Combination Gefitinib and Methotrexate to Treat Ectopic Pregnancy: Early Phase Clinical Trials

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ERRATA

p 131 para 2, 1st line: replace “minimization” with “minimisation”

p 136 para 5, 1st line: replace “(24/26)” with “(24/28)”

ADDENDUM

p 35 1st line: add “respectively,” after “100%”

p 70 para 2, 1st line: delete “a very rare” and replace with “an uncommon”

p 97 1st line: delete “Fallopian tube explants were collected at the time of surgery for either salpingectomy (ectopic pregnancy) or other surgery for treatment of benign gynaecological conditions (non-pregnant controls)” and replace with “Fallopian tube tissue was collected by salpingectomy (ectopic pregnancy) or during other surgery for treatment of benign gynaecological conditions (non-pregnant controls) and used as explants”

p 108 para 2, 1st line: delete “approximately” and read “Given that only 5-20%...”

p 129 para 2, line 10: delete “a minority of women diagnosed with the condition”

p 160 para 2, line 7: delete “interstitial lung disease (ILD)” and replace with “ILD”

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p 160 para 2 line 8: delete “non-small cell lung cancer”

Notice 1

Notice 2

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Abstract

This body of work examines novel diagnostics and therapeutics for ectopic pregnancy. The incidence of ectopic pregnancy is 1-2% of pregnancies, where implantation occurs outside of the endometrial cavity. It remains a leading cause of maternal death in early pregnancy. The management of ectopic pregnancy is predominantly surgical; this is mandated when rupture has occurred, however, only 5% of women present this way. The medical treatment option, consisting of single-dose methotrexate, has limited efficacy in treating more advanced ectopic pregnancies, so that only 25-30% of diagnosed women benefit from it.

The introduction of epidermal growth factor receptor inhibitors such as gefitinib into the pharmaceutical lexicon has created a potentially novel approach to the treatment of trophoblastic tissue disorders. Pre-clinical experiments have shown that combining gefitinib with methotrexate effects significant, supra-additive regression of trophoblastic cells and tissues. The main aim of this PhD is to translate these findings into clinical practice, thereby improving the medical management of ectopic pregnancy.

In a phase I dose-escalation toxicity study, we established the safety of combination gefitinib and methotrexate in 12 women with ectopic pregnancies currently eligible for medical management; combination treatment effected a more rapid decline in serum human chorionic gonadotrophin (hCG) levels and shortened the time to resolution compared to controls. We then applied combination treatment to larger and more complex non-tubal ectopic pregnancies; in a case series of 8 women, all were successfully treated with no undue toxicity caused. Subsequently, in a phase II study of 28 women, we confirmed combination gefitinib and methotrexate to be safe, well tolerated and effective at treating an extended range of ectopic pregnancies.

To further improve diagnosis and management of ectopic pregnancy, we identified and investigated two novel biomarkers – adrenomedullin and macrophage inhibitory cytokine-1 (MIC-1). We could

not confirm altered adrenomedullin expression in ectopic pregnancy, and correspondingly, it was not a useful biomarker of the condition. MIC-1 was able to exclude ectopic pregnancy above a certain threshold, demonstrating potential to form part of a panel of biomarkers for the diagnosis of the condition. We also validated that an early falling serum hCG is predictive of medical treatment success after single-dose methotrexate for ectopic pregnancy: in two studies examining 251 women, a fall in hCG between days 1-4 of treatment was 85-88% predictive of treatment success.

Finally, we examined the potential of combination gefitinib and methotrexate to improve treatment of persistent gestational trophoblastic disease (pGTD), a rare form of pregnancy with malignant progression; in a separate phase I dose-escalation toxicity study, we found the combination to again be safe and well tolerated in 6 women, despite higher doses of methotrexate administered.

Collectively, this work has contributed new approaches to the diagnosis and treatment of ectopic pregnancy and pGTD. It provides proof-in-principle evidence that combination gefitinib and methotrexate may be more effective in the treatment of ectopic pregnancy, underpinning future funding and investigation. It is my hope that this work will minimise the suffering of women diagnosed with these conditions.

PART A: General Declaration

Monash University

Declaration for thesis based or partially based on conjointly published or unpublished work

General Declaration

In accordance with Monash University Doctorate Regulation 17.2 Doctor of Philosophy and Research Master's regulations the following declarations are made:

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

This thesis includes 6 original papers published in peer reviewed journals and 2 traditional thesis chapters. The core theme of the thesis is improving the medical treatment of ectopic pregnancy. The ideas, development and writing up of all the papers in the thesis were the principal responsibility of myself, the candidate, working within the Ritchie Centre, Monash Institute of Medical Research, under the supervision of Professors Stephen Tong and Euan Wallace.

The inclusion of co-authors reflects the fact that the work came from active collaboration between researchers and acknowledges input into team-based research.

In the case of chapters 3, 4, 5 and 6, my contribution to the work involved the following:

Thesis chapter	Publication status	Publication	Nature and extent of candidate's contribution
3	Published	Decline in hCG levels between day 0 and day 4 after a single dose of methotrexate for ectopic pregnancy predicts treatment success: a cohort study	Conceived the study, collected the data, performed the data analysis and drafted the paper.
3	Published	Using a decline in serum hCG between days 0-4 to predict ectopic pregnancy treatment success after single-dose methotrexate: a retrospective cohort study	Conceived the study, performed the data analysis and drafted the paper.
4	Published	Adrenomedullin expression is not significantly altered in ectopic pregnancy.	Conceived the study, performed the laboratory experiments, performed the data analysis and drafted the paper.
4	Published	Maternal serum macrophage inhibitory cytokine-1 as a biomarker for ectopic pregnancy in women with a pregnancy of unknown location.	Conceived the study, performed the laboratory experiments, performed the data analysis and drafted the paper.
5	Published	Combination gefitinib and methotrexate compared with methotrexate alone to treat ectopic pregnancy.	Conceived the study, recruited and treated participants, performed the data analysis and drafted the paper.
6	Published	Combination gefitinib and methotrexate treatment for non-tubal ectopic pregnancies: a case series	Conceived the study, recruited and treated participants, performed the data analysis and drafted the manuscript.

I have renumbered sections of submitted or published papers in order to generate a consistent presentation within the thesis.

Signed: 

Date: 21/05/2014

Acknowledgements

I take this opportunity to thank the many people that have in their own way, contributed to this thesis, whether scientifically, professionally, financially and of course, personally. Primarily, however, I owe an enormous debt of gratitude to my principal supervisor, Professor Stephen Tong. For opening the door to a clinical research career and enriching my professional life, for years of mentoring and sound advice and for excellence in women's health research, thank you. I will undoubtedly continue to be inspired by you and your work, and trust this is not the end of our collaborative efforts. To my co-supervisor Professor Euan Wallace, thank you for fostering a keen research interest and for your support, both clinical and scientific; without it, I would not have been able to pursue either endeavour.

I thank past and present members of the Translational Obstetrics Group, my home away from home, who guided, taught, encouraged and supported me throughout my candidature. To Dr Kirsten Palmer and Dr Clare Whitehead, thank you for leading the way by impressively combining successful candidatures with clinical training; your example has been invaluable. To Dr Tu'uhevaha Kaitu'u-Lino, Dr Natalie Hannan, Dr Louie Ye and Laura Tuohey, thank you for your patience and assistance in the laboratory; this aspect of my work not only significantly enhanced my thesis, but also my scientific learning. Perhaps most importantly though, your collective work ethic, unashamed zeal for excellence, maintenance of work-life balance and happy dispositions have been truly inspiring. I will greatly miss the camaraderie, banter and peals of laughter.

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Publications, Presentations & Awards

Publications Relevant to Thesis

1. **SKUBISZ, M. M.** & TONG, S. 2011. Of leaves and butterflies: how methotrexate came to be the savior of women. *Obstet Gynecol,* 118**,** 1169-73.

2. **SKUBISZ, M.**, LI, J., WALLACE, E. & TONG, S. 2011. Decline in betahCG levels between days 0 and 4 after a single dose of methotrexate for ectopic pregnancy predicts treatment success: a retrospective cohort study. *BJOG,* 118**,** 1665-8.

3. **SKUBISZ, M. M.** & TONG, S. 2012. The evolution of methotrexate as a treatment for ectopic pregnancy and gestational trophoblastic neoplasia: a review. *ISRN Obstet Gynecol,* 2012**,** 637094.

4. **SKUBISZ, M.**,* DUTTON, P.,* DUNCAN, W. C., HORNE, A. W. & TONG, S. 2013. Using a decline in serum hCG between days 0-4 to predict ectopic pregnancy treatment success after single-dose methotrexate: a retrospective cohort study. *BMC Pregnancy Childbirth,* 13**,** 30.

5. **SKUBISZ, M.**, BROWN, J. K., TONG, S., KAITU'U-LINO, T. & HORNE, A. W. 2013. Maternal Serum Macrophage Inhibitory Cytokine-1 as a Biomarker for Ectopic Pregnancy in Women with a Pregnancy of Unknown Location. *PLoS One,* 8**,** e66339.

6. HORNE, A. W.,* **SKUBISZ, M. M.**,* DOUST, A., DUNCAN, W. C., WALLACE, E., CRITCHLEY, H. O., JOHNS, T. G., NORMAN, J. E., BHATTACHARYA, S., MOLLISON, J., RASSMUSEN, M. & TONG, S. 2013. Phase II single arm open label multicentre clinical trial to evaluate the efficacy and side effects of a combination of gefitinib and methotrexate to treat tubal ectopic pregnancies (GEM II): study protocol. *BMJ Open,* 3.

7. **SKUBISZ, M.**,* HORNE, A. W.,* JOHNS, T. G., NILSSON, U. W., DUNCAN, W. C., WALLACE, E. M., CRITCHLEY, H. O. & TONG, S. 2013. Combination Gefitinib and Methotrexate Compared With Methotrexate Alone to Treat Ectopic Pregnancy. *Obstet Gynecol,* 122**,** 745-751.

8. HORNE, A. W.,* **SKUBISZ, M. M.**,* TONG, S., DUNCAN, W. C., NEIL, P., WALLACE, E. M. & JOHNS, T. G. 2014. Combination gefitinib and methotrexate treatment for non-tubal ectopic pregnancies: a case series. *Hum Reprod*.

* Denotes joint first authors

Publications During Candidature

1. KAITU'U-LINO, T. J., TUOHEY, L., YE, L., PALMER, K., **SKUBISZ, M.** & TONG, S. 2013. MT-MMPs in pre-eclamptic placenta: relationship to soluble endoglin production. *Placenta,* 34**,** 168-73.

2. TUOHEY, L., MACINTIRE, K., YE, L., PALMER, K., **SKUBISZ, M.**, TONG, S. & KAITU'U-LINO, T. J. 2013. PLAC4 is upregulated in severe early onset preeclampsia and upregulated with syncytialisation but not hypoxia. *Placenta,* 34**,** 256-60.

Presentations

1. 'Usefulness of day 0-4 beta human chorionic gonadotropin in predicting treatment success after single-dose methotrexate for ectopic pregnancy.' **Poster** presented at the Royal Australian and New Zealand College of Obstetricians and Gynaecologists' (RANZCOG) Annual Scientific Meeting (ASM), Melbourne, Australia, 2011.
2. 'Combination gefitinib and methotrexate to treat ectopic pregnancy: a phase I clinical study.' **Poster** presented at the 59th ASM for the Society of Gynecologic Investigation (SGI), San Diego, California, USA, 2012.
3. 'Combination gefitinib and methotrexate to treat ectopic pregnancy: a phase I clinical study.' **Oral** presented at the Merck Sharp and Dohme (MSD) Organon Awards for Research in Women's Health Care, Melbourne, Australia, 2012.
4. 'Combination gefitinib and methotrexate to treat ectopic pregnancy: a phase I clinical study.' **Oral** presented at the ASM of the Endocrine Society of Australia and the Society for Reproductive Biology (SRB), Gold Coast, Queensland, Australia, 2012.
5. 'Combination gefitinib and methotrexate to treat ectopic pregnancy: a phase I clinical study.' **Oral** presented at Southern Health Research Week, Clayton, Victoria, Australia, 2012.
6. 'Combination gefitinib and methotrexate to treat ectopic pregnancy: a phase II clinical study.' **Poster** presented at the 60th ASM for the SGI, Orlando, Florida, USA, 2012.

Awards

1. RANZCOG ASM Poster Prize, Melbourne, Australia, 2011 – Runner-Up.
2. SGI Poster Prize, San Diego, California, USA, 2012 ($100).
3. Monash Institute of Medical Research (MIMR) Postgraduate Symposium, Clayton, Victoria, Australia 2012; Runner-Up ($250).
4. MSD Organon Women's Health Care Prize, Melbourne, Australia, 2012 – Winner ($2000).
5. Young Investigator Award, Southern Health Research Week, Clayton, Victoria, Australia, 2012 – Finalist.
6. SGI Poster Prize, Orlando, Florida, USA, 2013 ($100).
7. MIMR 3 Minute Thesis Competition, Clayton, Victoria, Australia, 2013 – Runner-Up ($150).

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Abbreviations

ADM	Adrenomedullin
ALL	Acute lymphoblastic leukaemia
ANZCTR	Australian and New Zealand Clinical Trials Registry
ART	Assisted reproductive technique
ASM	Annual scientific meeting
ATP	Adenosine triphosphate
CBI	Endocannabinoid receptor
cDNA	Copy deoxyribonucleic acid
CHM	Complete hydatidiform mole
CI	Confidence interval
CSE	Cigarette smoke exposure
CTCAE	Common Terminology Criteria for Adverse Events
CYP	Cytochrome P450
dEP	Definite ectopic pregnancy
DNA	Deoxyribonucleic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EIA	Enzyme immunoassay
EMA	Etoposide, methotrexate, actinomycin D
EMA-CO	Etoposide, methotrexate, actinomycin D - cyclophosphamide, vincristine
EP	Ectopic pregnancy
ETT	Epithelioid trophoblastic tumour
EVC	Extravillous cytotrophoblast
FDA	Food and Drug Administration
FIGO	International Federation of Gynecology and Obstetrics
FRHM	Familial recurrent hydatidiform mole
GEM	Gefitinib and methotrexate
GTD	Gestational trophoblastic disease
GTN	Gestational trophoblastic neoplasia
hCG	Human chorionic gonadotrophin

HER2	Human EGFR related-2
HOXA10	Homeobox protein A10
hPL	Human placental lactogen
HR	Hazard ratio
HREC	Human research ethics committee
ICC	Interstitial cell of Cajal
IL-8	Interleukin 8
ILD	Interstitial lung disease
iNOS	Inducible nitric oxide synthase
INTEREST	Iressa NSCLC Trial Evaluating Response and Survival versus Taxotere
IPASS	Iressa Pan-Asia Study
ISEL	Iressa Survival Evaluation in Lung Cancer
IUD	Intrauterine device
IUGR	Intrauterine growth restriction
IUP	Intrauterine pregnancy
IVF	*In vitro* fertilisation
LIF	Leukaemia inhibitory factor 1
MAC	Methotrexate, actinomycin D, chlorambucil
MAPK	Mitogen-activated protein kinase
MIC-1	Macrophage inhibitory cytokine 1
MIMR	Monash Institute of Medical Research
mRNA	Messenger ribonucleic acid
MUC1	Mucin 1
NF-κB	Nuclear factor kappa-light- chain enhancer of activated B cells
NGF	Nerve growth factor
NPV	Negative predictive value
NSCLC	Non-small cell lung cancer
PAPP-A	Pregnancy-related plasma protein A
pEP	Probable ectopic pregnancy
pGTD	Persistent gestational trophoblastic disease
PHM	Partial hydatidiform mole
PIP3/Akt	Phosphotidylinositol 3 kinase/protein kinase B
PPV	Positive predictive value

PROKR	Prokineticin receptor
PSI	Prognostic score index
PSTT	Placental-site trophoblastic tumour
PUL	Pregnancy of unknown location
RANZCOG	Royal Australian and New Zealand College of Obstetricians and Gynaecologists
RCOG	Royal College of Obstetricians and Gynaecologists
RCT	Randomised controlled trial
RNA	Ribonucleic acid
RWH	Royal Women's Hospital, Melbourne, Australia
SCID	Severe combined immunodeficiency
SGI	Society for Gynecologic Investigation
SRB	Society for Reproductive Biology
TLR2	Toll-like receptor 2
TVUS	Transvaginal ultrasound/ultrasonography
UK	United Kingdom
US	United States
UV	Ultraviolet
VEGF	Vascular endothelial growth factor
WHO	World Health Organisation

Chapter 1: Introduction

1.1 Synopsis

This thesis details the scientific to clinical translation of a new therapeutic approach – combination gefitinib and methotrexate – to the treatment of two disorders of trophoblastic tissue. In particular, it seeks to improve the diagnosis and medical treatment of ectopic pregnancy and persistent gestational trophoblastic disease (pGTD), both potentially life-threatening gynaecological conditions with devastating reproductive consequences.

