 10 seconds and 72°C for 10 seconds. All samples were assayed in triplicate and a six point standard and an internal control were assayed in triplicate on each plate. Relative mRNA expression was determined by the Standard Curve method [28].

**Table 1 Sequence of primers used for qRT-PCR**

<b>Gene</b>	<b>Sequence</b>	<b>PCR product length (bp)</b>
<i>VEGFA</i> -FP <i>VEGFA</i> -RP	5'-CTGGAGTGTGTGCCCACTGA-3' 5'-TCCTATGTGCTGGCCTTGGT-3'	96
<i>PGF</i> -FP <i>PGF</i> -RP	5'-AATCTGCACTGTGTGCCGG-3' 5'-TCCCCAGAACGGATCTTTAGG-3'	67
<i>FLT1</i> -FP <i>FLT1</i> -RP	5'-TCCCTTATGATGCCAGCAAGT-3' 5'-CCAAAAGCCCCTCTTCCAA-3'	79
<i>KDR</i> -FP <i>KDR</i> -RP	5'-CTTCGAAGCATCAGCATAAGAACT-3' 5'-TGGTCATCAGCCCACTGGAT-3'	156
<i>18s</i> -FP <i>18s</i> -RP	5'-AGAAACGGCTACCACATCCAA-3' 5'-CCTGTATTGTTATTTTTCGTCACCT-3'	91

FP, Forward primer; RP, Reverse primer; bp, base pair

## Statistics

The Chi square test was used to compare categorical variables and ANOVA was used to compare continuous variables that were normally distributed. Placental expression of VEGF family angiogenic growth factors in complicated pregnancy was compared to the expression in uncomplicated term pregnancy using Kruskal Wallis test since these data were not normally distributed. All data analyses were performed using PASW version 17.02 (SPSS, Inc, Cary, North Carolina). Results are reported as N (%), mean  $\pm$  standard deviation (SD) or median and range.  $P < 0.05$  was considered statistically significant.

## Results

Characteristics of the study population are detailed in table 2 [29]. In the study cohort, 50 (58.1%) women had unassisted vaginal delivery, 17 (19.8%) had operative vaginal delivery, 17 (19.8%) underwent Caesarean section after the onset of labour and 2 (2.3%) underwent elective Caesarean section. Of the preeclamptic women, 9 (50%) had severe hypertension and delivered SGA infants. Four women in the preeclampsia group and one woman in the normotensive SGA group delivered prior to 37 weeks gestation. There was no significant difference in placental mRNA expression of *VEGFA*, *PGF*, *KDR* and *FLT1* based on the mode of delivery (data not presented). Placental mRNA levels were unaffected by smoking or alcohol intake at 15 weeks gestation (data not presented). Placental weight was not related to mRNA expression of any gene (data not presented).

**Table 2 Characteristics of the study population**

Characteristic	Control n = 30	Preeclampsia n =18	<i>P</i>	GH n = 15	<i>P</i>	NSGA n = 13	<i>P</i>	PTB n = 10	<i>P</i>
Maternal age (years)	23.6 ± 4.8	24.1 ± 4.7	0.7	23.9 ± 4.5	0.8	23.7 ± 4.8	0.9	21.6 ± 4.7	0.3
Maternal BMI (kg/m)	27.4 ± 5.1	30.1 ± 7.1	0.2	28.4 ± 5.1	0.5	28.1 ± 5.9	0.7	24.8 ± 6.2	0.2
Neonatal birthweight (g) *	3547 ± 370	3212 ± 660	<b>0.04</b>	3466 ± 381	0.4	2844 ± 265	<b>&lt;0.001</b>	3110 ± 430	0.2
Customised birthweight centile*	53 ± 23	32 ± 31	<b>0.03</b>	44 ± 24	0.2	5.0 ± 3.0	<b>&lt;0.001</b>	44 ± 38	0.6
Gestational age at delivery (weeks) **	40.1 (38-41)	38.0 (32-41)	<b>&lt;0.001</b>	39.3 (37-41)	<b>0.03</b>	39.4 (36-41)	0.06	35.4 (34-36)	<b>&lt;0.001</b>
Placental weight*	621 ± 125	570 ± 148	0.9	523 ± 79	0.07	478 ± 94	<b>0.005</b>	474 ± 93	0.6
Birthweight : Placental weight ratio*	6.0 ± 0.9	5.5 ± 1.1	0.1	6.7 ± 0.7	0.06	5.8 ± 0.9	0.4	5.6 ± 0.8	0.1

Data are either N (%) or Mean ± SD, comparisons are with uncomplicated term pregnancy using ANOVA, \*adjusted for gestational age at delivery, \*\* mean (range), bold indicates significant *p* values

### **Placental VEGF family mRNA expression in preeclampsia**

Placental expression of *VEGFA*, *PGF*, *KDR* and *FLT1* mRNA were reduced by 53% ( $p = 0.006$ ), 60% ( $p < 0.001$ ), 55% ( $p < 0.001$ ) and by 45% ( $p = 0.02$ ) respectively, in preeclamptic placentae compared to those from uncomplicated pregnancy (Figure 1). The results were unchanged when placentae from the women who delivered preterm and those who delivered SGA infants were excluded from the analyses.