Methotrexate is an anti-folate drug which inhibits cell division by interfering with deoxyribonucleic acid (DNA) replication (Cronstein and Bertino, 2000). It is used clinically in medicine to treat a range of malignant and non-malignant conditions. Methotrexate is currently used in gynaecology to treat ectopic pregnancy (where a fertilised ovum implants outside of the uterine endometrial cavity) and gestational trophoblastic neoplasia (GTN) (disordered growth of trophoblastic tissue with malignant change). Whilst the incidences of these conditions in pregnancy are relatively rare (in Australia ranging from 0.7% for gestational trophoblastic disease (GTD) (Cancer Australia, 2009) to 1-2% for ectopic pregnancy (Boufous et al., 2001), their impact on the lives of young women of reproductive age, both in terms of mortality and morbidity are significant.

Methotrexate alleviates some of the disease burden of ectopic pregnancy and GTN, however, it is not always a successful or suitable treatment option in these conditions. In the treatment of ectopic pregnancy, methotrexate is only used clinically where the pre-treatment serum human chorionic gonadotrophin (hCG) levels are less than 5000 IU/L; above this level, the rates of failure of medical treatment are approximately 15%, making initial surgical management preferable (Menon et al., 2007). There are no other medical (drug-based) treatments for ectopic pregnancy in clinical use (Lipscomb, 2007). This means that for the estimated 75% of women who present with unruptured,

stable ectopic pregnancies and serum hCGs >5000 IU/L, the only treatment option is surgical excision (Farquhar, 2005, Jurkovic and Wilkinson, 2011). A drug treatment with better efficacy and a similar or improved toxicity profile could therefore help increase the number of women who can avoid surgery for the treatment of their ectopic pregnancy.

Methotrexate is a well recognised and widely used medical treatment of GTN. It is used as either a mono-chemotherapy in low-risk disease or as part of a multiple-drug regimen in high-risk disease to achieve 100% and 84% cure rates, respectively (Seckl et al., 2010). However, there is still scope to improve medical treatment of GTN, both in terms of reducing exposure to and length of treatment with methotrexate in low-risk disease, and improving cure rates with combination chemotherapy in high-risk disease.

The development and clinical availability of small molecule therapeutics specifically targeting the epidermal growth factor receptor (EGFR) has potentially created a new class of drugs with which to treat disorders of trophoblastic tissue. Publically available microarray assays show that the EGFR is expressed in human placenta at over thirty times its mean expression in all human tissues (Su et al., 2004). Furthermore, preclinical data generated by the Translational Obstetrics Group suggests that the EGFR antagonist gefitinib is indeed effective at neutralising trophoblastic tissue, but more excitingly, that combining it with methotrexate produces a superior, supra-additive treatment effect compared to using either drug alone.

The aim of this PhD is to clinically test the hypothesis that combination gefitinib and methotrexate is a potential new therapeutic approach for disorders of trophoblastic tissue. I proposed to undertake early phase human clinical trials to assess whether the combination of gefitinib and methotrexate is safe and efficacious in treating two trophoblastic tissue disorders – ectopic pregnancy and pGTD – given that the two drugs in combination produced a supra-additive treatment effect in preclinical studies. Ancillary aims of my work were to improve the diagnosis and use of existing medical treatment protocols for ectopic pregnancy. Altogether this work aspires to provide new insights to

clinicians who frequently manage these conditions, to optimise the safe and non-interventional treatment of ectopic pregnancy and pGTD, and to ultimately reduce the personal costs suffered by women who are diagnosed with these afflictions.

1.2 Chapter Outline

This thesis is presented as a hybrid of peer-reviewed, published manuscripts (chapters 3-6) and traditional chapters (7 and 8), in accordance with university guidelines. The methodologies range from basic science techniques, retrospective clinical studies, a case series through to early phase clinical studies. This chapter provides a synoptic view of the entire work; chapter 2 is a detailed review of the literature and the current state of scientific and clinical research as it relates to the two conditions being studied (ectopic pregnancy and pGTD), the two pharmaceuticals proposed as the intervention (methotrexate and gefitinib), and the combination of the two drugs in preclinical and clinical settings.

All but one of the chapters focus on the condition of ectopic pregnancy, the commonest of the three types of trophoblastic tissue disorders. Chapter 3 examines the current medical treatment protocol for ectopic pregnancy, and utilising retrospective clinical data, establishes that it can provide meaningful, predictive clinical information sooner than is currently practised. In chapter 4 I investigate the potential of two novel biomarkers – adrenomedullin (ADM) and macrophage inhibitory cytokine-1 (MIC-1) to diagnose ectopic pregnancy in a cohort of women presenting with early pregnancy complications; furthermore, I seek to establish the whether serum and tubal ADM expression is affected by exposure to *Chlamydia trachomatis* or cigarette smoke, the biggest risk factors for ectopic pregnancy, as a means by which it may contribute to the pathophysiology of ectopic pregnancy.

Chapter 5 is the first of the early phase clinical studies, designed as a first-in-human phase I dose-escalation toxicity study combining gefitinib and methotrexate for the treatment of small, stable

ectopic pregnancies which are currently eligible for medical treatment according to single-dose methotrexate protocols. Chapter 6 presents a case series of women with non-tubal ectopic pregnancies, a rarer and more complicated subset of patients where mortality and loss of fertility are even more at risk, and where features of the ectopic pregnancy make it less amenable to single-agent methotrexate treatment. In chapter 7 I seek to extend the possible therapeutic application of combination gefitinib and methotrexate, by conducting a phase II study in stable women with ectopic pregnancies with clinical features that would presently exclude them from the option of medical treatment.

Chapter 8 seeks to further extend the application of this work to other disorders of trophoblastic tissue, namely pGTD; this is again designed as a phase I toxicity study due to the combination of gefitinib and with a high-dose methotrexate protocol. Chapter 9 summarises the findings and conclusions drawn from my PhD, the clinical application of this work and the future translational and research directions that have arisen as a result of thesis.

Chapter 2: Literature Review

2.1 Ectopic Pregnancy

2.1.1 Definition and Early History

An ectopic pregnancy occurs when a fertilised ovum implants outside of the endometrial cavity, most commonly in one of the Fallopian tubes (98%), but potentially anywhere in the abdomen and pelvis (Fritz, 2011). The name comes from the Greek word *ektopos,* meaning 'out of place' (Soanes and Stevenson, 2005). Figure 2.1 illustrates the common sites of ectopic pregnancy implantation.

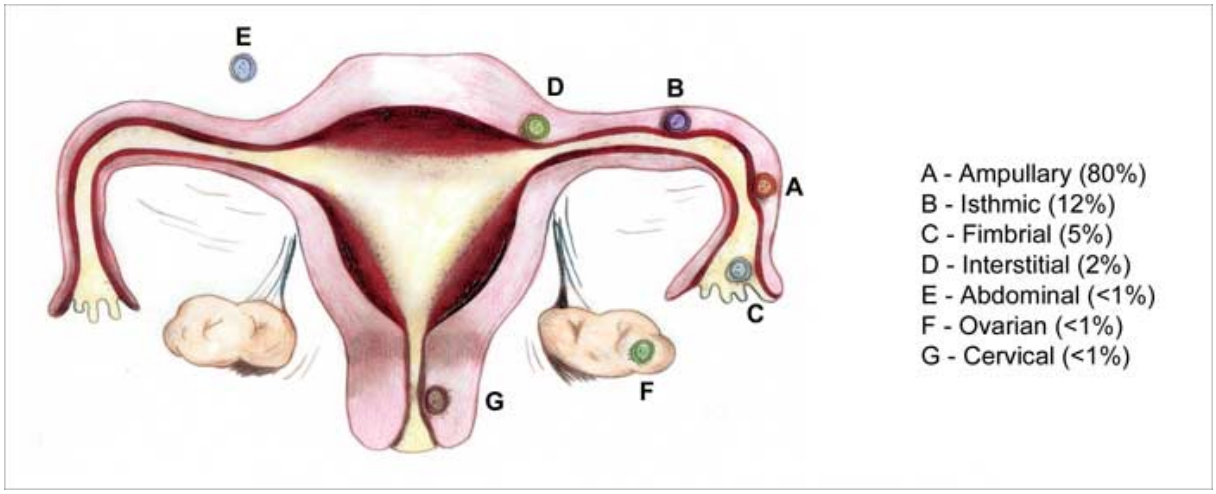

Figure 2.1: The common sites of ectopic pregnancy implantation.
Reproduced with permission from The Ectopic Pregnancy Trust, P.O. Box 70187, London, WC1A 9JD, United Kingdom. Available at: http://www.ectopic.org.uk/professionals/clinical-features/. Retrieved May 23, 2014.

The first known reference to ectopic pregnancy is found in the 11th century writings of the Arabic physician Abulcasis, who describes extracting a fetal skeleton through 'suppuration and drainage' of an abdominal swelling directly through the abdominal wall (Lurie, 1992). During the medical Enlightenment of the 16th to 18th centuries, physicians progressively came to know the body through cadaveric dissection; cases of ectopic pregnancy were increasingly described during this

time, both at post-mortem examinations of women and in early attempts at surgical management (Lurie, 1992, Marion and Meeks, 2012). Despite this, ectopic pregnancy was known as a 'universally fatal accident' until the late 19th century, when medical treatments such as 'vaginal section', "starvation, purging and bleeding of the mother, administration of strychnine, passage of electromagnetic, galvanic or Faradic currents through the ectopic mass and injection of morphine into the fetal sac" were employed (Lurie, 1992). The reported fatality rate of ectopic pregnancy at this time was between 72-99% (Thomas and Mundé, 1880).

2.1.2 Incidence, Significance and Mortality

The incidence of ectopic pregnancies today is between 1-2% of all pregnancies (Cantwell et al., 2011, Creanga et al., 2011, Boufous et al., 2001), and as high as 4% where assisted reproductive techniques (ARTs) have been used to conceive (Fernandez and Gervaise, 2004). The incidence of ectopic pregnancy increased dramatically during the late 20th century, more than doubling between the 1970s and the 1990s (Farquhar, 2005, Chang et al., 2003). Factors thought to have contributed to this global phenomenon include increased uptake of smoking by women, increasing use of ARTs and greater awareness of the condition, as women were increasingly treated in specialised early pregnancy centres (Sivalingam et al., 2011). The incidence of ectopic pregnancy has since stabilised and even declined, a trend which perhaps most tellingly, appears to shadow the incidence of pelvic inflammatory disease (Kamwendo et al., 2000).

Ectopic pregnancy remains a potentially life-threatening condition, as the unregulated, invasive and angiogenic nature of trophoblastic tissue outside of the endometrial cavity means that the growing pregnancy can disrupt maternal vasculature and cause catastrophic haemorrhage. The advent of modern surgical techniques, anaesthesia, blood transfusions and antibiotics in the early 20th century (Fritz, 2011), and more recently, the development of a sensitive and specific

serum human chorionic gonadotrophin (hCG) enzyme immunoassay (EIA) and the capacity for ultrasound diagnosis in the late 20th century (Lurie, 1992), have seen the mortality of ectopic pregnancy fall to 0.5 deaths per 100,000 live births in the United States, 0.26 per 100,000 maternities in the United Kingdom and 0.13 per 100,000 births in Australia (Creanga et al., 2011, Cantwell et al., 2011, Sullivan et al., 2007). Ectopic pregnancies are still, however, the leading cause of maternal death in early pregnancy, and account for between 5-6% of all maternal deaths (Creanga et al., 2011, Cantwell et al., 2011). The case fatality rates of ectopic pregnancy in the developing world are likely to be much higher, with one in 10 diagnosed African women succumbing to the condition (Leke et al., 2004).

2.1.3 Risk factors for Ectopic Pregnancy

Risk factors for ectopic pregnancy fall into two categories: those attributable to contraceptive failure and those resulting from reproductive failure. Although the use of any contraception dramatically lowers a woman's risk of ectopic pregnancy by reducing overall pregnancy rates, if pregnancy does occur, some forms of contraception significantly increase the likelihood of an ectopic pregnancy. The risk of ectopic pregnancy is highest in women where tubal sterilisation has failed, with an odds ratio of 9.3 (95% confidence interval (CI) 4.9-18) compared to pregnant controls (Mol et al., 1995). A pregnancy that occurs despite the presence of an intra-uterine device (IUD) is significantly more likely to be ectopic (1 in 25-30 or 3-4%) (Treiman et al., 1995), and progestin-only contraceptives are also more likely to result in ectopic pregnancy (Mol et al., 1995, Furlong, 2002). Combined oestrogen-progestin contraceptives, vasectomy and condom use are associated with the lowest risk of ectopic pregnancy (Mol et al., 1995).

Risk factors for ectopic pregnancy related to reproductive failure can further be categorized into high, moderate and low risk (Table 2.1) (Ankum et al., 1996, Farquhar, 2005).

Table 2.1: Relative risk factors for ectopic pregnancy.

Degree of risk	Risk factors	Odds ratio
High	Previous ectopic pregnancy	9.3-47
	Previous tubal surgery	6.0-11.5
	Tubal ligation	3.0-139
	Tubal pathology	3.5-25
	In utero diethylstilbestrol exposure	2.4-13
	Current IUD use	1.1-45
Moderate	Infertility	1.1-28
	Previous cervicitis (gonorrhoea, chlamydia)	2.8-3.7
	History of pelvic inflammatory disease	2.1-3.0
	Multiple sexual partners	1.4-4.8
	Smoking	2.3-3.9
Low	Previous pelvic/abdominal surgery	0.93-3.8
	Vaginal douching	1.1-3.1
	Early age of intercourse (<18 years)	1.1-2.5

Adapted from Ankum et al. 1996 and Farquhar 2005.

A history of previous ectopic pregnancy is the single-most important risk factor, with a recurrence risk of 10% after 1 previous ectopic pregnancy, rising to 25% after two or more (Fritz, 2011). Tubal injury from either surgical intervention or pathological processes represents a cluster of high-risk factors, whereas a history of sexually transmitted infection and/or pelvic inflammatory disease confers a moderate risk (Farquhar, 2005, Ankum et al., 1996). Behaviours that might predispose to genital infection also moderately increase the risk of ectopic pregnancy, along with a history of infertility and smoking. A case-control study comparing 803 women with ectopic pregnancy and 1683 controls, found the highest risk factors for ectopic pregnancy to be a history of pelvic infection (OR 3.4; 95% CI 2.4-5) and smoking (in a dose-dependent manner) (OR 3.9; 95% CI 2.6-5.9) (Bouyer et al., 2003). Advancing maternal age, previous miscarriages, infertility, previous IUD use and medical terminations were also strongly associated with ectopic pregnancy (Bouyer et al., 2003). Evidently, many of these risk factors are interrelated, however, up to 50% of women have no identifiable risk factors upon diagnosis of ectopic pregnancy (Fritz, 2011).

2.1.4 Pathophysiology of Ectopic Pregnancy

Impaired embryo-tubal transport and changes to the tubal environment facilitating implantation are the two main mechanisms implicated in the pathophysiology of ectopic pregnancy (Shaw et al., 2010a), and there is a growing body of research to support and characterise this.

Upon ovulation, the oocyte-cumulus complex is normally swept off the ovary by the fimbriae and actively transported along the Fallopian tube towards the endometrial cavity (Fritz, 2011). This passage is facilitated by tubal smooth muscle contractions and ciliary beating (Knoll and Talbot, 1998, Shaw et al., 2010a). Reduced expression of inducible nitric oxide synthase (iNOS), Interstitial Cells of Cajal (ICCs), endocannabinoid receptors (CBIs) and prokineticin receptors (PROKRs), as well as increased expression of activated macrophages and activins in Fallopian tubes affected by ectopic pregnancy, are thought to mediate impaired (reduced) tubal contractility and reduced cilial beat frequency (Shaw et al., 2010a). Other maternally controlled factors that favour the implantation of an embryo in the Fallopian tube include increased levels of interleukin 8 (IL-8) (which either mediates or reflects tissue damage from pelvic infections) and reduced expression of mucin 1 (MUC1) (which allows the implantation of abnormal embryos) (Shaw et al., 2010a).

Embryo-controlled factors that favour tubal implantation include increased levels of uteroglobin, leukaemia inhibitory factor 1 (LIF), homeobox protein A10 (HOXA10), vascular endothelial growth factor (VEGF), trophinin and integrins, mediating levels of tubal inflammation, receptivity, angiogenesis and adhesion (Shaw et al., 2010a). Additionally, tubal ectopic pregnancies resulting from *in vitro* fertilisation (IVF) are known to express increased levels of E-cadherin, a cell adhesion molecule essential for implantation, compared to spontaneous tubal ectopic pregnancies (Revel et al., 2008). This may be due to subtle differences in the culture media used to develop IVF embryos compared to the tubal milieu in *in vivo* conceptions (Revel et al., 2008).

Recent work has focussed on elucidating the mechanisms by which the main risk factors of ectopic pregnancy, namely pelvic infection with *Chlamydia trachomatis* and smoking, might be involved in the pathogenesis of ectopic pregnancy and in particular, tubal implantation.

Investigating the role of PROKRs in tubal implantation, a group from the Queen's Medical Research Institute in Edinburgh have found that the expression of messenger ribonucleic acid (mRNA) encoding PROKR1 is significantly higher in Fallopian tubes of smoking women affected by ectopic pregnancy compared to that of non-smokers (Shaw et al., 2010b). Furthermore, by treating healthy tubal explants with cotinine, a stable metabolite of nicotine, they were able to increase the PROKR1 mRNA expression 2-3 fold compared to controls; this was not related to previous *C. trachomatis* infection, as screened for in the sera of participants (Shaw et al., 2010b). Similarly, the group showed that PROKR2 mRNA levels are increased in the Fallopian tubes of women with evidence of past infection with *C. trachomatis* (Shaw et al., 2011). Accordingly, *in vitro* exposure of both Fallopian tube explants and oviductal epithelial cell lines (OE-E6/7) to the bacterium up-regulated PROKR2 mRNA expression (Shaw et al., 2011). This is likely mediated by *C. trachomatis* ligation of toll-like receptor 2 (TLR2) and subsequent downstream activation of nuclear factor kappa-light- chain enhancer of activated B cells (NF-κB), which in turn regulates PROKR2 mRNA expression (Shaw et al., 2011).

More recently, a group from the University of Hong Kong have investigated the possible role of adrenomedullin (ADM) in the pathogenesis of ectopic pregnancy. The have confirmed the presence of ADM in the human Fallopian tube, and that ADM expression levels vary in relation to the hormonal cycle (Li et al., 2010). Furthermore, they have shown that ADM stimulates ciliary beating in the Fallopian tube, and that this effect is enhanced by contact with human sperm (Li et al., 2010). In a subsequent study, the group showed that plasma ADM levels were significantly lower in women diagnosed with ectopic pregnancy compared to women with a normal pregnancy; similarly, ADM mRNA expression was significantly lower in the Fallopian tube

explants of women with ectopic pregnancy compared to pregnancy-simulated controls (Liao et al., 2012). Ciliary beat frequency was also significantly reduced in Fallopian tubes where ectopic pregnancy had occurred compared to pregnancy-simulated controls; treatment of the Fallopian tube explants with ADM was able to increase ciliary beat frequency in both groups, however, the significant difference between groups remained despite treatment (Liao et al., 2012). Treatment with ADM also increased the basal tone and frequency of contraction of both affected and control Fallopian tube explants, however, the amplitude of these contractions was decreased in both groups (Liao et al., 2012).

In Chapter 3 of my thesis, I explore the potential of ADM as a biomarker of ectopic pregnancy and furthermore, seek to determine whether there is an association between past exposure to *C. trachomatis* infection and/or cigarette smoke exposure (CSE) and tubal ADM expression.

2.1.5 Diagnosis of Ectopic Pregnancy

The most important clinical tool in the diagnosis of ectopic pregnancy is a strong index of suspicion: to consider and exclude the diagnosis in any woman presenting with abdominal pain in her reproductive years. The 2003-2005 Confidential Enquiry into Maternal Deaths in the UK found that one third of deaths attributable to ectopic pregnancy were initially misdiagnosed as a gastrointestinal problem, after women presented with abdominal pain and diarrhoea (Lewis, 2007). Ectopic pregnancy is typically heralded by a triad of symptoms; abdominal pain is a feature in almost all cases (99%), amenorrhoea in 74% and vaginal bleeding in 56% of cases (Alsuleiman and Grimes, 1982). The amount of vaginal bleeding varies, and the intensity of abdominal pain does not correlate with the volume of intra-abdominal bleeding (Jurkovic and Wilkinson, 2011). The two key diagnostic aids developed in the latter half of the 20th century that allow clinicians to confidently and non-invasively diagnose ectopic pregnancy prior to rupture, are the serum hCG EIA and transvaginal ultrasonography (TVUS) (Ankum et al., 1993).

hCG is initially produced by the extravillous cytotrophoblast (EVC) and after implantation, by the syncytiotrophoblast cells of the placenta, and is detectable in the maternal serum 8-10 days after ovulation where conception has occurred (Fritz, 2011, Visconti and Zite, 2012). hCG promotes invasion of the EVC and development of anchoring villi, is involved in spiral artery angiogenesis and subsequently drives progesterone production by the corpus luteum until the placenta itself can produce sufficient quantities of the hormone to sustain pregnancy (Visconti and Zite, 2012). hCG has also been shown to play a role in the development of maternal immunotolerance, facilitating trophoblastic invasion (Kayisli et al., 2003). hCG radio-immunoassays were first developed in the 1970s, targeting the unique β sub-unit of the molecule (Lurie, 1992). Modern 'sandwich' EIAs target 2 or more regions of the β sub-unit, providing a highly sensitive and specific test for pregnancy with detection limits below 5 IU/L (Fritz, 2011).

Serum hCG detection is not only useful in establishing whether or not a woman is pregnant: the trend of quantitative serial hCG measurement provides information pertaining to the viability of a pregnancy. Viable intra-uterine pregnancies (IUPs) demonstrate at least a doubling of serum hCG levels over 48 hours (Barnhart, 2009, Horne et al., 2011), whereas a failing pregnancy or miscarriage is strongly associated with fall in serum hCG of >13% (Condous et al., 2006). In contrast, 71% of women subsequently diagnosed with an ectopic pregnancy demonstrate a suboptimal rise or a sub-diagnostic fall in their serum hCG levels over 48 hours (Barnhart, 2009). Nevertheless, an ectopic pregnancy can demonstrate a 'normal' rise in serum hCG levels, and serial hCG trends should not be used in isolation to guide management.

The serum hCG level also guides the appropriate use of TVUS in the diagnosis of ectopic pregnancy. The so-called 'discriminatory zone' is the serum hCG level above which TVUS detection of an IUP approaches 100% (Barnhart, 2009). This is postulated to be between 1500 and 3000 IU/L, with a trade-off between sensitivity and specificity at lower and higher hCG levels, respectively (Barnhart, 2009). Depending on the skill of the operator, TVUS has a sensitivity of

between 87-99% and specificity of between 94-99% for diagnosis of ectopic pregnancy (Kirk et al., 2014). When TVUS fails to demonstrate an IUP at serum hCG levels above the discriminatory zone, this is highly suggestive of a failing or ectopic pregnancy.

TVUS criteria for diagnosis of an ectopic pregnancy include visualisation of an extra-uterine gestational sac (bagel sign) with a yolk sac and/or an embryo, with our without cardiac motion, or more liberally, an extra-uterine inhomogenous adnexal mass (blob sign) (Barnhart et al., 2011). According to a recent consensus statement, these are classified as a definite ectopic pregnancy (dEP) and probable ectopic pregnancy (pEP), respectively (Barnhart et al., 2011).

Whilst serum hCG level quantification and TVUS have vastly improved the non-invasive diagnosis and management of ectopic pregnancy, hCG is not specific to the condition and only approximately 74% of women will have an ectopic pregnancy visualised by TVUS at first presentation (Kirk et al., 2007b). This leaves a substantial number of women with a suspicion of ectopic pregnancy raised, who after TVUS, are left with the interim diagnosis of pregnancy of unknown location (PUL). PULs must be followed up with serial hCG measurement and TVUS. Studies have shown that only a minority of these women will in fact be subsequently diagnosed with an ectopic pregnancy (up to 20%), whereas 30-47% will have an ongoing viable IUP and the majority (50-70%), will have had a failed pregnancy whose location was never confirmed (Kirk et al., 2007b). Occasionally, PULs may persist (as demonstrated by plateauing hCG levels) and require treatment. An interim diagnosis of PUL thus represents a range of possible pregnancy outcomes, with various levels of associated risk.

A strong research focus in the field is to identify a unique biomarker of ectopic pregnancy, to avoid the ambiguity of a diagnosis of PUL when a woman presents with abdominal pain and/or bleeding in early pregnancy. A range of molecules have been suggested, grouped according to biological functional role in Table 2.2 (Rausch and Barnhart, 2012).

Table 2.2: Proposed biomarkers of ectopic pregnancy grouped by functional roles.

Abnormal Implantation	Trophoblast Function	hCG Activin A Pregnancy specific β1 glycoprotein PAPP-A Human Placental Lactogen
	Corpus Luteal Function	Progesterone Inhibin A Oestradiol Relaxin Renin
	Endometrial Function	Glycodelin Activin B Leukaemia Inhibitory Factor
Growth in Fallopian Tube	Angiogenesis	VEGF
	Muscle Cell Damage	Myoglobin Smooth muscle heavy-chain myoglobin Creatine kinase
Inflammation & Peritoneal Irritation		Interleukin-6 Interleukin-8 Tumour necrosis factor α CA-125

Adapted from Rausch and Barnhart 2012.

To date, no single molecule has demonstrated sufficient discriminatory value to diagnose ectopic pregnancy. For example, serum progesterone measurement, whilst highly predictive of a non-viable pregnancy at levels of <20nmol/L (Banerjee et al., 2001), is poorly predictive of the location of a pregnancy and therefore not sufficiently discriminatory to assist with the diagnosis of ectopic pregnancy (Mol et al., 1998). Research groups are turning their attention to panels of biomarkers selected for differential expression in ectopic compared to normal pregnancy, to achieve better diagnostic specificity. The two most successful examples include a group from Switzerland, who combined VEGF, pregnancy-associated plasma protein-A (PAPP-A) and progesterone in a diagnostic algorithm achieving a sensitivity of 97.7% and specificity of 92.4% for the diagnosis of ectopic pregnancy (Mueller et al., 2004). More recently, a group from the US selected progesterone, VEGF, inhibin A and activin A in an alternate algorithm, to achieve a

sensitivity and specificity of 98% and 100% for the diagnosis of ectopic pregnancy; this quadruple test, however, was only able to diagnose or exclude ectopic pregnancy in 42% of 200 tested samples (Rausch et al., 2011).

In chapter 3, I identify and investigate the potential of another molecule, macrophage inhibitory cytokine-1 (MIC-1) to be used as a biomarker of ectopic pregnancy. MIC-1 is a divergent member of the transforming growth factor-β superfamily, with very high expression in the human placenta, maternal serum and amniotic fluid (Moore et al., 2000). MIC-1 maternal serum levels increase with advancing gestation, and MIC-1 expression is associated with macrophage activation, suggesting a role in immunotolerance at the materno-fetal interface for the establishment and maintenance of pregnancy (Marjono et al., 2003). MIC-1 levels have been shown to be significantly reduced in the sera of asymptomatic women who go on to miscarry compared to women with ongoing gestations, weeks prior to pregnancy loss when fetal viability is recorded on TVUS (Tong et al., 2004, Tong et al., 2012). Given its association with failed pregnancy, I investigated whether there is a relationship between MIC-1 expression and ectopic pregnancy compared to other pregnancy outcomes, and whether or not such an association could be used to diagnose the condition.

2.1.6 Expectant Management of Ectopic Pregnancy

Expectant management of ectopic pregnancy is an active process that requires close monitoring of a woman's serial serum hCG levels and vigilance for the possibility of tubal rupture and clinical deterioration. There is little research comparing expectant management to common surgical and medical treatment options for ectopic pregnancy. Two small, randomised studies compared expectant management of ectopic pregnancy with local and systemic prostaglandin injection, and with low-dose systemic methotrexate administration (van Mello, 2009). These studies provide little meaningful clinical guidance, however, because the comparison treatments and

doses are not routinely used to treat ectopic pregnancy (van Mello, 2009). Given the possibility of fatal complications, most clinicians feel obliged to treat ectopic pregnancy actively. Practice guidelines recommend clinicians reserve expectant management for women diagnosed with an ectopic pregnancy who are haemodynamically stable and have an initial serum hCG level of <1000 IU/L, with studies suggesting the lower the initial serum hCG and the more rapidly declining it is, the greater the likelihood that it will spontaneously resolve (2008, RCOG, 2010).

A recently published prospective, observational study from the United Kingdom (UK) recruited 146 women with small (<3cm diameter), stable ectopic pregnancies with serum hCG levels of <1500 IU/L to expectant management of their condition. Their follow up protocol consisted of serial serum hCG measurements between 2-7 days apart, with a sustained rise in serum hCG levels or a rise to greater than 2000 IU/L prompting recommendation for surgical management. An inability to continue with follow up or a presentation with severe abdominal pain also prompted withdrawal from the study and progression to surgical management. The study found that a third of expectantly managed women with stable ectopic pregnancies and serum hCG levels <1500 IU/L could be managed successfully without any intervention (Mavrelos et al., 2013).

2.1.7 Surgical Management of Ectopic Pregnancy

The first isolated reports of surgical management of ectopic pregnancy are from the 17th century, with increasingly documented attempts in France and the United States in the 18th century. Despite this, the survival rate of women who were operated on at this time was 5 in 30, worse than the 1 in 3 who survived despite no intervention (Fritz, 2011). In 1884, the Scottish surgeon Robert Lawson Tait (1845-1899) reported the first successful salpingectomy for treatment of ectopic pregnancy (Tait, 1884). His continued success with this approach in saving the lives of thousands of women with ectopic pregnancy meant that the procedure was widely adopted, and

by 1913, treatment of the condition was exclusively surgical. The development of a successful surgical treatment for ectopic pregnancy significantly reduced to mortality rate, so that in the early 20th century, 85% of women undergoing a salpingectomy could expect to be cured, compared to the 86% fatality rate associated with conservative treatment (Lurie, 1992).

Later in the 20th century, survival of women undergoing surgical management of ectopic pregnancy benefitted greatly from peri-operative advances such as aseptic technique, anaesthesia, antibiotics and blood transfusions (Fritz, 2011). Operative techniques also improved, and in the latter half of the 20th century, laparoscopy replaced laparotomy as the preferred surgical approach. Laparoscopy confers significant cost savings, a result of shorter operating times, less intra-operative blood loss, shorter hospital stays and shorter associated convalescence (Mol et al., 2008). These factors make laparoscopy more acceptable to patients as well. Systematic reviews, however, suggest that the laparoscopic approach is significantly less successful than laparotomy in eliminating ectopic pregnancy (OR 0.28, 95% CI 0.09-0.86), mainly due to a higher incidence of persistent trophoblastic tissue after salpingostomy (see below) (OR 3.5, 95% CI 1.1-11) (Hajenius et al., 2000).

Two techniques are described to remove the ectopic pregnancy from the Fallopian tube; 1) salpingectomy, where the pregnancy is removed *en bloc* with the tube and 2) salpingostomy, where an incision is made on the anti-mesenteric border of the Fallopian tube overlying the ectopic pregnancy, which is then carefully removed with forceps or irrigation and the resultant incision either closed or left to heal by secondary intention (Fritz, 2011). Surgical management is mandated when there is significant maternal haemorrhage and/or hypovolaemic shock, and the preferred technique in these circumstances is salpingectomy. Salpingectomy is perhaps also more appropriate when there is extensive damage to the tube, when an ectopic pregnancy has recurred in the ipsilateral Fallopian tube, and when future pregnancies are not desired (Fritz, 2011).

A recently published large, multi-centre randomised controlled trial investigated the subsequent spontaneous IUP rates after either salpingectomy or salpingostomy treatment of stable ectopic pregnancy. After randomisation of 446 women, the cumulative ongoing pregnancy rate was 60.7% after salpingostomy and 56.2% after salpingectomy (fecundity rate ratio 1.06, (95% CI 0.81-1.38; log-rank p=0.678) (Mol et al., 2014). Persistent trophoblastic tissue requiring subsequent management was significantly more likely to occur in women who had a salpingostomy, whereas the ectopic pregnancy recurrence rate was not significantly different between the two groups (Mol et al., 2014).

2.1.8 Medical Management of Ectopic Pregnancy

Medical management of ectopic pregnancy centres on the use of methotrexate (see section 2.3). Methotrexate was first used in the treatment of ectopic pregnancy in the 1960s, to aid safe surgical removal of the placenta from its abdominal implantation sites in second and third trimester cases (Lathrop and Bowles, 1968). In the 1980s, the use of methotrexate to treat ectopic pregnancies became independent of surgical excision, with treatment based on protocols used in gestational trophoblastic neoplasia (GTN) i.e. a fixed, multi-dose methotrexate regimen with intervening folinic acid rescue (Bagshawe et al., 1989, Goldstein et al., 1976). These were full chemotherapeutic doses, which although achieving cure, also produced significant side effects in women.

Stovall *et al.* first explored the potential of methotrexate/folinic acid therapy as an outpatient treatment for ectopic pregnancy in 1989. At this time, however, the gold standard of diagnosis of ectopic pregnancy was by direct visualization at surgery, as ultrasound was still too crude an instrument to rely upon in this potentially life-threatening situation. Surgery as part of the diagnostic algorithm of ectopic pregnancy, however, heavily negated the benefits of then proceeding to medical management, both in terms of cost effectiveness and acceptability to

patients (Stovall and Ling, 1993a). The rapid technological advancement and improvement in image quality of TVUS though, meant that by the mid to late 1990s it became the preferred means of diagnosing ectopic pregnancy (Ankum et al., 1993).

Stovall *et al.* remained the pioneers of outpatient treatment of ectopic pregnancies with methotrexate, and undertook much of the research that underpins the current 'single-dose' treatment and monitoring protocol used worldwide today (Appendix A). Their first trial with the aim of using a single dose of methotrexate to treat ectopic pregnancy was published in 1991, with a cure rate of 96.7% (Stovall et al., 1991), and by 1993, had evolved into an entirely non-surgical treatment approach when TVUS was incorporated into the diagnostic algorithm (Stovall and Ling, 1993b).