### **Placental VEGF family mRNA expression in pregnancies complicated by gestational hypertension**

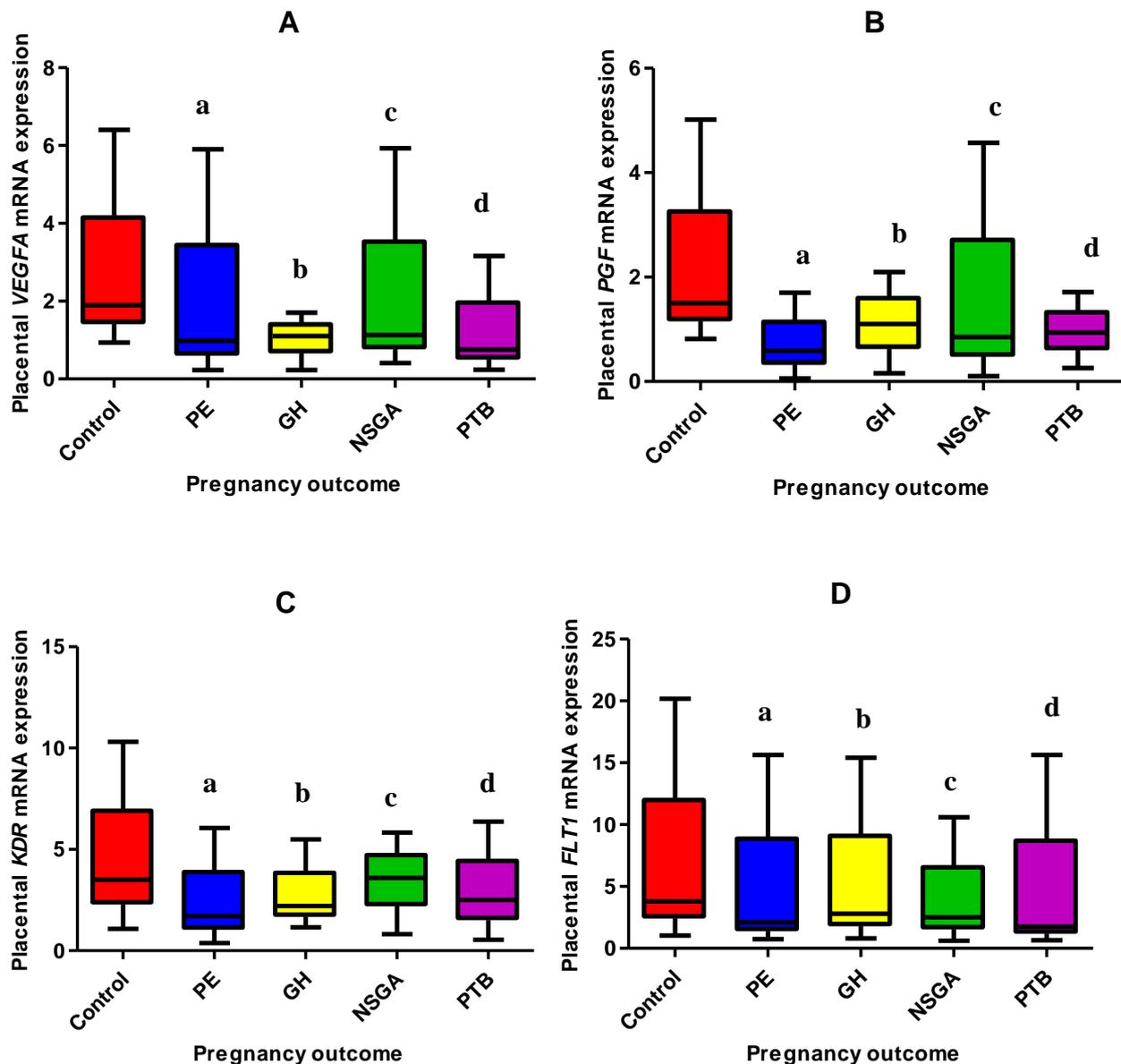
Placental expression of *VEGFA*, *PGF* and *KDR* mRNA were reduced by 47% ( $p < 0.001$ ), 27% ( $p = 0.01$ ), and by 39% ( $p = 0.008$ ) respectively, in pregnancies complicated by gestational hypertension compared to uncomplicated pregnancy (Figure 1). *FLT1* mRNA expression was similar between the two groups ( $p = 0.4$ ).

### **Placental VEGF family mRNA expression in normotensive SGA pregnancies**

Placental expression of *VEGFA* mRNA was reduced by 42% ( $p = 0.03$ ) in normotensive SGA compared to uncomplicated pregnancy (Figure 1). Significant differences were not observed in the expression of *PGF* ( $p = 0.08$ ), *KDR* ( $p = 0.5$ ) and *FLT1* ( $p = 0.5$ ) mRNA between the two groups. The results were unchanged when the placentae from the woman who delivered preterm was excluded from the analyses.