As part of developing a single-dose outpatient treatment protocol for ectopic pregnancy, Stovall *et al.* also proposed a monitoring protocol; this consisted of a baseline serum hCG level on day 1 of treatment, and subsequent serum hCG measurements on day 4 and day 7. Single-dose methotrexate treatment of ectopic pregnancy was considered effective if a ≥15% fall in serum hCG was observed between day 4 and day 7, and serum hCG levels could then continue to be monitored weekly until complete resolution occurred (serum hCG <15 IU/L) (Stovall and Ling, 1993b). This definition of single-dose methotrexate treatment efficacy has been independently validated; a fall of ≥15% fall in serum hCG between day 4 and day 7 has a positive predictive value (PPV) of 93% for medical treatment success, with a sensitivity of 93% and a specificity of 84.2% (Kirk et al., 2007a).

Where single-dose methotrexate treatment of ectopic pregnancy is not successful, i.e. where a <15% fall in serum hCG occurs between day 4 and day 7, a second (and subsequently third) intramuscular injection of methotrexate (50mg/m^2) can be given, with monitoring recommencing at day 1 with each dose. Approximately 20% of patients will require more than one dose of methotrexate to achieve a cure of their ectopic pregnancy (Lipscomb, 2007).

In 2010, a small study of 30 patients medically treated with single-dose methotrexate for ectopic pregnancy suggested that a falling serum hCG level by day 4 is 100% predictive of method success (Nguyen et al., 2010). In Chapter 4, I examine the prognostic value of a fall in serum hCG level between day 1 and day 4 to predict the likelihood of medical treatment success, 3 days earlier than this current monitoring protocol allows.

The use of methotrexate in the treatment of ectopic pregnancy is limited by its efficacy, which drops significantly from 96% at serum hCG levels between 2000-4999 IU/L (n=106), to 86% for serum hCG levels between 5000-9999 IU/L (n=49) (OR 3.76, 95% CI 1.16-12.33), as based on the findings of a systematic review (Menon et al., 2007). This necessarily limits the number of women with ectopic pregnancies eligible for medical management at the time of diagnosis, with only an estimated 25-30% of diagnosed women satisfying criteria (Jurkovic and Wilkinson, 2011).

Methotrexate has been combined with mifepristone (RU486), a progesterone antagonist, in the treatment of stable ectopic pregnancies. Two trials have shown that single-dose methotrexate was slightly less successful at curing ectopic pregnancy than when 600mcg of mifepristone was added orally (OR 0.84, 95% CI 0.71-1.0) (van Mello, 2009). In addition to the borderline significance of this finding, the cohorts of women studied featured relatively low starting serum hCG levels (between 346-1679 IU/L), itself is the strongest prognostic indicator of medical treatment success (van Mello, 2009, Lipscomb et al., 1999). There may be a role for combination mifepristone and methotrexate to treat larger ectopic pregnancies, but at this time, the data is lacking.

Other medical treatments such as prostaglandins and/or hyperosmolar glucose have been compared to treatment with systemic methotrexate alone. Prostaglandins, alone or in combination with hyperosmolar glucose, showed no difference in treatment success or side-effects compared to methotrexate, and a study comparing methotrexate and hyperosmolar glucose was abandoned due to a high failure rate in the latter group (OR 0.30, 95% CI 0.05-2.0)

(Hajenius et al., 2000). Alternative means of administering methotrexate have also been studied, for example by direct injection into the tubal mass at laparoscopy or with TVUS guidance. Both methods require clinicians with a higher skill set than required for systemic administration of methotrexate and are less successful treatments than laparoscopic salpingectomy (Hajenius et al., 2000). Hence, systemic methotrexate, particularly the 'single' dose regimen, remains the only medical treatment of ectopic pregnancy in common clinical use.

Most clinical centre protocols and guidelines recommend criteria to select appropriate women for medical outpatient treatment of their ectopic pregnancies (Appendix B) (RCOG, 2010). This is determined primarily by a woman's haemodynamic stability and lack of clinical evidence of ectopic pregnancy rupture: women must demonstrate a normal blood pressure, pulse rate and oxygen saturation, have no evidence of guarding or rigidity on abdominal examination, normal red blood cell indices on blood tests and minimal or no evidence of blood in the Pouch of Douglas on TVUS. Additional determinants of likelihood of medical treatment success are assessed when deciding on whether a woman is suitable for medical treatment of her ectopic pregnancy and generally include: a serum hCG of <5000 IU/L, a gestational sac size of less than 3-4cm and no fetal heart visualised on TVUS. Finally, a judgement must be made as to the reliability of the woman to attend for follow up and the availability and their proximity to emergency services.

Systemic methotrexate has been compared to laparoscopic salpingostomy as a treatment for tubal ectopic pregnancy. Multi-dose methotrexate was comparable to laparoscopic salpingostomy in a multi-centre study (n=100) in terms of treatment success in women with ectopic pregnancies of any size or starting serum hCG level (OR 1.8, 95% CI 0.73-4.6), however, multi-dose methotrexate was associated with a significantly greater incidence of side effects (60% vs. 12%) and poorer health related quality of life ($p<0.05$) (Hajenius et al., 2000). Multi-dose methotrexate was also a more expensive treatment option (Hajenius et al., 2000). Single-

dose systemic methotrexate compared to laparoscopic salpingostomy in women with selected ectopic pregnancies (less than 4cm in size on TVUS and with starting serum hCG levels less than 10,000 IU/L) was a far less effective treatment for ectopic pregnancy (OR 0.38, 95% CI 0.20-0.71), however, when allowing for additional doses of methotrexate where serum hCG was falling inadequately, there was no difference in treatment success compared with laparoscopic salpingostomy (OR 1.1, 95% CI 0.52-2.3) (Hajenius et al., 2000).

It is estimated that only around 25-30% of women who present with an ectopic pregnancy are eligible for medical management with methotrexate (Farquhar, 2005, Jurkovic and Wilkinson, 2011), whereas fewer than 5% present in hypovolaemic shock (Fritz, 2011). Thus, a large percentage of women with diagnosed, stable ectopic pregnancies could additionally avoid surgery if a more efficacious medical treatment for ectopic pregnancy existed.

The main body of research I have undertaken as part of my PhD centres on the investigation of a new, combination medical treatment comprising gefitinib (Iressa™, Astra Zeneca), an epidermal growth factor receptor (EGFR) inhibitor (see section 2.4) and methotrexate (see section 2.3). Building on pre-clinical data that showed this combination treatment to have a supra-additive effect on regression of trophoblastic tissues (Nilsson et al., 2013) (see section 2.5.2), I have conducted a number of early phase human clinical trials investigating the safety and efficacy of combination gefitinib and methotrexate in the treatment of ectopic pregnancy. Chapter 5 reports on a phase I dose-escalation study ascertaining the safety of combination gefitinib and methotrexate in women with ectopic pregnancies that would currently be eligible for medical treatment. In chapter 6, I present a case series of women with non-tubal ectopic pregnancies, investigating the potential of combination gefitinib and methotrexate to treat more complex and potentially resistant cases of ectopic pregnancy. Finally, chapter 7 details the findings of a phase II study extending the indication for medical management of ectopic pregnancy, investigating whether combination gefitinib and methotrexate could increase the

number of women with the condition who can avoid surgery and preserve their reproductive potential.

2.1.9 Reproductive Sequelae

For women who wish to conceive again after treatment for ectopic pregnancy, there is little evidence to recommend one treatment modality over another. Data from observational studies suggested that subsequent spontaneous IUP conception rates were higher after salpingostomy (73%) compared to salpingectomy (57%), but so were ectopic pregnancy recurrence rates (15% vs. 10%) (Fritz, 2011). Methotrexate treatment was associated with the lowest likelihood of recurrence of ectopic pregnancy (8%) (Fritz, 2011). The most recent and only randomised, prospective study in the field has, however, demonstrated no difference in either the subsequent pregnancy rate (60.7% vs. 56.2%) or ectopic pregnancy recurrence rate (8% vs. 5%) when comparing salpingostomy with salpingectomy (Mol et al., 2014). Observational data has shown that overall, between 38-89% of women who suffer an ectopic pregnancy will conceive a subsequent spontaneous IUP (Farquhar, 2005); in one prospective observational study conducted in France, the cumulative IUP rate was 56% at 1 year and 67% at 2 years, with maternal age >35 years, a history of infertility and previous tubal damage negatively associated with subsequent IUP (Ego et al., 2001). Parous women and those with a healthy contralateral tube are the most likely to carry a successful IUP after treatment for ectopic pregnancy (Lundorff et al., 1992, Ory et al., 1993).

2.2 Gestational Trophoblastic Disease

2.2.1 Definition and Brief History

Gestational trophoblastic disease (GTD) consists of a range of premalignant and malignant tissues that arise from trophoblastic cells in association with pregnancy. The premalignant forms of the disease are called hydatidiform moles or molar pregnancies. There are two types of molar pregnancy, differentiated by histopathological and genetic features – complete hydatidiform moles (CHMs) and partial hydatidiform moles (PHMs); together they comprise over 90% of all GTD. Gestational trophoblastic neoplasia (GTN) is the collective term for malignant forms of GTD. Malignant change can occur after molar pregnancy and is termed invasive mole or persistent GTD (pGTD). Choriocarcinoma is the most aggressive form of GTN, occurring most commonly after CHMs (50%), but in 25% of cases after a normal term gestation and in the remaining 25%, after a spontaneous miscarriage or ectopic pregnancy (Soper et al., 2004). The rarest forms of GTN are the placental-site trophoblastic tumour/epithelioid trophoblastic tumour (PSTT/ETT), which can occur after any gestational event (Seckl et al., 2013).

Hippocrates himself was most likely referring to GTD when he described 'dropsy' of the uterus around 400BC (Seckl et al., 2010), and there are mostly anecdotal accounts of molar pregnancy until the 19th century (Ober, 1986). Hans Chiari (1851-1916), a professor of pathology in Vienna, documented three cases of likely choriocarcinoma in 1877 and correctly identified the tumour as epithelial in origin; but despite being impressed by the tumour's occurrence in relation to pregnancy in each of the three cases, Chiari failed to recognise the fetal origin of the disease (Ober, 1986). In 1888, Max Sänger (1853-1903), a German obstetrician and gynaecologist, wrongly described GTD as a sarcoma due to its origins in the uterus, explaining the unusual multinucleated syncytiotrophoblast as giant tumour cells (Ober, 1986). Nonetheless, his became

the pervasive school of thought until 1895, when the association between pregnancy and GTD was confirmed by German pathologist Felix Marchand (1846-1928), who convincingly illustrated that the tumours arose from fetal cell lines (Figure 2.2) (Seckl et al., 2010, Ober, 1986).

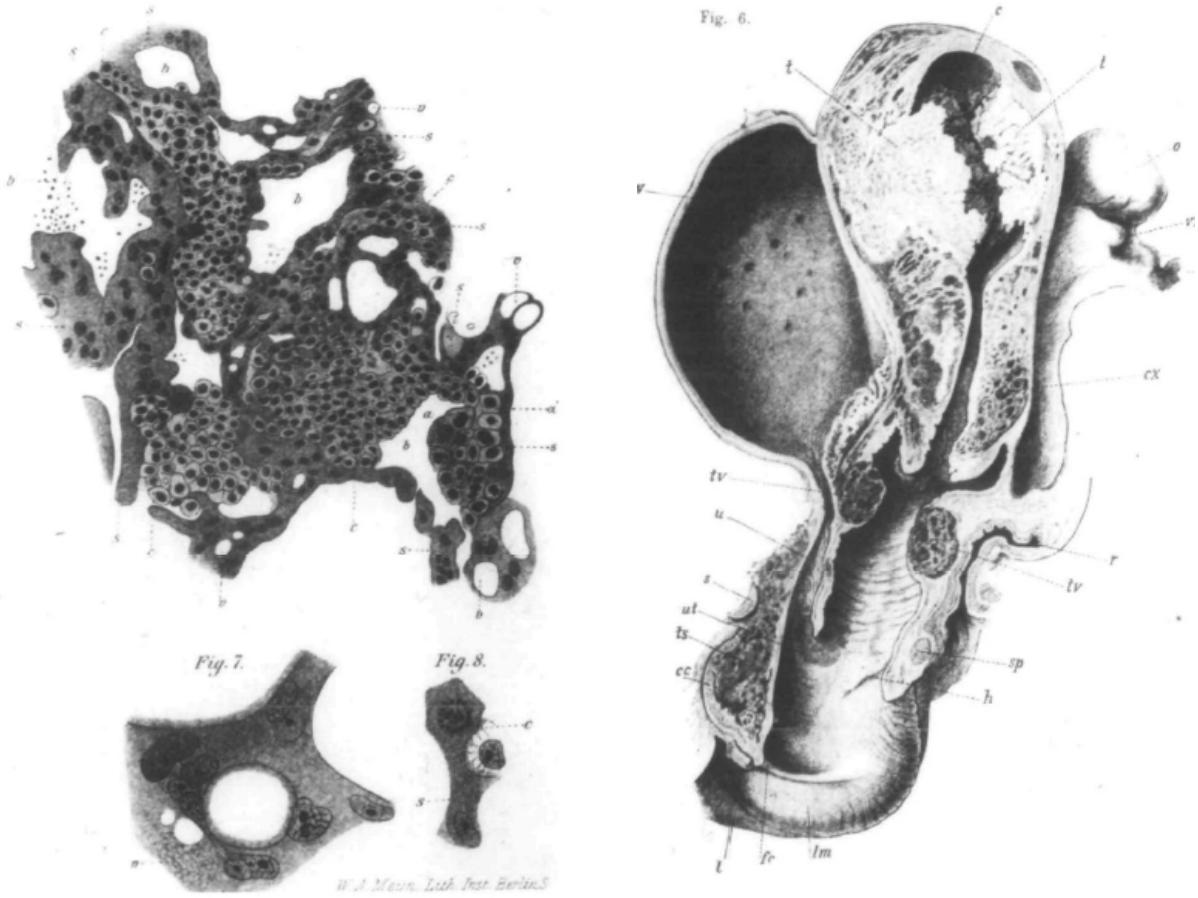

Figure 2.2: Historical drawings of the histological and macroscopic appearance of choriocarcinoma.
Reproduced with permission from Ober (© Oxford University Press, 1986).

Prior to the 1950s and the era of chemotherapy, metastatic choriocarcinoma - the most aggressive form of GTN - was fatal in 90-95% of cases (Ober, 1986). Surgical resection, and in particular hysterectomy for locally confined disease, was the only effective treatment. In 1956, the anti-folate drug methotrexate (see section 2.3) was experimentally administered to a terminally ill 24-year old woman with advanced metastatic choriocarcinoma, after it was earlier observed that methotrexate resolved urinary hCG levels in a patient unsuccessfully treated for

melanoma (Yarris and Hunter, 2003, Li, 1979). The woman not only got better, but was discharged home four months later with no identifiable disease; she is recognised as the first known cure of a solid tumour in humans using chemotherapy (Chabner and Roberts, 2005).

2.2.2 Incidence, Significance and Mortality

The incidence of molar pregnancies is notoriously difficult to determine, as it relies on highly specialised histopathological diagnosis and as such, is hospital- rather than population-based. Furthermore, estimates of the incidence of molar pregnancies are calculated against either total number of pregnancies, or deliveries, or both, creating confusion, and these statistics themselves are prone to under-reporting (Steigrad, 2003). Estimates of the incidence of molar pregnancy in the Western world including Australia range from 0.5-1/1000 pregnancies, with a much higher incidence in Asia (1-2/1000 pregnancies in Japan and China and up to 12/1000 pregnancies in Indonesia) (Steigrad, 2003, Seckl et al., 2013). The incidence of choriocarcinoma is estimated as 1:20,000-50,000 deliveries, and PSTT/ETT accounts for 0.2% of GTD in the UK (Seckl et al., 2013, Soper et al., 2004).

Molar pregnancies have malignant potential, with local invasion a feature in 15% of cases and metastatic disease in 4% (Berkowitz and Goldstein, 1996). CHMs have a higher rate of persistence and neoplastic potential with 15% progressing to pGTD, compared to 0.5-1% of PHMs (Seckl et al., 2010). All forms on GTN require treatment, the basis of which is chemotherapy. The cure rate for high- and low-risk (see below) GTN today is approximately 86% and 100%, respectively (Soper, 2006). There are still, however, improvements that can be made in the management of GTN. The mortality from choriocarcinoma after non-molar pregnancy is 21% compared to 6% after molar pregnancy, mainly due to late diagnosis and advanced disease at diagnosis, and women still die from drug resistant disease (Tidy et al., 1995). Cost-effective screening or surveillance measures to identify women with GTN after non-molar pregnancies

would potentially help to further improve survival by diagnosing the condition earlier in this cohort (Tidy et al., 1995, Seckl et al., 2010). Newer, more effective and less toxic drug treatments would also greatly improve the care of women with GTN, who suffer extended periods of chemotherapy with the attendant side effects and loss of reproductive function.

2.2.3 Risk Factors for GTD

Extremes of maternal age and previous molar pregnancy are the two most strongly proven risk factors for GTD (Palmer, 1994). Pregnancies occurring during teenage years carry a 1.5-2 fold increased risk of molar pregnancy, whereas in women greater than 40 years old, the risk is increased 5-7.5 fold (Steigrad, 2003). If a woman has had one previous molar pregnancy, her risk of recurrence in a future pregnancy is 1% compared to 0.1% of the general population (Berkowitz et al., 2000), and if she has had two or more, this risk rises to between 16-28% (Bagshawe et al., 1986, Sand et al., 1984, Altieri et al., 2003). Recurrent disease may be due to familial recurrent hydatidiform mole (FRHM), an autosomal recessive condition (Seckl et al., 2013). Other risk factors such as maternal blood group, smoking, parity and oral contraceptive pill use have been proposed, but have not been consistently proven (Altieri et al., 2003). There does, however, appear to be an association between dietary consumption of vitamin A (beta carotene) and the incidence of GTD, with areas of deficiency strongly correlating with a high incidence of GTD, and increased ingestion protective of the risk of GTD (Berkowitz et al., 1985, Parazzini et al., 1988). Risk factors for developing invasive, persistent disease (pGTD) after a molar pregnancy has been diagnosed are: serum hCG levels >100,000IU/L at diagnosis, associated large ovarian theca lutein cysts (>6cm), uterine size larger than expected for dates, maternal age >35 years and heterozygous CHMs (Ayhan et al., 1996, Baasanjav et al., 2010).

2.2.4 Pathophysiology of GTD

Molar pregnancies occur after an abnormal fertilization event, leading to the development of abnormally proliferative trophoblastic tissue. There are two types of molar pregnancies: 1) CHM (complete hydatidiform mole), where an ovum devoid of maternal genetic material is fertilized by either one sperm which replicates its DNA (androgenetic monospermic – 80% of CHMs), or two sperm (androgenetic dispermic – 20% of CHMs); and 2) PHM (partial hydatidiform mole), where an apparently normal ovum with a haploid set of maternal chromosomes is fertilized by two sperm, resulting in triple the normal amount of chromosomal material (biparental triploid) (Seckl et al., 2013). A rare form of biparental CHM is associated with an autosomal recessive disorder, resulting most commonly from a mutation on the NLR family, pyrin domain containing 7 (*NLRP7*) gene on chromosome 19q, and more rarely, from a mutation in the KH domain containing 3-like, subcortical maternal complex member (*KHDC3L*) gene on chromosome 6q; these mutations are responsible for FRHM (Seckl et al., 2013). It is worth noting that even when all the nuclear genetic material is paternal in origin, the mitochondrial DNA is still maternally derived (Azuma et al., 1991). Figure 2.3 illustrates the different forms of hydatidiform moles and their genetic make-up (Seckl et al., 2013).

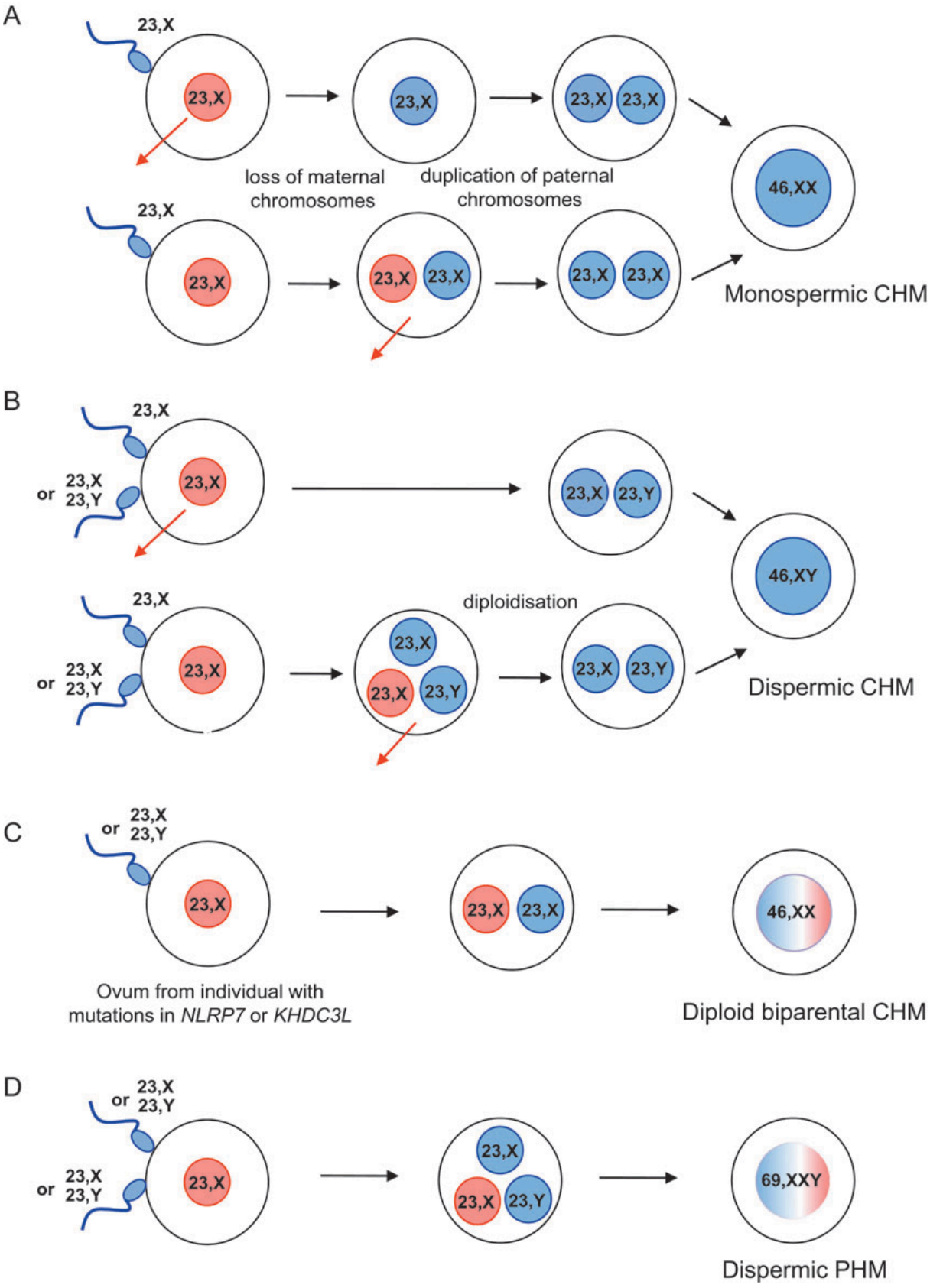

Figure 2.3: Schematic representation of the genetic make-up of molar pregnancies.
Reproduced with permission from Seckl *et al.* (© Oxford University Press, 2013)

In normal pregnancy, the outer cell mass of the blastocyst differentiates into specialised epithelial trophoblastic cells, which implant into the maternal endometrium. Shortly before implantation, the trophoblast further differentiates into two cell lines, the outer syncytiotrophoblast, and the inner cytotrophoblast; the villous syncytiotrophoblast initially invades the endometrium to allow implantation, then loses its proliferative capacity and instead becomes an endocrinological and respiratory organ in direct contact with maternal blood. The cytotrophoblast develops an invasive phenotype and produces a constant supply of extravillous trophoblasts (EVCs), which infiltrate the decidua, the myometrium and spiral arteries to anchor the placenta and form the materno-fetal circulation (Altieri et al., 2003, Fitzgerald et al., 2008). This is a tightly regulated process with many parallels to tumorigenesis, and is controlled by numerous cytokines, but in particular by LIF, which promotes trophoblast invasion, and by its physiological antagonist, suppressor of cytokine signalling 3 (Fitzgerald et al., 2008).

Molar pregnancies and choriocarcinoma are both derived from villous cell line, whilst PSTT/ETT arise from the EVC (Seckl et al., 2013). CHMs are morphologically characterised by a villous architecture associated with abnormal trophoblast hyperplasia, stromal hypercellularity, stromal karyorrhectic debris and collapsed villous blood vessels, whereas PHMs feature patchy villous hydropic change, scattered, abnormally shaped irregular villi with trophoblastic pseudoinclusions and patchy trophoblast hyperplasia (Seckl et al., 2013). PHMs are further differentiated from CHMs by the presence of fetal development, including fetal red blood cells (Soper, 2006). Ancillary techniques such as immunostaining with $p57^{KIP2}$, ploidy analysis and molecular genotyping may be required to differentiate PHM from hydropic non-molar miscarriage, where the morphological features are sometimes indistinct. Unfortunately there are no histopathological or immunohistochemical features which can differentiate or predict which molar pregnancies will progress to pGTD (Seckl et al., 2013).

Choriocarcinoma is a malignant epithelial tumour of a villous trophoblast phenotype, which secretes hCG. It exhibits central necrosis and a characteristic biphasic architecture of recapitulating cytotrophoblast-type cells and multinucleate, pleomorphic syncytiotrophoblast-like areas (Seckl et al., 2013). It is differentiated from pGTD by the absence of villi (Altieri et al., 2003). PSTT is instead differentiated along an extravillous line, with histological features of locally infiltrating nests and sheets of monomorphic interstitial-type trophoblast, moderate pleomorphism and mitotic activity and expression of human placental lactogen (hPL) and other EVC markers (Seckl et al., 2013). ETT is thought to be a variant of PSTT with distinctive hyalinisation and a slightly differing immunohistochemical profile (Seckl et al., 2013). Despite these nuanced differences, the management of GTN incorporating pGTD and choriocarcinoma is the same, and based rather on risk profiling than on actual histopathological diagnosis. The exception to this is the extremely rare PSTT/ETT.

2.2.5 Management of GTD

First line treatment for molar pregnancies is suction curettage, and indeed the majority of molar pregnancies are diagnosed after histopathology testing of products of conception from this procedure (Kohorn, 2004). This is because GTD presents similarly to and is frequently misdiagnosed as a miscarriage, and even ectopic pregnancy. Once the products of conception have been surgically removed and the diagnosis is confirmed as either CHM or PHM, patients are followed up with serial serum and/or urinary hCG levels (Wolfberg et al., 2004, Wolfberg et al., 2006). Serum/urinary hCG levels should be measured at least every 2 weeks during this time (Seckl et al., 2010, Soper, 2006). Once hCG levels are normalised (<5 IU/L), women are followed up for a further 6 months and if their levels remain normal, they are discharged from surveillance. Where hCG normalises within 56 days, there is a reduced risk of developing GTN. Conversely, for women whose hCG levels take longer than 56 days to normalise, extended

surveillance for 2 years picked up only 1 additional woman who went on to develop pGTD and required treatment; hence since 2007, these women are followed up for 6 months from the time of hCG normalization (Sebire et al., 2007).

If hCG follow up for GTD detects plateauing or rising levels, invasive and therefore malignant forms of GTD are assumed (Kohorn, 2001). The Federation of Gynaecologists and Obstetricians (FIGO) proposed the following consensus diagnostic criteria for pGTD using hCG surveillance: 1) an hCG level plateau of four values ±10% over 3 weeks; 2) an hCG level increase of more than 10% of three values over 2 weeks and 3) persistence of detectable hCG for more than 6 months after curettage (Soper, 2006).

2.2.6 Staging, Classification and Management of GTN

Repeat suction curettage for pGTD is generally discouraged. It carries a high risk of uterine perforation and in particular, of significant haemorrhage, as trophoblastic tissue is highly vascular and prone to arterio-venous malformations in GTN (Lim et al., 2002). More than 50% of pGTD patients will still need chemotherapy after a repeat curettage, therefore chemotherapy is the preferred first-line treatment (Seckl et al., 2010). For women who have completed their families, hysterectomy is an option to treat pGTD apparently confined to the uterus, however, because of pGTDs ability to micro-metastasise very early, this does not obviate the need for hCG surveillance or the possibility of requiring chemotherapy (Seckl et al., 2010).

Staging investigations include a baseline serum hCG level to guide and monitor treatment response, as well as a TVUS, including Doppler studies, to exclude a viable pregnancy and characterise the volume, spread and vascularity of the disease (Seckl et al., 2013). A chest X-ray is performed to exclude pulmonary metastases, the most common first site of metastasis in GTN; if any pulmonary metastases are identified, further imaging including a magnetic resonance image study of the brain and computed tomography study of the chest, abdomen and

pelvis are warranted, to locate and characterise all metastases which will guide subsequent management (Seckl et al., 2013).

The most appropriate form of chemotherapy (single agent versus multiple agents) is determined by classification of a patient's risk as either low- or high-risk, as assessed by the 2000 revised FIGO staging system, incorporating a modified World Health Organisation (WHO) Prognostic Score Index (PSI) (Table 2.3) (FIGO, 2009). Unlike other cancers where the staging (i.e. anatomical spread) of the tumour best determines management and correlates to prognosis, it is the presence of certain risk factors that has been shown to correlate most highly with treatment outcome and prognosis in GTN (Goldstein et al., 1998, DuBeshter et al., 1987). Low-risk assessment indicates the patient is likely to respond favourably to single-agent chemotherapy, whereas high-risk patients require more aggressive, multi-agent chemotherapy to achieve disease remission (Ngan et al., 2003). The FIGO staging of GTN does, however, still incorporate anatomical spread of the disease; this is denoted as a Roman numeral, followed by a semi-colon and then the PSI as an Arabic numeral.

Table 2.3: 2000 FIGO staging and classification of GTN.

FIGO Anatomical Staging

Stage I	Disease confined to uterus
Stage II	GTN extends outside of the uterus, but is limited to the genital structures
Stage III	GTN extends to the lungs, with or without known genital tract involvement
Stage IV	All other metastatic sites

Modified WHO PSI as adapted by FIGO

Score	0	1	2	4
Age	<40	≥40		
Antecedent pregnancy	Molar	Abortion	Term	
Interval months from index pregnancy	<4	4-6	7-12	>12
Pre-treatment serum hCG (IU/L)	$<10^3$	10^3-10^4	10^4-10^5	$>10^5$
Largest tumour size (cm) (including uterus)	<3	3-4	>5	-
Site of metastases	Lung	Kidney*	GIT**	Liver/Brain
Number of metastases	-	1-4	5-8	>8
Previous failed chemotherapy	-	-	Single drug	≥2 drugs

* Or spleen
**Gastrointestinal

Adapted from FIGO 2009.

Low risk disease is defined as a PSI of 0-6 and represents approximately 95% of GTN patients (Seckl et al., 2010). These women are highly likely to respond to single agent chemotherapy with either methotrexate (see section 2.3) or actinomycin D (an old antibiotic with anti-cancer activity through inhibition of DNA replication), the two most widely used first-line agents (Alazzam et al., 2009). Various methotrexate regimens are used, with little evidence of the superiority of one regimen over the other (Alazzam et al., 2009). The remission rate resulting from methotrexate therapy ranges from 50-90% depending on the route, dose, frequency of administration and patient selection criteria used (Seckl et al., 2010). Some studies suggest that actinomycin D is more likely to induce remission than methotrexate (Seckl et al., 2010), however, it also seems more likely to cause toxicity such as alopecia (Alazzam et al., 2009).

An 8-day multi-dose methotrexate protocol with intervening folinic acid (to minimize toxicity) was first suggested by Bagshawe and Wilde in 1964, and this regimen is the first line treatment for low-risk disease, used by large centres for trophoblastic disease management and research in the both the United States (US) and the UK; hence it is the most widely used regimen in the world (Alazzam et al., 2009, Bagshawe and Wilde, 1964). The 8-day methotrexate protocol achieves remission in 90% of low risk stage I patients and 70% of low-risk stage II-III patients and is associated with low toxicity; <15% patients experience nausea, <5% vomiting and approximately 2% develop mouth ulcers, sore eyes or chest or abdominal pain from pleuritic or peritoneal serositis (McNeish et al., 2002). If first line treatment fails, treatment with the alternate first-line agent (actinomycin D) or even multi-agent chemotherapy is used to attain an overall survival rate of nearly 100% (Seckl et al., 2010). Treatment is continued until the serum hCG remains normalised (<5IU/L) for at least three consecutive weeks (Alazzam et al., 2009).

Multi-agent chemotherapy is used to treat high risk GTN, defined as stage IV disease or stage II-III disease with a PSI of 7 or above, as well as PSTT/ETT and treatment-refractory low-risk disease (Seckl et al., 2013). Again, a wide variety of regimens are employed worldwide, with very little evidence to show superiority of any one regimen. The most widely used multi-agent chemotherapy regimen – EMA-CO – consists of: etoposide, methotrexate, actinomycin D, cyclophosphamide and vincristine (Oncovin™, Eli Lilly and Company) (Seckl et al., 2013). It achieves a remission rate of 90.6%, and the reported 5 year overall survival rate ranges between 75-90% (Seckl et al., 2013, Kim et al., 1998). EMA-CO is a relatively well tolerated regimen, with alopecia being the commonest side-effect, high-grade haematologic toxicities experienced by less than 2% of patients and more than half of patients retaining their fertility. There are no randomized controlled clinical trials comparing EMA-CO to other combination chemotherapies (Deng et al., 2009). Other combination therapies include EMA (etoposide, methotrexate and actinomycin D), with one retrospective study suggesting similar efficacy but less toxicity

compared to EMA-CO, and MAC (methotrexate, actinomycin D and chlorambucil), which in a retrospective study achieved much less durable remission rates compared to EMA-CO and required a greater number of cycles to achieve remission (Deng et al., 2009).