### **Placental VEGF family mRNA expression in spontaneous preterm birth**

Placental expression of *VEGFA*, *PGF*, *KDR* and *FLT1* mRNA were reduced by 58% ( $p = 0.008$ ), 40% ( $p = 0.01$ ), 29% ( $p = 0.04$ ) and by 53% ( $p = 0.02$ ) respectively, in spontaneous preterm birth compared to those from uncomplicated pregnancy (Figure 1).



**Figure 1 Placental expression of VEGF family mRNA in adverse pregnancy outcomes compared to uncomplicated term pregnancy.** PE, preeclampsia; GH, gestational hypertension; NSGA, normotensive SGA, PTB, preterm birth; Data are presented as median (range) and are analysed using Kruskal Wallis test; (A) *VEGFA* mRNA expression <sup>a</sup> $p = 0.006$ , <sup>b</sup> $p < 0.001$ , <sup>c</sup> $p = 0.03$ , <sup>d</sup> $p = 0.008$ ; (B) *PGF* mRNA expression <sup>a</sup> $p < 0.001$ , <sup>b</sup> $p = 0.01$ , <sup>c</sup> $p = 0.08$ , <sup>d</sup> $p = 0.01$ ; (C) *KDR* mRNA expression <sup>a</sup> $p < 0.001$ , <sup>b</sup> $p = 0.008$ , <sup>c</sup> $p = 0.5$ , <sup>d</sup> $p = 0.04$ ; (D) *FLT1* mRNA expression <sup>a</sup> $p = 0.02$ , <sup>b</sup> $p = 0.4$ , <sup>c</sup> $p = 0.5$ , <sup>d</sup> $p = 0.02$ .

## Discussion

This is the first study to report placental expression of *VEGFA*, *PGF*, *KDR* and *FLT1* genes in several pregnancy complications within one cohort. The overall results of our study demonstrate that placental mRNA expression of the VEGF family of angiogenic growth factors is reduced in pregnancies complicated by preeclampsia, gestational hypertension, SGA infants and spontaneous preterm birth compared to uncomplicated term pregnancy.

Placental expression of *VEGFA* mRNA has been localized to villous and extravillous trophoblasts, villous mesenchyme, Hofbauer cells and maternal decidual cells [13-17] throughout gestation. In the first trimester, *PGF* is mainly expressed in extravillous trophoblasts within the maternal decidua but towards term the expression is abundant in villous trophoblasts [17, 18, 30]. The expression pattern of *FLT1* is similar to that of *VEGFA* while abundant *KDR* expression is localized to vascular endothelial cells [13, 31].

*VEGFA* exerts its effects via both FLT-1 and KDR while PlGF acts only through FLT-1 [32]. VEGF-A acting through FLT-1 and KDR is known to regulate early placental villous vasculogenesis and branching angiogenesis which continues up to 25 weeks of gestation [33, 34]. From then onwards, angiogenesis switches from branching to non-branching which continues to term and is known to be regulated by PGF acting through FLT-1 [7]. Placentae from pregnancies complicated by growth restricted fetuses, as well as those complicated by preeclampsia plus growth restriction are characterised by a reduction in the number of chorionic villi and their accompanying vasculature [4, 35].

In addition to its role in villous angiogenesis, the VEGF family is known to regulate maternal spiral artery remodelling [36]. Early pregnancy is associated with an influx of leukocytes into the decidua including uterine natural killer cells (uNK) and

macrophages. Uterine NK cells isolated from first trimester decidua secrete many angiogenic growth factors including VEGF-A and PlGF and un-remodelled spiral arteries express KDR [37]. Uterine NK cells are a major source of angiogenic growth factors at the maternal-fetal interface during early pregnancy and play an important role in vascular remodelling [38]. Impaired maternal spiral artery remodelling is demonstrated in pregnancies complicated by preeclampsia, SGA infants and preterm birth [3-6].

We found that placental expression of *VEGFA*, *PGF*, *KDR* and *FLT1* were significantly reduced in the preeclamptic group compared to the uncomplicated pregnancy group. To our knowledge this is the first study to evaluate placental *PGF* mRNA expression in pregnancy complications. There is consistent evidence that maternal plasma PlGF concentration is decreased as early as the first trimester of pregnancy and that there is a marked reduction prior to the clinical onset of the disease [39]. As the placenta is a major source of PlGF during pregnancy, our findings suggest that reduced placental expression and hence production of PlGF may contribute to the pathogenic vascular defects seen in preeclamptic pregnancy. A previous study has investigated placental KDR expression in preeclampsia and has demonstrated that expression of both *KDR* mRNA and protein are decreased in preeclampsia compared to uncomplicated pregnancy [40]. Our finding of reduced *KDR* mRNA expression in preeclampsia concurs. Consistent with our results, two other groups have shown that placental expression of *VEGFA* is reduced in preeclamptic placentae compared to normal placentae [19, 20] while others have demonstrated an increase [21] and no difference [22, 23]. The discrepant results observed among these studies may be due to the differences in the gene expression quantification method, sample size, the many genetic and environmental factors that may alter gene expression and, importantly, phenotypic differences in classification of preeclampsia.

Previously it has been shown that VEGF-A immunostaining in the syncytiotrophoblast layer is reduced in growth restricted placentae compared to controls [35]. Consistent with this, we found that the placental expression of *VEGFA* mRNA was reduced in normotensive SGA pregnancies compared to uncomplicated pregnancy suggesting that the reduced mRNA may correlate with reduced protein expression and contribute to impaired villous vasculogenesis.

We found that *VEGFA*, *PGF*, *KDR* and *FLT1* mRNA expression were reduced in placentae from spontaneous preterm deliveries. At present there is a paucity of data on the role of the VEGF family in preterm birth, although a failure of physiological transformation of spiral arteries has been reported in this complication [5, 6]. A recent study revealed that in the setting of inflammation (elevated high sensitivity C-Reactive Protein), women experiencing preterm birth had reduced serum PIGF levels [41] which is consistent with our findings of reduced placental *PGF* mRNA expression in the preterm birth group.

Our finding of reduced expression of *VEGFA*, *PGF* and *KDR* in the gestational hypertension group was surprising considering that placentation defects are not established in women with gestational hypertension. However, it is proposed that all these pregnancy complications constitute a continuum of disorders with similar underlying pathogenic abnormalities. The cross talk between fetus, placenta and mother may modulate these effects and the ability of the mother to respond may be a particularly important determinant of outcome.

The VEGF family of angiogenic growth factors gained much interest in the field of preeclampsia research recently due to the consistent and repeated findings of reduced maternal plasma PIGF in women destined to develop preeclampsia. There is debate on whether the low level observed is the consequence of low production or the result of inhibition by the endogenous inhibitor sFLT1 which is increased in the maternal

circulation in preeclamptic pregnancy. There is debate also on whether the low level of circulating PlGF is the cause or the consequence of the disease. *VEGFA* mRNA expression and protein production are oxygen dependant and are known to be up-regulated by hypoxia [42]. Generally it has been assumed that the placentae in pregnancies complicated by preeclampsia and growth restricted fetuses are hypoxic. If these placentae were hypoxic due to the consequence of the disease, we would expect *VEGFA* mRNA expression to be up-regulated. We have found that *VEGFA* mRNA expression is reduced in all these pregnancy complications at term. Potentially their expression was also reduced earlier in gestation and may have contributed to impaired villous angiogenesis. We have recently shown that polymorphisms in the VEGF family genes that are associated with reduced gene expression are also associated with preeclampsia and SGA [43, 44]. Inherited susceptibility to reduced placental angiogenic gene expression together with other environmental and life style factors may contribute to the reduced placental expression seen in this study.

The strengths of our study include a well characterized study population, diagnosis of pregnancy complications based on current international guidelines and quantifying gene expression using qRT-PCR. A limitation in our study is that it was not designed to evaluate the expression of VEGF family proteins which would have been beneficial in correlating with the mRNA levels. We also acknowledge that our study groups were relatively small and that the controls were not matched for gestational age. Selection of controls for studies on human placental studies is a vexed issue. Unless placentae are collected from terminations of normal pregnancies in mid to late gestation but prior to 37 weeks, pre-term placentae cannot be considered to be normal. In our hospital we are unable to collect normal placentae earlier than term. However, with the exception of the preterm group almost all of the placentae collected for our study were from term (>37 weeks gestation) pregnancies. Exclusion of the preterm deliveries from the analyses did

not change the results. There was also no relationship between the weight of the placenta and angiogenic gene mRNA expression. Since a fixed amount of RNA was reverse-transcribed, this is not surprising. However, lower expression per volume of RNA would translate to lower total mRNA transcription which would be reduced again when the placenta is small. This may contribute to the pathogenesis of pregnancy complications possibly through reduced secretion in to the maternal circulation and maternal maladaptation to pregnancy.

In conclusion, our study demonstrates that placental mRNA expression of VEGF family angiogenic factors is reduced in gestational hypertensive disorders, SGA and in preterm birth. Identification of this common pathway may be important for future screening and therapeutics.

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## **General Discussion**

The primary aim of this project was to investigate the association of functional single nucleotide polymorphisms in angiogenic and anti-angiogenic genes in pregnancies complicated by preeclampsia, small for gestational age infants and spontaneous preterm birth. The secondary aims were to explore the association of these polymorphisms with markers of disease pathogenesis, to identify potential gene-environmental interactions that modify the risk for these pregnancy complications and to identify differences in angiogenic gene mRNA expression in the placenta in complicated compared to uncomplicated pregnancy. To our knowledge the SCOPE study is one of the largest studies conducted on pregnancy complications to date with data on parent-infant trios, and the first study to explore angiogenesis regulating gene polymorphisms in the father.