The management of PSTT takes some exception from that of pGTD and choriocarcinoma. A population-based study found the FIGO scoring system to not be prognostic of survival for these tumours, with only time since antecedent pregnancy remaining predictive on multivariate analysis (Schmid et al., 2009). These tumours are resistant to combination chemotherapy, and surgical treatment with hysterectomy with pelvic lymph node dissection is considered first-line for localised disease (Schmid et al., 2009). Multi-agent chemotherapy is recommended for metastatic disease, in combination with surgery, as residual masses can harbour microscopic disease and hCG levels do not correlate as well with burden of disease (Kohorn, 2009, Schmid et al., 2009). At 10 years, the probability of overall survival for women with PSTT is 70% (Schmid et al., 2009). ETT is presumed to mimic the behaviour of PSTT, but is so rare that there is little data to support this or recommend best management (Seckl et al., 2013).

In Chapter 8 I apply the principles of combination gefitinib and methotrexate treatment of trophoblastic tissue to the treatment of GTN. The chapter reports on the findings of a phase I dose-escalation study combining gefitinib with the 8-day methotrexate protocol for the treatment of women diagnosed with low-risk pGTD.

2.2.7 Reproductive and Long-term Sequelae

Women treated for GTN should remain on life-long serum/urinary hCG surveillance, initially weekly and with decreasing frequency of testing to 6-monthly after 5 years (Seckl et al., 2010). The estimated risk of recurrence is between 3-9%, with the majority of relapses occurring in the first 12 months (Seckl et al., 2013, Matsui et al., 2004, Ngan et al., 2006). Pregnancy should therefore be avoided until at least such time, with whatever contraceptive method the woman

chooses. The oral contraceptive pill in particular is safe, with no proven association between its use and increased risk of development of pGTD (Costa and Doyle, 2006). There does not appear to be any appreciable impact of chemotherapy on fertility, with subsequent pregnancy rates of over 83% after either single- or multi-agent treatment (Woolas et al., 1998). EMA-CO does, however, appear to advance menopause by an average of 3 years (Bower et al., 1998).

2.3 Methotrexate

2.3.1 History and Development

Methotrexate was one of the first drugs synthesised for a specific chemotherapeutic purpose – *in situ* folic acid inhibition for the treatment of acute lymphoblastic leukaemia (ALL) in children (Cronstein and Bertino, 2000). Methotrexate's history therefore, is closely related to the discovery and characterisation of folic acid. The existence of a nutritional factor later proved to be folic acid was first revealed by English haematologist Lucy Wills (1888-1964) in India in the 1930s (Hoffbrand and Weir, 2001). Wills went to Bombay to study the high prevalence of megaloblastic anaemia, then known as 'pernicious anaemia of pregnancy,' in impoverished, pregnant textile workers, where she speculated on the possible causative effects of diet (Roe, 1978). By experimenting on nutritionally deprived rats, rhesus monkeys and patients, Wills observed that a nutritional factor present in vitamin B-rich crude liver and yeast extracts (Marmite) was able to prevent and treat megaloblastic anaemia. Furthermore, the factor was distinct to vitamin $B_{12,}$ known to treat pernicious anaemia, and the patients exhibited no signs of achlorydia, signalling a separate disease entity (Roe, 1978).

In 1941 this so-called 'Wills factor' was first isolated from spinach leaves and called folic acid (*folium* being Latin for leaf), and shortly thereafter in 1943, was synthesised in crystalline form by biochemical nutritionist Robert Stokstad (1913-1995) working at the Lederle Laboratories in New York (Hoffbrand and Weir, 2001). At this time, American pathologist and paediatrician Sidney Farber (1903-1973) had noted the morphological similarities between ALL and megaloblastic anaemia, and subsequently trialled folic acid in the treatment of children with ALL; unfortunately, folic acid supplementation paradoxically accelerated ALL progression (Cronstein and Bertino, 2000). Diet-induced folic acid deficiency was subsequently used as a strategy to slow ALL

progression (Heinle and Welch, 1948). Utilising the Krebs principle that similarity of structure can lead to biological antagonism, Farber collaborated with biochemists at the Lederle Laboratories to synthesise folate analogues, the second of which was methotrexate (amethopterin), developed in 1949 (Figure 2.4) (Cronstein and Bertino, 2000, Hoffbrand and Weir, 2001).

Methotrexate, although managing to induce remission of ALL in children, was not able to cure the disease (Yarris and Hunter, 2003). Min C. Li (1919-1980), a Chinese-American oncologist and cancer researcher, subsequently experimented with the use of methotrexate to treat metastatic melanoma; whilst it produced no effect on the melanoma, Li noted that the urinary hCG levels of his patient resolved with treatment (Li, 1979). In 1955, Li, now working at the National Cancer Institute in collaboration with reproductive endocrinologist Roy Hertz (1909-2002), administered methotrexate as a palliative measure to a terminally ill 24 year old woman with metastatic choriocarcinoma, an hCG producing tumour (Yarris and Hunter, 2003). Ongoing administration of methotrexate saw this young US Navy dental technician not only improve, but leave hospital 4 months later with no remaining clinical or radiographic evidence of the disease (Li, 1979, Yarris and Hunter, 2003). This was the first recognised cure of a solid tumour in humans using chemotherapy (Yarris and Hunter, 2003, Chabner and Roberts, 2005).

2.3.2 Chemical Structure and Mechanism of Action

Methotrexate was created as an analogue of folic acid. As such, its chemical structure closely resembles that of folic (pteroylglutamic) acid, consisting of pteridine ring, paraminobenzoic acid and a glutamic acid (Hoffbrand and Weir, 2001). The differences between the two molecules equate to a hydroxyl group being substituted by an amino acid and the insertion of an additional methyl group (Figure 2.4) (Hoffbrand and Weir, 2001, Schroder and Stein, 2003).

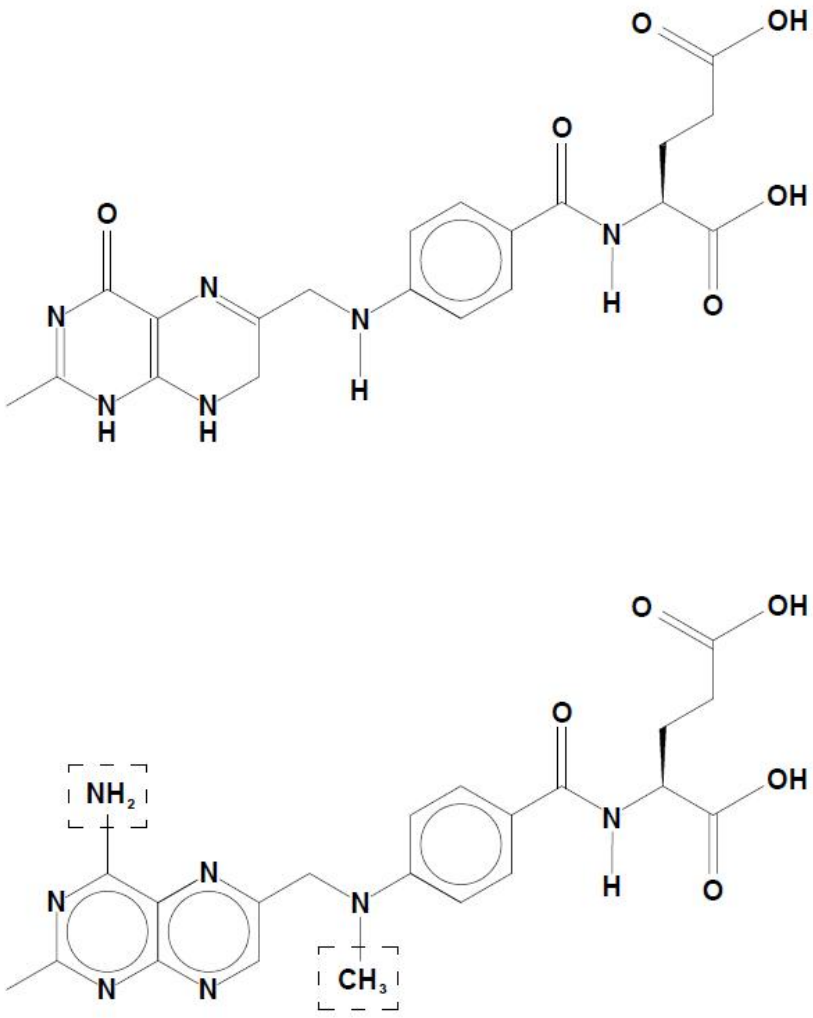

Figure 2.4: The chemical structure of dihydrofolic acid and methotrexate.
Reproduced with permission from Skubisz and Tong (© Obstetrics & Gynecology, 2011).

Methotrexate is therefore able to effectively compete with folic acid for biological activity; it does this by binding with the enzyme dihydrofolate reductase with a thousand times greater affinity than dihydrofolate (Goodsell, 1999). This prevents the conversion of dihydrofolate to tetrahydrofolate, which is essential in the de novo synthesis of purine nucleotides and thymidylate, themselves essential substrates of DNA/RNA synthesis, repair and cell proliferation (Figure 2.5) (Cronstein and Bertino, 2000, Bleyer, 1978). Methotrexate thus blocks cell proliferation in the S-phase of the cell cycle during which DNA replication occurs, making rapidly dividing cells such as trophoblast especially susceptible to its action (Cronstein and Bertino,

2000, Bleyer, 1978). Nevertheless, because of its ability to impede RNA synthesis also, methotrexate at higher doses also causes cell death at other stages of the cell cycle (Stika, 2012). Affected cells are thought to subsequently undergo apoptosis (Cronstein and Bertino, 2000).

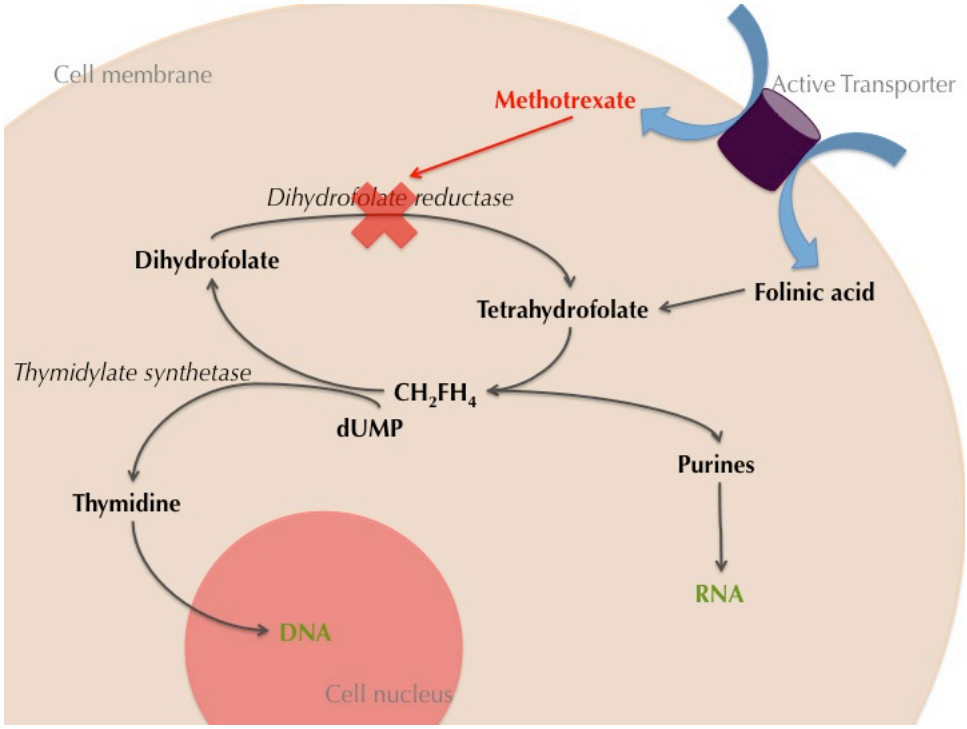

Figure 2.5: Diagrammatical representation of the mechanism of action of methotrexate.
Active transport includes the reduced folate carrier and an endocytic pathway activated by a folate receptor; dUMP: deoxyuridine monophosphate; CH_2FH_4: methylenetetrahydrofolate

2.3.3 Pharmacology

Methotrexate may be administered orally, intramuscularly, intrathecally or intravenously, and there is a direct relationship between dose and plasma concentrations with the latter (Barnhart et al., 2001). Methotrexate is rapidly and completely absorbed at low doses (<80mg/m^2) after oral and intramuscular administration, with peak serum levels achieved 1-4 hours post-oral administration and within 0.5-2 hours after intramuscular and intravenous administration (Shen and Azarnoff, 1978). The plasma disposition of methotrexate is triphasic: there is an initial rapid total body water distribution phase, a renal clearance phase with a half-life of 3.5 hours, and the

third terminal clearance phase with a half-life of 10-12 hours, which may reflect methotrexate's enterohepatic circulation (Stika, 2012). Approximately 50% of the drug is bound to plasma proteins, and other drugs such as salicylates, sulfonamides and phenytoin can displace protein-bound methotrexate and cause drug toxicity (Barnhart et al., 2001, Shen and Azarnoff, 1978). The majority of methotrexate (80%) is excreted in the urine by glomerular filtration and active tubular secretion, which again can be affected by concomitant administration of salicylates (Barnhart et al., 2001, Shen and Azarnoff, 1978). Approximately 10% of methotrexate is eliminated by biliary excretion (Shen and Azarnoff, 1978). Methotrexate enters the cell through an active transporter (the reduced folate carrier) and by diffusion. Intracellularly, it is converted to its polyglutamate form with the addition of up to 5 glutamate groups by folylglutamylsynthetase; in this way methotrexate is better retained within the cell and has a stronger affinity for folate-dependent enzymes (Schroder and Stein, 2003, Stika, 2012). This may also explain the delayed clearance phase of methotrexate (Stika, 2012).

2.3.4 Clinical Application and Side Effects

Methotrexate has a wide range of indications, which extend well beyond its original development for the treatment of haematological malignancy. It is still used for the treatment of neoplasms such as leukaemias and lymphomas, but also lung cancers, breast cancer, head and neck cancers, osteosarcomas, bladder and ovarian cancer and GTN (Cronstein and Bertino, 2000). It has been found to have an immunomodulatory effect in autoimmune conditions such as rheumatoid arthritis, where methotrexate is thought to cause an adenosine-mediated anti-inflammatory effect, and psoriasis, where it is thought to impede the rapid turnover of skin cells characteristic of the condition (Cronstein and Bertino, 2000, Tian and Cronstein, 2007). Methotrexate is also occasionally used in Crohn's disease, multiple sclerosis and psoriatic

arthritis, and as discussed previously, methotrexate is also used in the treatment of ectopic pregnancies (Cronstein and Bertino, 2000).

As methotrexate's mechanism of action is non-specific, the corollary of this is the potential for numerous side effects. Methotrexate has a list of possible toxicities that includes every organ system. The exact likelihood and incidence of side effects vary depending on the condition being treated, the route of administration, the dosage and length of treatment (Hoffbrand and Weir, 2001). Through its mechanism of action, methotrexate preferentially targets rapidly dividing cells, therefore the haematological, gastrointestinal and dermatological systems are the most likely to display signs and symptoms of toxicity such as: neutropenia and generalised myelosuppression, nausea, vomiting, diarrhoea and gastrointestinal inflammation as well as generalised erythema, rash, photosensitivity and alopecia (Barnhart et al., 2001). These side effects have been greatly ameliorated in the field of gynaecology by the development of a low, single-dose protocol in the treatment of tubal ectopic pregnancies, and to some extent, with the use of folinic acid rescue (a form of tetrahydrofolate) in GTN chemotherapy protocols (Stovall et al., 1991, Bagshawe et al., 1989).

2.4 Gefitinib

2.4.1 History and Development

Gefitinib (Iressa™, AstraZeneca) is a selective inhibitor of the EGFR. Growth factor research began in 1952 when neurologist Rita Levi-Montalcini (1909-2012) identified a substance secreted by murine tumours which promoted neurite outgrowth in chicken embryos, and called it nerve growth factor (NGF) (Gschwind et al., 2004). In collaboration with biochemist Stanley Cohen (1922), NGF protein was purified from snake venom and murine salivary-gland extracts, and Cohen went on to isolate another salivary-gland protein that was able to stimulate proliferation of epithelial cells – epidermal growth factor (EGF). In 1978, Cohen identified the putative EGFR and speculated that phosphorylation of this membrane-bound protein produced intracellular signals that regulated cell proliferation (Gschwind et al., 2004, Carpenter and Cohen, 1979). In 1979, separate research revealed that proteins could in fact be modified by phosphorylation of their tyrosine residues and in 1980, Cohen established that the EGFR was one of a number of protein tyrosine kinase growth factor receptors (Eckhart et al., 1979, Ushiro and Cohen, 1980). Levi-Montalcini and Cohen were awarded the Nobel Prize for Physiology and Medicine in 1986 for their growth factor research (Gschwind et al., 2004).