### **Placental expression of VEGF family mRNA is reduced in pregnancy complications**

There are extensive data to confirm that the angiogenic protein PlGF and to a lesser extent VEGF-A are reduced, and the anti-angiogenic sFLT-1 is increased, in the maternal circulation in preeclampsia. A similar phenotype has been reported in pregnancies complicated by small for gestational age infants. Although there are limited data on the role of these growth factors in spontaneous preterm birth, a decrease in plasma PlGF has been demonstrated in women experiencing spontaneous preterm birth. There is debate on whether the low level of angiogenic proteins observed in pregnancy complications is the consequence of low production or the result of inhibition by the endogenous inhibitor sFLT-1.

We found that the expression of VEGF family mRNA in term placenta was reduced in preeclampsia, small for gestational age pregnancies and spontaneous preterm birth

compared to uncomplicated pregnancy suggesting that the reduction may be in part at the transcriptional level (manuscript 8).

### **Polymorphisms in angiogenesis regulating genes are associated with pregnancy complications**

Many genetic polymorphisms in the angiogenic pathway have been discovered and some are known to regulate gene expression and protein production. The polymorphisms studied in this project, *VEGFA* -2578C/A (rs699967), *VEGFA* +936C/T (rs3025039), *PGF* 642C/A (rs1042886), *KDR* -604T/C (rs2071559), *KDR* Val297Ile (rs2305948), *ANGPT1* 1414T/A (rs2507800) and *TSP1* 2210A/G (rs2228262), were selected because of known functional effects.

We demonstrated that homozygosity for the C allele of the *KDR* -604T/C polymorphism in both the father and the infant was associated with preeclampsia. *KDR* -604T/C is a promoter polymorphism with the C allele exhibiting 68% lower transcriptional activity compared to the T allele and the CC homozygotes having significantly lower KDR levels compared to TT homozygotes [1]. We also found that the maternal *ANGPT1* 1414T/A TT genotype was associated with a reduced risk of preeclampsia [2]. This polymorphism is located in the microRNA-211 (miRNA-211) target site in the 3' UTR of the *ANGPT1* gene. The A allele is known to suppress angiopoietin-1 translation by facilitating miRNA-211 binding while the T allele is resistant to miRNA -211 induced reduction in translation. The TT genotype is associated with higher plasma angiopoietin-1 levels compared to the AA genotype [3].

The above two polymorphisms were also associated with SGA pregnancies. Homozygosity for the C allele of the *KDR* -604T/C polymorphism in both the father and the infant was also associated with increased risk for SGA and SGA with abnormal uterine and/ or umbilical artery Doppler [4] while the maternal *ANGPT1* 1414T/A TT

genotype was associated with a reduced risk for hypertensive SGA and SGA with abnormal Doppler [4]. We also demonstrated that the *VEGFA* +936C/T [CT+TT] genotype model in the infant was associated with increased risk for SGA and SGA with abnormal Doppler [5]. The *VEGFA* +936C/T polymorphism is located in the 3'-untranslated region (3'UTR) of the VEGF gene and the T allele has previously been shown to be associated with reduced plasma VEGF-A levels [6]. We also found that the *TSP1* 2210A/G GA and GG genotypes in both the father and the infant were associated with increased risk for SGA pregnancies [7]. This polymorphism has been extensively studied for its functional effects on plasma TSP-1. The G allele is known to be associated with lower Ca<sup>2+</sup> binding capacity and conformational stability, enhanced interaction with fibrinogen on platelet surfaces and a higher rate and extent of platelet aggregation [8-10]. The expression of the anti-angiogenic TSP-1 on the surface of platelets from carriers of the G allele is also known to be increased [10].

These findings demonstrate that genotypes conferring a decreased pro-angiogenic and an increased anti-angiogenic phenotype increase the risk, and that a genotype that confers a pro-angiogenic phenotype decreases the risk of both preeclampsia and SGA pregnancies.

### **Paternal angiogenesis regulating gene polymorphisms are associated with pregnancy complications**

There is growing evidence that the father plays a significant role in the causation of pregnancy complications. The risk of fathering a preeclamptic pregnancy is increased among men who fathered a preeclamptic pregnancy with a different partner [11] and among men who were themselves the product of a preeclamptic pregnancy [12]. A paternal contribution to SGA is suggested by a positive correlation of paternal birthweight with infant birthweight [13-15]. It is also known that men who were SGA at

birth are more likely than those with a normal birthweight to parent an SGA infant [13, 16]. In this study, we found two paternal polymorphisms in the angiogenic pathway that were associated with preeclampsia (*KDR -604T/C*) and SGA pregnancies (*KDR -604T/C* and *TSP1 2010A/G*). All three associations were significant after adjusting for established maternal and paternal risk factors for both disorders, demonstrating an independent association between these paternal polymorphisms and pregnancy complications. To our knowledge, this is the first study to investigate paternal polymorphisms in angiogenesis regulating genes in adverse pregnancy outcomes. Our findings provide further evidence for the role that the father plays in these pregnancy disorders. It seems likely that this contribution is mediated via functional effects on the placenta.

#### **Angiogenesis regulating gene polymorphisms may have a role in the pathogenesis of pregnancy complications**

Impaired maternal spiral artery remodelling is implicated in the pathogenesis of pregnancies complicated by preeclampsia, SGA infants and preterm birth and impaired placental villous vascularisation has been demonstrated in SGA pregnancies [17-20]. These abnormalities result in increased vascular impedance in the uterine and/or umbilical circulations which is detected during the antenatal period by increased uterine and umbilical artery resistance indices on Doppler velocimetry [21, 22]. We found that the [CT+TT] genotype model of the *VEGFA +936C/T* polymorphism in the mother was associated with an increased resistance index in the umbilical artery and that the TT genotype in the infant was associated with an increased resistance index in the uterine artery. In addition we also demonstrated that the placental [CT+TT] genotype model of the *VEGFA +936C/T* polymorphism was associated with reduced first trimester placental *VEGFA* mRNA expression [5]. We demonstrated similar results for the

*ANGPT1 1414T/A* polymorphism. The TT genotype in the mother was associated with a reduced risk for abnormal uterine artery resistance index suggesting a protective effect of the genotype [2]. These findings suggest that these polymorphisms may contribute to impaired maternal spiral artery remodelling and villous vascularisation in the placenta (figure 1).

### **Gene-environment interactions modify the risk of pregnancy complications**

Smoking and overweight/obesity are known risk factors for pregnancy complications and are also known to regulate angiogenic gene expression. We identified three polymorphisms in the angiogenic pathway that interact with these risk factors to modify the risk of pregnancy complications. We demonstrated that the CC genotype of the *KDR -604T/C* polymorphism in the mother was associated with preeclampsia, SGA and SGA with abnormal uterine and/or umbilical artery Doppler in non-smokers. This may be due to the increase in expression of angiogenic growth factors in smokers [23] which is likely mediated by hypoxia.

In overweight and obese women, the *VEGFA -2578C/A* AA genotype that is known to be associated with a lower transcriptional activity and plasma VEGF-A levels than the CC genotype [24] was associated with an increased risk for spontaneous preterm birth, whereas, no association was detected in women of normal weight. We also found that in women with a normal BMI, the *ANGPT1 1414T/A* AA genotype that is known to be associated with lower plasma angiopoietin-1 levels compared to the TT genotype [3] was associated with an increased risk for spontaneous preterm birth, whereas, no association was detected in overweight/obese women. To our knowledge, this is the first study to report interactions between polymorphisms in angiogenesis regulating genes and clinical/lifestyle risk factors that may modify the risk for pregnancy complications.

Our findings point to interesting potential mechanisms contributing to pregnancy disorders that need to be further explored.

### **Evidence for a genetic contribution to vascular diseases**

There is growing evidence to support the theory that preeclampsia, SGA pregnancies and later life vascular diseases represent a spectrum of disorders of vascular origin that manifest at different time points throughout life. Women who develop preeclampsia or deliver SGA infants are known to be at increased risk for later life hypertension, coronary artery disease and stroke [25-32]. Small for gestational age infants, as well as those born of a preeclamptic pregnancy are also known to be at increased risk for hypertension, coronary artery disease and stroke in adult life [33-40].

The relationship between these pregnancy complications and later life vascular disease involves shared risk factors for both, including chronic endothelial dysfunction [41, 42]. However, familial segregation of preeclampsia, as well as cardiovascular and cerebrovascular disease, suggest that these diseases may share a common genetic predisposition that interacts with the environment to predispose individuals to vascular disorders which manifest at different time points throughout the life course. In support of this theory, few groups have demonstrated polymorphisms in candidate genes that are associated with both pregnancy complications and later life vascular diseases [43-45]. In this project, we have identified three polymorphisms (*KDR -604T/C*, *TSP1 2210A/G* and *ANGPT1 1414T/A*) in the angiogenic pathway that have previously been shown to be associated with coronary artery disease or stroke [1, 3, 46, 47] to be associated with preeclampsia and/or SGA infants [2, 4, 7], providing further evidence for potential shared genetic factors between these disorders.

### **Limitations in candidate gene association studies**

A major limitation in candidate gene association studies on pregnancy complications is lack of reproducibility of results. This is due to many factors, including differences in genotype frequencies among ethnic groups, phenotypic differences in classification of pregnancy complications and small sample size in many studies. In this project we investigated the same polymorphisms in a Caucasian population and a Sri Lankan population of women with preeclampsia. We found that the maternal *KDR* -604T/C and *ANGPT 1414T/A* polymorphisms were associated with preeclampsia in Caucasian women and that the *PGF* 642C/A polymorphism was associated with preeclampsia in Sri Lankan women. There are several possible reasons for the lack of reproducibility of the results. The genotype frequencies of the polymorphisms were different between the two ethnicities. In addition the preeclamptic phenotype was different between the two populations. The Caucasian population consisted of mainly late preeclamptic women whereas, 42% of the the Sri Lankan preeclamptic women had early onset disease. It is increasingly being recognised that early and late preeclampsia are two distinct subtypes with different underlying pathogenic abnormalities [48]. This may in part be responsible for the inconsistency in the results.