Scientific endeavour around EGFR in the 1980s focussed on sequencing the copy deoxyribonucleic acid (cDNA) of EGFR, and through this, increasing associations were made between the receptor and its oncogenic potential (Gschwind et al., 2004). EGFR exhibited high peptide sequence homology with a recently described avian oncogene v-*erbB*, and Southern blot analysis showed its expression to be increased 25-fold in human A431 epidermal cancer cells (Ullrich et al., 1984, Downward et al., 1984). Overexpression of EGFR was subsequently noted in other human epithelial cancers, and EGFR mutations resulting in increased catalytic tyrosine

kinase activity of the receptor were discovered (Gschwind et al., 2004). Ultimately today, the EGFR family is known to consist of 4 members: EGFR, human EGFR related-2 (HER2 or neu/ERBB2), HER3 and HER4 (Gschwind et al., 2004, Cataldo et al., 2011). HER2 was noted to have the same chromosomal location as the rat *neu* oncogene, whose effects could be dampened by targeted monoclonal antibodies; when HER2 was found to be overexpressed in 30% of invasive breast cancers and related to reduced patient survival and time to relapse, the era of receptor tyrosine kinase based cancer therapies began (Gschwind et al., 2004).

Initially tyrosine kinase directed therapy was based on monoclonal antibody treatment of breast and colorectal cancer, but a targeted, small-molecule pharmacological approach was also sought. In 1987 it was reported that mutations in the adenosine triphosphate (ATP)-binding pocket of EGFR abrogate its tyrosine-kinase function (Gschwind et al., 2004, Honegger et al., 1987), and in 1994, quinazolines were shown to inhibit tyrosine kinase function (Fry et al., 1994, Osherov and Levitzki, 1994). Subsequently in 1996, gefitinib was presented as a potent and selective inhibitor of EGFR tyrosine kinase activity (Gschwind et al., 2004, Wakeling et al., 1996). A significant number of trials have since been conducted seeking the most beneficial application of gefitinib. The signalling pathway of EGFR in non-small cell lung cancer (NSCLC) is activated by either protein over-expression, increased gene copy number or genetic mutations in over half of sufferers; the median overall survival of patients with metastatic NSCLC is approximately 1 year, with only 3.5% surviving 5 years (Cataldo et al., 2011). NSCLC makes up 85-90% of all lung cancer cases and is the leading cause of cancer-related death worldwide (Cataldo et al., 2011).

The Iressa Survival Evaluation in Lung Cancer (ISEL) phase III study showed a delayed time to treatment failure compared to placebo (3.0 vs. 2.6 months, $p<0.001$), and in a subsequent non-inferiority trial with docetaxel – the Iressa NSCLC Trial Evaluating Response and Survival versus Taxotere (INTEREST) – gefitinib treatment resulted in non-inferior overall survival (7.6 vs. 8

months; hazard ration (HR) for death 1.02 (96% CI 0.91-1.15)) (Thatcher et al., 2005, Kim et al., 2008).

Sub-group analyses from these studies revealed certain patient characteristics that were associated with improved treatment response to gefitinib, such as Asian ethnicity, female sex, non-smoking status and a diagnosis of adenocarcinoma (Cataldo et al., 2011). The Iressa Pan-Asia Study (IPASS) selected out patients with adenocarcinoma with minimal or no smoking history and found that compared to carboplatin and paclitaxel treatment, the rate of progression-free survival at 1 year was significantly better for the gefitinib treatment group (24.9% vs. 6.7%; HR for progression or death 0.74 (95% CI 0.65-0.85; $p<0.001$)); furthermore, the quality of life for gefitinib treated patients was improved, despite similar overall survival between the two groups (Mok et al., 2009).

The presence of EGFR mutations causing intrinsic activation of the receptor has been the most positive predictive factor of favourable NSCLC treatment response to gefitinib to date (Cataldo et al., 2011). Two Japanese randomised controlled trials (RCTs) comparing gefitinib with either carboplatin and paclitaxel or cisplatin and docetaxel showed significantly improved median progression-free survival in the gefitinib treatment groups: 10.8 vs. 5.4 months; HR 0.30; 95% CI 0.22-0.41; $p<0.001$ compared to carboplatin and paclitaxel, and 9.2 vs. 6.3 months; HR 0.49; 95% CI 0.34 -0.71; $p<0.001$ compared to cisplatin and docetaxel (Maemondo et al., 2010, Mitsudomi et al., 2010).

2.4.2 Chemical Structure and Mechanism of Action

EGFR is a prototypical cell surface receptor with an intracellular tyrosine kinase domain. It is activated by numerous ligands including epidermal growth factor and transforming growth factor alpha; ligand binding induces dimerisation and subsequent ATP-driven phosphorylation of intracellular tyrosine residues (Cataldo et al., 2011). This signal transduction initiates several

downstream signalling cascades, including mitogen-activated protein kinase (MAPK) and phosphotidylinositol 3 kinase/protein kinase B (PIP3/Akt), which initiate cell proliferation, angiogenesis and anti-apoptotic cell mechanisms (Figure 2.6) (Cataldo et al., 2011, Herbst et al., 2004).

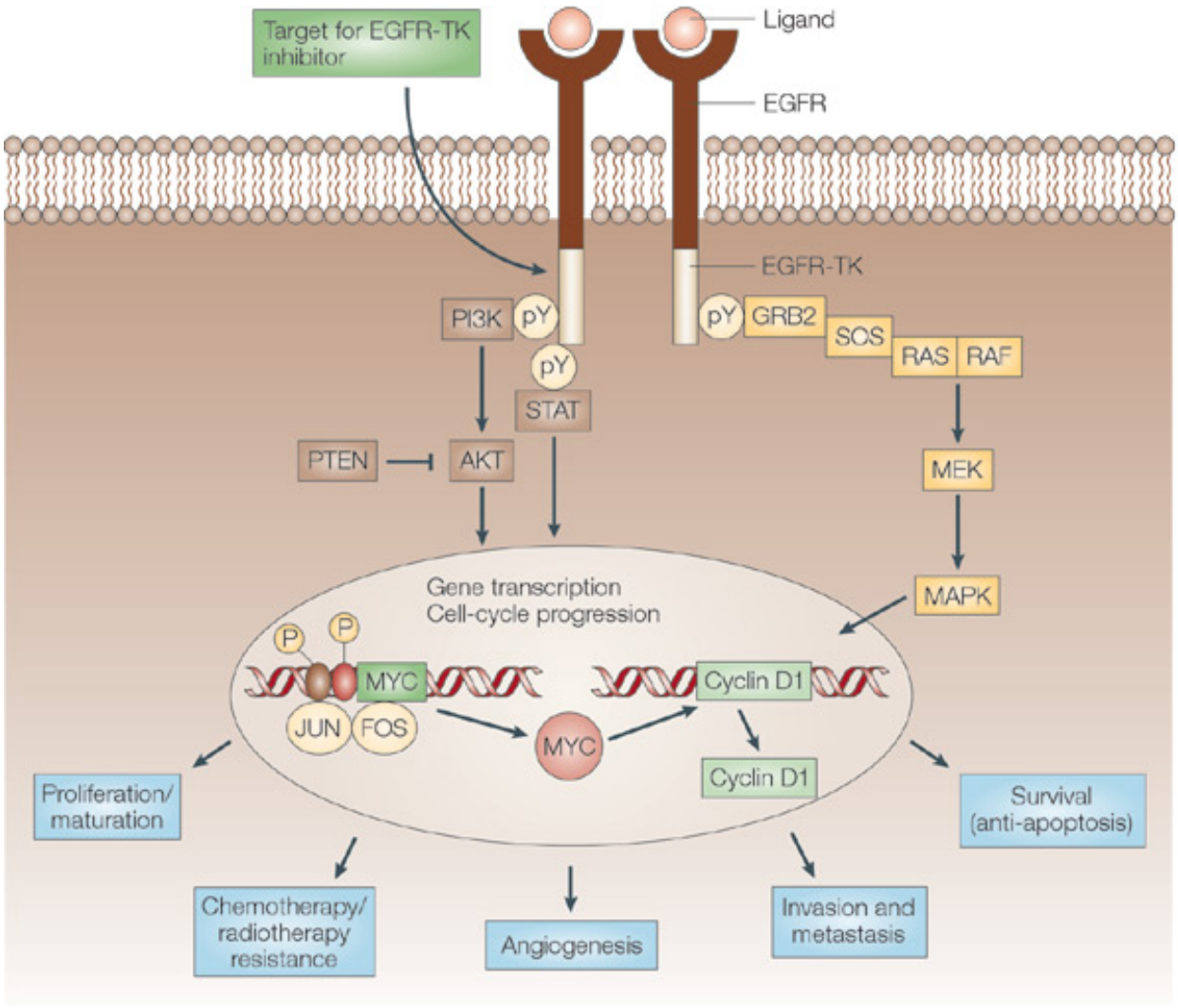

Figure 2.6 EGFR and its intracellular signal transduction cascade.
Reproduced with permission from Herbst *et al.* (© Nature Publishing Group, 2004).

Gefitinib was selected as a likely inhibitor of EGFR because of its structural similarity to ATP, and in fact inhibits ATP-driven phosphorylation by binding to the EGFR tyrosine kinase ATP binding site with much greater affinity than ATP itself (Cohen et al., 2004). Gefitinib is thus a signal transduction inhibitor of EGFR tyrosine kinase, and in particular, is thought to inhibit the anti-

apoptotic Ras signalling transduction cascade. Although gefitinib is marketed as a selective or specific EGFR inhibitor, the ATP binding site of tyrosine kinases is a highly conserved, and there is evidence that it inhibits the activity of other intracellular trans-membrane tyrosine-specific protein kinases at similar concentrations to which is mediates its effect on EGFR; hence gefitinib may exert its anti-tumour effect through signal cascades other than downstream of EGFR (Cohen et al., 2004).

Gefitinib is a low molecular weight synthetic anilinoquinazoline whose chemical structure is shown in Figure 2.7.

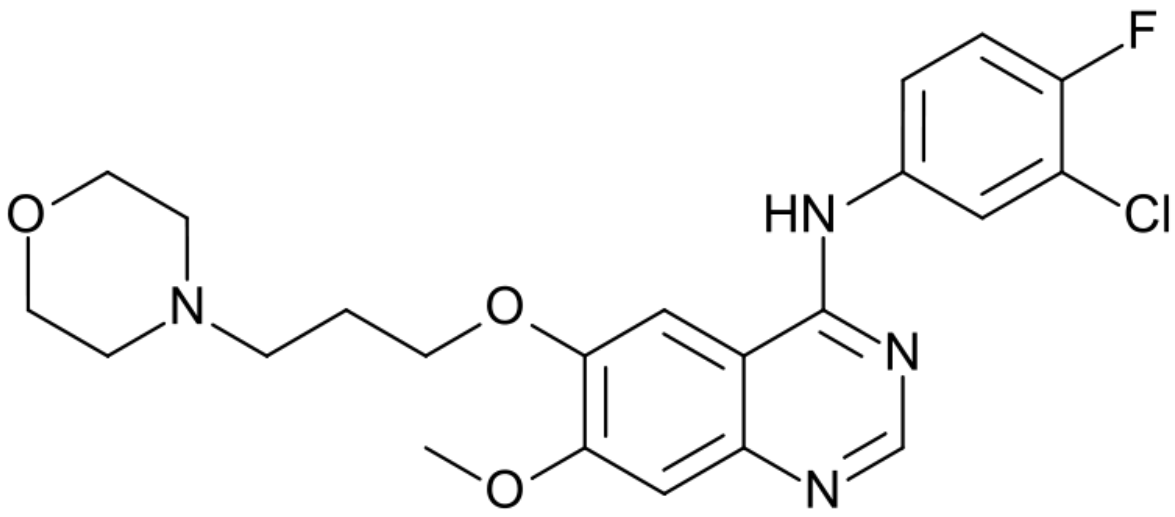

Figure 2.7: The chemical structure of gefitinib.
Reproduced with permission from Cohen *et al.* (© Amercian Association for Cancer Research, 2004).

2.4.3 Pharmacology

Based on phase I clinical trials monitoring toxicity and biological effect, the standard clinical dose of gefitinib is 250mg daily, administered orally (Herbst et al., 2004). Peak plasma levels occur 3-7 hours after a single oral dose, which has a bioavailability of 60% (Cohen et al., 2004). Gefitinib is widely distributed throughout the body after a single 50 mg intravenous bolus dose, with 91% total binding to human plasma proteins (Cohen et al., 2004). Gefitinib has a half-life of 48 hours,

with the majority (approximately 86%) being excreted in faeces; <4% is excreted renally (Cohen et al., 2004). Gefitinib is extensively metabolised in the liver, mainly by cytochrome P450 (CYP) 3A4, which it weakly inhibits along with CYP 1A2 and CYP 2C9. Gefitinib more markedly inhibits the activity of CYP 2C19 and CYP 2D6 (24% and 43% respectively) (Cohen et al., 2004).

2.4.4 Clinical Application and Side Effects

NSCLC remains the only approved indication for gefitinib worldwide. The US Food and Drug Administration (FDA) granted accelerated approval for gefitinib treatment of locally advanced or metastatic NSCLC after failure of both platinum-based and docetaxel chemotherapies in 2003 (Cohen et al., 2004). The FDA withdrew this approval in 2005 after subsequent studies failed to demonstrate a survival benefit, and limited gefitinib use in the US to patients who are currently benefiting from or who have previously benefited from treatment (Cataldo et al., 2011). In Europe in 2009, the European Medicines Agency approved gefitinib for all-stage treatment of locally advanced or metastatic NSCLC in patients with a proven EGFR-activating mutation, with an identical indication issued by Therapeutic Goods Administration in Australia in 2010 and the Pharmaceutical Management Agency in New Zealand in 2012. Gefitinib is being trialled in the treatment of other cancers such as gliomas, breast, head and neck, colon and prostate cancers (Penne et al., 2005).

Gefitinib is well tolerated and compared to conventional chemotherapeutics, has a relatively benign side effect profile. Data is available from post marketing surveillance representing over 92,000 patients, and has shown gefitinib to be largely free of serious side effects such as myelosuppression, neuropathy, alopecia and intractable nausea (Cataldo et al., 2011, van Zandwijk, 2003). Mild diarrhoea in up to 35% and a skin rash in 33% are the most common side effects experienced by patients (Cataldo et al., 2011). Diarrhoea was the dose-limiting toxicity in phase I trials, but rarely requires admission at the set therapeutic dose (Cataldo et al., 2011).

The EGFR-inhibitor associated skin rash is described as an acneiform eruption and can be severe, but is generally self-limiting and resolves with cessation of treatment (Cataldo et al., 2011). It generally occurs within the first 2 weeks of treatment and most commonly involves regions rich in sebaceous glands and those commonly exposed to ultraviolet (UV) light, such as the face, neck and décolletage, suggesting a possible mechanism (Jacot et al., 2004, Peus et al., 2000). The development of an EGFR-inhibitor associated rash is associated with improved therapeutic response, a meta-analysis showing it to be an independent predictor of survival (HR 0.30; 95% CI 0.21-0.43; $p<0.001$) and disease progression (HR 0.50; 95% CI 0.41-0.61; $p<0.001$) (Petrelli et al., 2012). Management is symptomatic with use of alcohol- and fragrance-free pH neutral moisturisers and soaps, minimised sun exposure with use of sunscreen, avoidance of acne preparations such as benzoyl peroxide and tepid baths or showers. Topical treatment with cream-based antibiotics and occasional topical steroid preparations are recommended for mild-moderate cases, progressing to systemic treatment with oral tetracyclines for their immunomodulating and anti-inflammatory effects and occasionally steroids for severe cases (Potthoff et al., 2011).

Interstitial lung disease (ILD) is a very rare but serious side effect of gefitinib. It is a thickening of the lung parenchyma that is fatal in approximately a third of cases (Cohen et al., 2004, Herbst et al., 2004). From available data, the incidence of ILD is as high as 5% in Japanese populations, and <1% in other populations including South-East Asians (Cataldo et al., 2011, Herbst et al., 2004). Risk factors for developing ILD with gefitinib treatment include male sex, age >55 years, smoking status, previous chemotherapy or radiation therapy to the lungs, pre-existing parenchymal lung disease, metastases and pulmonary infection (Cataldo et al., 2011, Cohen et al., 2004, Herbst et al., 2004). It should be noted, however, that ILD is a known complication of lung cancer, and is generally associated with chemotherapy and radiotherapy (Herbst et al., 2004). The median time to onset of ILD was 24 days in Japan and 42 days in the US (Cohen et

al., 2004). The onset of respiratory symptoms such as cough and dyspnoea during gefitinib treatment should initiate prompt work-up of the patient for ILD and permanent discontinuation of EGFR-inhibition therapy (Cohen et al., 2004, Herbst et al., 2004).

2.5 EGFR and the Placenta

2.5.1 EGFR Expression and Function in Trophoblastic Tissues

Trophoblastic cells and cancer cells share many similarities, not only in terms of functional behaviour such as proliferation, migration, invasion and lack of cell-contact inhibition, but also in terms of shared molecular circuits (Ferretti et al., 2007). The duality of the EGFR's importance in the proliferation and survival of both placental and malignant cells was perhaps evident from the start, given whence it was isolated: human trophoblastic cells and A431 epidermal carcinoma cells; even then (1984) it was noted that EGFR expression was 25-fold higher in the cancer cells compared to normal trophoblastic cells (Ullrich et al., 1984). EGFR is a known proto-oncogene, which once constitutively expressed, leads to unregulated cell growth and neoplasia (Ferretti et al., 2007).