Another possible reason may be the interactions of genes with environmental and life style risk factors. In manuscript 6, we demonstrated interactions between *VEGFA* and *ANGPT1* gene polymorphisms with maternal BMI. These findings suggest that the risk is modified by the interactions and provides a plausible explanation to the low risk associated with many single polymorphisms in most candidate gene association studies as well as to the non-reproducibility of results among different populations and ethnicities.

Interpretation of the results of genetic association studies on multiple polymorphisms also becomes complicated due to the issue of correction for multiple testing. A

commonly used method to control for multiple testing is Bonferroni correction which is considered highly conservative and may result in true positives being overlooked [49]. The false discovery rate correction is considered less conservative and is increasingly being used in many genetic association studies [50]. In the work described in this thesis, we did not perform an overall correction for multiple testing which may be a limitation of the study. However, in manuscripts 4 and 6 where several genotype models as well as several outcomes were considered, we have performed a false discovery rate (FDR) test to adjust for multiple comparisons controlling the FDR at 15%. In this study, the polymorphisms were selected based on known functional effects and only a few polymorphisms were explored. We also aimed to explore the potential role of these polymorphisms in the pathogenesis of these pregnancy disorders and hence, provide mechanistic evidence for the associations. Therefore, we believe that an overall correction for multiple testing will have a limited role in our study.

### **Future implications**

Research on angiogenic growth factors to date has shown consistent evidence of their role in pregnancy complications, and at present the VEGF family appears to have a pivotal role in the pathogenesis of preeclampsia. In our review on “VEGF family angiogenic growth factors in adverse pregnancy outcomes” we demonstrated that although PlGF and sFLT-1 in combination with other clinical and biochemical markers in late first or second trimester appear to predict early onset preeclampsia with a high sensitivity and specificity, these molecules do not appear to have sufficient power to accurately predict late onset preeclampsia, small for gestational age pregnancies or preterm birth. Therefore, at present there is no effective screening test that will accurately predict the risk of these pregnancy complications in the majority of pregnant women.

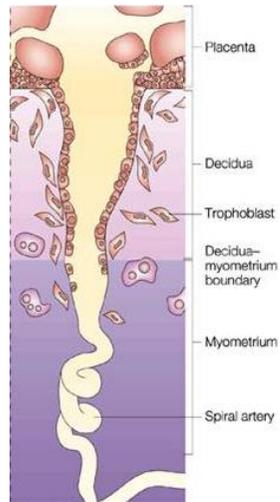
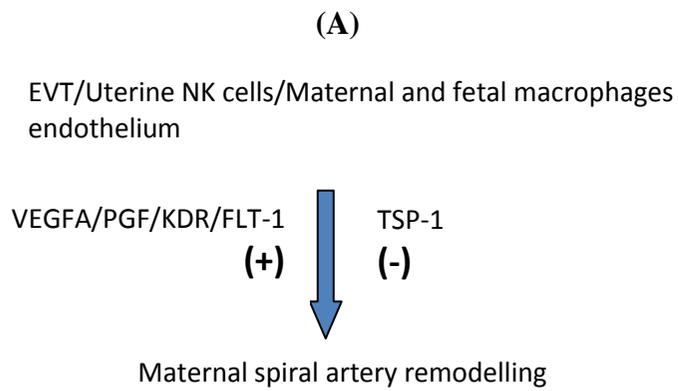
In our study, we have shown that functional polymorphisms in angiogenesis regulating genes are associated with the risk of pregnancy complications, as well as disease pathogenesis. Our findings are novel associations that need to be replicated in other independent large cohorts. Assuming our results can be replicated, these polymorphisms in combination with other clinical variables may be useful in developing algorithms to predict the risk of pregnancy complications very early in pregnancy or even prior to pregnancy. Although the SCOPE study is a prospective cohort, our candidate gene association study was a nested case control study. The rationale for performing an initial case-control study was to identify genotypes of polymorphisms that were different between pregnancy complications compared to uncomplicated pregnancies. For these results to be utilised in a clinical setting to predict the risk of pregnancy complications, these associations need to be replicated in a cohort study design. In this study, we demonstrated that some polymorphisms were associated with multiple pregnancy complications. The *KDR -604T/C* was associated with both preeclampsia and SGA pregnancies. The *ANGPT 1414T/A* polymorphism was associated with preeclampsia and SGA pregnancies and the interaction between maternal *ANGPT 1414T/A* and maternal BMI was associated with spontaneous preterm birth. In addition, we found that the placental VEGF family mRNA expression at term was reduced in all the pregnancy complications that were studied compared to uncomplicated pregnancy. These findings suggest that there is some overlap between these pregnancy complications that may be due to the similar underlying pathogenic mechanisms. The development of a screening test to differentiate one pregnancy complication from another may be complicated due to these reasons.

Considering the rapid advances in the field of VEGF family proteins in pregnancy complications, only a very few groups have studied the role of polymorphisms in these genes in pregnancy complications. Therefore, more studies possibly incorporating the

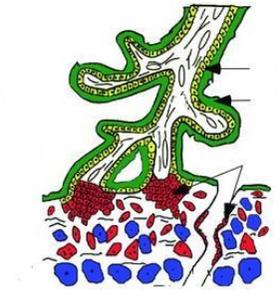
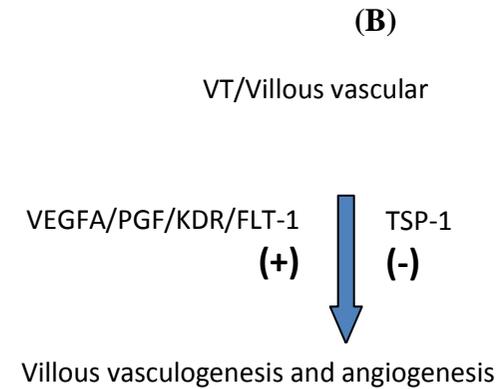
sequencing of these genes may provide potential candidate polymorphisms that may be useful in predicting pregnancy complications. Our results on the *ANGPT1* and *TSP1* polymorphisms also suggest that in addition to the VEGF family, other angiogenesis regulating gene polymorphisms also need to be explored. The association of angiogenic gene polymorphisms with pregnancy complications and later life vascular diseases also merits future research that may identify other potential polymorphisms that may be useful in screening for vascular diseases.

### **Conclusions**

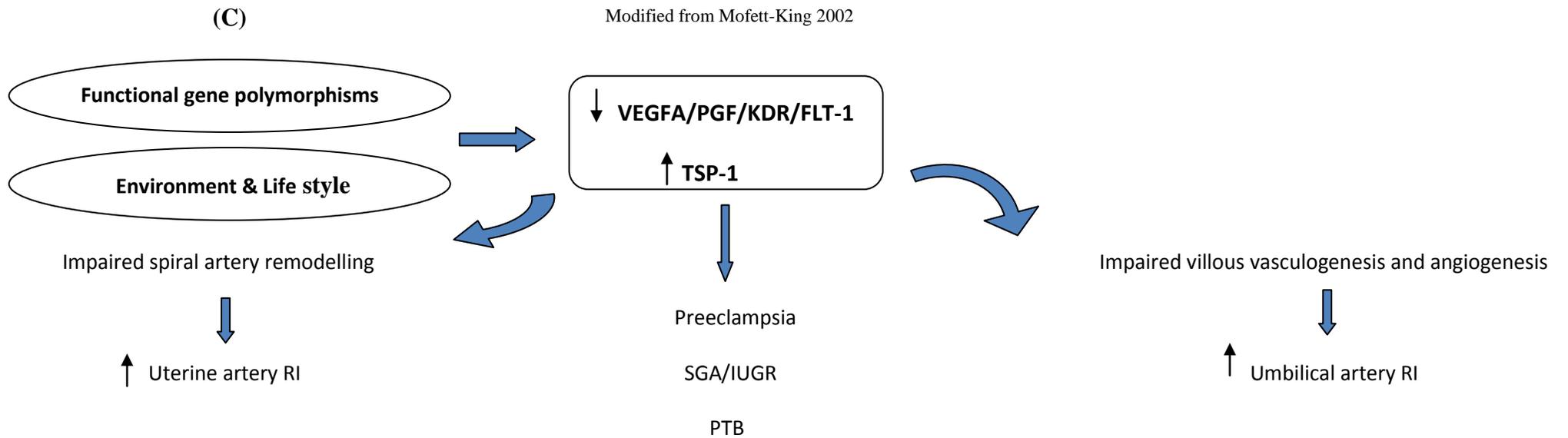
Experimental evidence strongly supports the implication of angiogenic growth factors in the pathogenesis of pregnancy complications mainly preeclampsia and SGA infants. Angiogenic growth factors regulate placental villous angiogenesis and maternal spiral artery remodelling. In the work presented in this thesis, we have demonstrated that functional polymorphisms in angiogenesis regulating genes that confer a reduction in a pro-angiogenic phenotype and those that confer an increase in an anti-angiogenic phenotype contribute to the risk of pregnancy complications and their pathogenesis (Figure 1). The work published from this thesis will contribute to the advancement of research on the genetic basis of pregnancy complications and may be beneficial in the development of predictive models in the future.



Modified from Mofett-King 2002



Modified from Springer Link Images



**Figure 1** Potential role of angiogenesis regulating gene polymorphisms in pregnancy complications

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