In the mouse model, EGFR nullizygosity causes strain specific abnormalities in placental development that result in fetal death before or shortly after birth, and reduced EGFR expression causes reduced placental development across all strains, as well as variable but increased incidence of intra-uterine growth restriction (IUGR) and fetal death (Dackor et al., 2009). In the human placenta, EGFR signalling has been shown to promote cytotrophoblast motility, block apoptosis and is an important defence mechanism against cell death when the placenta is exposed to hypoxia or alcohol (LaMarca et al., 2008, Wolff et al., 2007, Johnstone et al., 2005). There is also a growing association between EGF mutations and the incidence of pre-eclampsia and IUGR in affected women (Dissanayake et al., 2007, Chenthuran et al., 2014).

The potential of EGFR inhibition as a treatment of disorders of trophoblastic tissues is underlined by its significantly increased expression in this cell type. Publically available micro-array assays

reveal the expression of EGFR mRNA in human placenta to be over 30 times its median expression in all other human tissues (Figure 2.8) (Su et al., 2004).

Figure 2.8: Relative expression of EGFR in human tissues.
Reproduced with permission from BioGPS (© The Scripps Research Institute, 2014). Available at: http://biogps.org/#goto=genereport&id=1956. Retrieved May 23, 2014.

2.5.2 EGFR Inhibition to Treat Trophoblastic Tissue Disorders – Preclinical Data

The first research investigating the potential of EGFR inhibition to treat disorders of trophoblastic tissue focussed on the condition of ectopic pregnancy (see section 2.1). After developing the idea and acquiring funding, my supervisor Professor Stephen Tong and colleagues in the Translational Obstetrics Group initially confirmed the expression of EGFR in tubal ectopic pregnancy implantation sites; they found the receptor to be highly expressed in the syncytiotrophoblast, and to a lesser degree, in the cytotrophoblast (Figure 2.9) (Nilsson et al., 2013). EGFR was also expressed in the placental cell lines BeWo and JEG3 to be used for subsequent *in vitro* experiments.

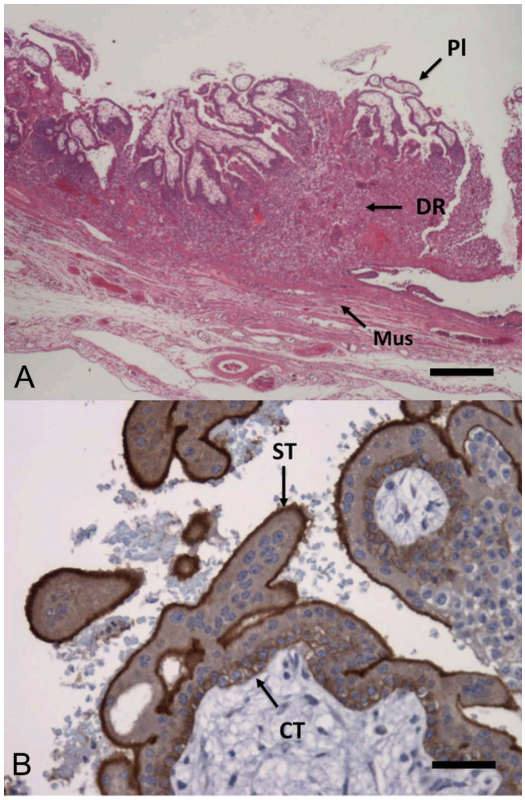

Figure 2.9: Immunohistochemistry staining demonstrating EGFR expression in ectopic pregnancy implantation sites.
A: Haematoxylin and eosin stain of an ectopic pregnancy implanted in a Fallopian tube. PI: placenta; DR: decidual-like reaction; Mus: muscularis of the Fallopian tube. Scale bar is 500 micrometres. B: EGFR staining (brown) of the placenta from a tubal ectopic pregnancy. ST: syncytiotrophoblast; CT: cytotrophoblast. Scale bar is 250 micrometres. Reproduced with permission from Nilsson et al. (© The American College of Obstetricians and Gynecologists, 2013).

They then examined the effect of gefitinib and methotrexate treatment, both alone and in combination, on JEG3 cells, using the xCELLigence system; electrical impedance is continuously measured in real-time, with a greater number of living cells causing greater resistance. Methotrexate alone inhibited growth of JEG3 cells in dose-dependent manner, whereas gefitinib treatment had little effect on placental cell growth. Combining increasing concentrations of gefitinib to a fixed dose of methotrexate (100 micromolar), however, produced a significant supra-additive treatment effect (Figure 2.10 A, B and C) (Nilsson et al., 2013).

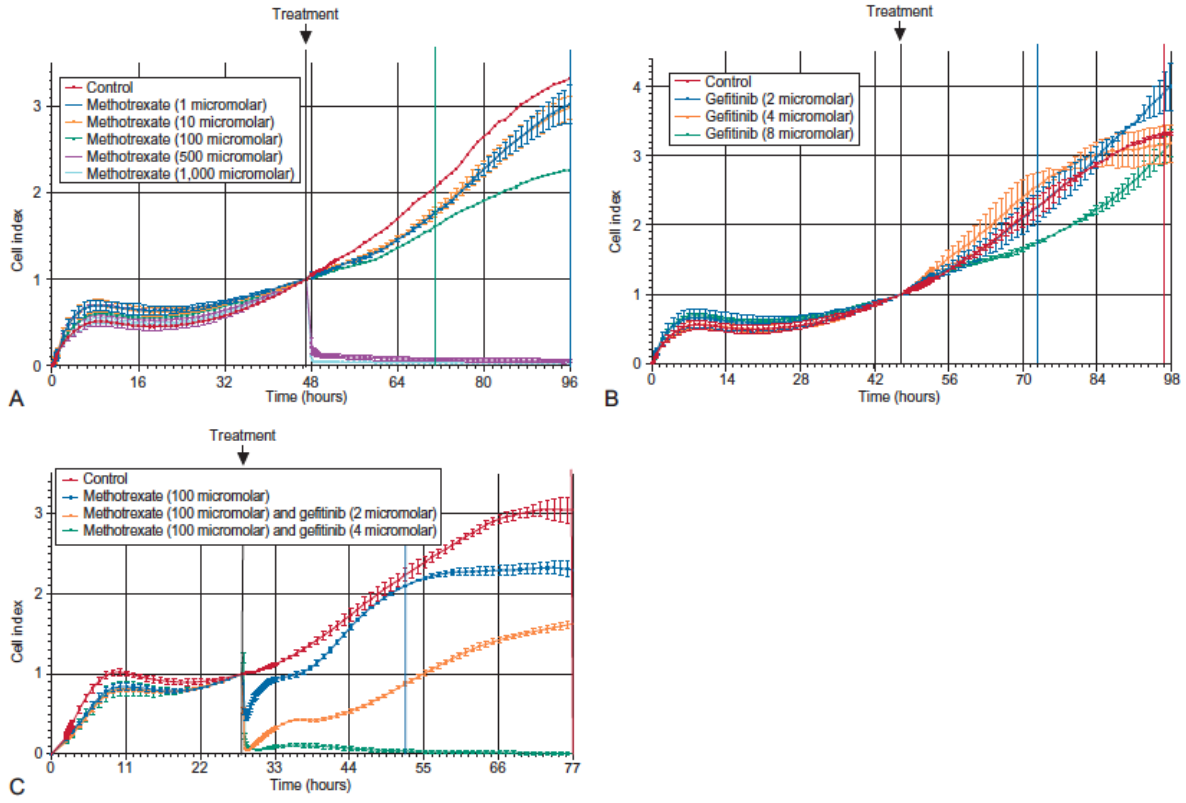

Figure 2.10: Effect of gefitinib, methotrexate and combined treatment of JEG3 cells.
A: Methotrexate only; B: Gefitinib only; C: Combination gefitinib and methotrexate, showing JEG3 growth over time using the xCELLigence system. Mean of triplicates ± standard error of the mean (SEM) shown.
Reproduced with permission from Nilsson et al. (© The American College of Obstetricians & Gynecologists, 2013).

This finding was confirmed in a further 2 placental cell types: BeWo and purified first trimester trophoblast cells. The group also showed that combination treatment more potently decreased EGFR phosphorylation and additionally inhibited Akt phosphorylation, an effect not seen with

methotrexate alone. Combination treatment significantly increased the apoptotic markers Annexin V and caspase 3. Together this data suggests that combination gefitinib and methotrexate treatment of placental cell lines is able to induce cell death as well as inhibit cell growth (Nilsson et al., 2013). An *in vivo* model of ectopic pregnancy was developed, implanting JEG3 xenografts into severe combined immunodeficient (SCID) mice. Treatment with combination gefitinib and methotrexate again produced a significant supra-additive treatment effect on tumour volume and serum hCG levels, compared to treatment with either drug alone (Nilsson et al., 2013).

In summary, this pre-clinical work provided compelling evidence for the improved efficacy of combination treatment with gefitinib and methotrexate for the treatment of ectopic pregnancy. In chapters 5, 6, 7 and 8 of my PhD I have sought to translate this into clinical practice by conducting early phase clinical trials, seeking to verify the safety and efficacy of combination gefitinib and methotrexate to better treat both ectopic pregnancy and pGTD in humans.

2.6 Combination Gefitinib and Methotrexate in Humans

There are no reported studies in humans that combine the use of gefitinib and methotrexate. There has developed a literature combining the use of two agents from the same drug classes as methotrexate and gefitinib: pemetrexed, a newer-generation multi-targeted folic acid antagonist and erlotinib, an alternative marketed small-molecule EGFR inhibitor, in the treatment of various cancers, including NSCLC. In an early phase I study, 42 patients with a range of solid tumours, including 16 participants with NSCLC, were treated with pemetrexed 500mg/m^2 intravenously every 21 days, and various doses of intermittent erlotinib: in Arm A, patients received 800-1400mg erlotinib on days 2, 9 and 16, and in Arm B, 150-250mg on days 2-16 (Davies et al., 2009). The study reported two incidents of dose-limiting toxicity, one in each Arm; both were related to grade 3 infection (fever in Arm A and neutropenia in Arm B) (Davies et al., 2009). The most recently published study used pemetrexed 500mg/m^2 intravenously 3 times a week and erlotinib 150mg daily to treat NSCLC in 159 patients; it showed significant improvements in progression-free survival, overall survival and time to treatment failure in the combination erlotinib/pemetrexed group compared to those treated with pemetrexed alone, but again reported increased rates of severe febrile neutropenia, including one death, in the combined treatment arm (Dittrich et al., 2014).

The available evidence therefore suggests a possible increase in myelosuppressive toxicity in humans when the two classes of drug (folic acid and EGFR inhibitors) are combined. Of note, this is in clinical studies where high-dose intravenous pemetrexed was administered, an agent which featured myelosuppression as dose-limiting toxicity in its own phase I studies (Rollins and Lindley, 2005). Given that gefitinib and methotrexate have not specifically been given in combination to humans before, a cautious, well-monitored toxicity study is an essential step in the translation of the theory of combination treatment into clinical practice, as both the treatment

of ectopic pregnancies and GTN are novel indications for gefitinib and occur in a population of generally fit and healthy young women of reproductive age. We therefore designed two separate phase I dose-escalation studies, combining gefitinib with low- and high-dose methotrexate in the treatment of ectopic pregnancy and pGTD, respectively (Chapters 5 and 8).

PART B: Declaration for Thesis Chapter 3

Monash University

Declaration by Candidate:

In the case of Chapter 3, the nature and extent of my contribution to the work was the following:

Section	Nature of Contribution	Contribution (%)
3.3	Conceived the study, collected the data, performed the data analysis and drafted the paper.	70%
3.4	Conceived the study, performed the data analysis and drafted the paper.	60%

The following co-authors contributed to the work. If co-authors are students at Monash University, the extent of their contribution in percentage terms must be stated:

Name	Nature of Contribution	Contribution (%)
Jourena Li	Collected the data.	
Euan Wallace	Supervised the study, provided critical input.	
Stephen Tong	Conceived and supervised the study, performed the data analysis and drafted the paper.	
Philip Dutton	Collected the data.	
Colin Duncan	Supervised the study and provided critical input.	
Andrew Horne	Supervised the study and provided critical input.	
Stephen Tong	Conceived and supervised the study, performed the data analysis and drafted the paper.	

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-author's contributions to this work:

Candidate's Signature: [redacted] Date: 21/05/2014

Main Supervisor's Signature: [redacted] Date: 21/05/2014

Chapter 3: Early Prediction of Medical Treatment Success in Ectopic Pregnancy

3.1 Introduction

We know human chorionic gonadotrophin (hCG) is produced by the syncytiotrophoblast cells of the developing placenta, and that serum levels are correlated to the amount of functional trophoblastic tissue (Goldstein, 1976). Early pregnancy levels double every 1.5 days and can provide an approximation of gestational age (Fritz and Guo, 1987). Treatment response of trophoblastic tissues can therefore be observed by serial serum hCG measurement, where a decline may be interpreted as evidence that the placenta in the ectopic conceptus is involuting and undergoing cell death.

The current clinical monitoring protocol for assessment of ectopic pregnancy treatment response was proposed with the first outpatient use of single-dose methotrexate treatment for the condition (Stovall et al., 1991). Medical treatment success was defined as a fall in serum hCG of ≥15% between day 4 and day 7 of treatment, and this measure has been validated to have a positive predictive value (PPV) of 93% for medical treatment success with single-dose methotrexate in ectopic pregnancy (Kirk et al., 2007). Clinicians and patients must therefore wait a full week before establishing whether or not their chosen treatment approach is successful; this is a significant period of relative inaction in the management of a potentially life-threatening condition.

3.2 Early Serum hCG Trends and Prediction of Medical Treatment Success

In 2010 a small study suggested that a falling serum hCG level between the baseline and day 4 measures was 100% predictive of medical treatment success in 30 women given single-dose methotrexate for their ectopic pregnancies (Nguyen et al., 2010). This was a clinically significant finding, suggesting that clinicians can provide meaningful advice to their patients about how their

treatment is progressing 3 days earlier than in the current monitoring protocol. These results, however, required independent validation in a much larger cohort of women. To this end, we conducted a retrospective study of women treated with single-dose methotrexate for ectopic pregnancy to determine if an early falling serum hCG level between day 1 and day 4 of treatment was indeed predictive of medical treatment success; the results of this study have been published in the *British Journal of Obstetrics and Gynaecology* and are presented in section 3.3 (Manuscript 1).

In collaboration with international colleagues, we then further sought to validate these findings with a much larger cohort of women. The subsequent study presented in section 3.4 (Manuscript 2) was published in *BioMed Central Pregnancy & Childbirth* and confirmed that an early falling serum hCG level is predictive of medical treatment success, though not with 100% specificity as suggested by Nguyen et al. (2010). Cumulatively our research examined this hypothesis in 251 women and can confidently recommend to clinicians that where there is a falling serum hCG level between day 1 and day 4 of single-dose methotrexate treatment of ectopic pregnancy, the woman is highly likely to experience medical treatment success with no further intervention required.

3.3 Manuscript 1

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www.bjog.org

Short communication

Decline in βhCG levels between days 0 and 4 after a single dose of methotrexate for ectopic pregnancy predicts treatment success: a retrospective cohort study

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In current protocols for the medical management of ectopic pregnancies, the first indication of treatment response is obtained no sooner than day 7. We examined whether human chorionic gonadotrophin (βhCG) trends between days 0 and 4 after methotrexate provide an earlier indication of the likely outcome. Of 33 patients where serum βhCG dropped between days 0 and 4 after methotrexate, the ectopic pregnancy was resolved in 88% of cases without further treatment. Of 12 women where serum βhCG rose between days 0 and 4, only 42% had treatment success. A fall in βhCG between days 0 and 4 after treatment with methotrexate for ectopic pregnancy predicts a high likelihood of treatment success.

Keywords Biomarker, ectopic pregnancy, human chorionic gonadotrophin, medical management, methotrexate.

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Introduction

Until recently, ectopic pregnancy was considered an exclusively surgical condition. In 1989, Stovall et al.[1] showed that outpatient medical management of women with an ectopic pregnancy who were clinically stable, using methotrexate, was safe and acceptable, and by 1991 had developed the single-dose outpatient regimen commonly used today.[2]

Integral to this regimen is a protocol for the strict monitoring of treatment response based on serial monitoring of serum βhCG levels as a surrogate marker of trophoblast/ectopic pregnancy viability. Additional doses of methotrexate are indicated if βhCG does not fall by ≥15% between days 4 and 7 after treatment, or in any subsequent weekly βhCG measurement. Success is defined by the complete resolution of βhCG to <15 iu/L, without recourse to surgery, whether or not additional methotrexate doses are required. Overall, about nine out of 10 women with a small, clinically stable ectopic pregnancy can be successfully managed medically by the use of a 'single' dose of methotrexate (with or without additional doses, which are required in around 15% of cases).[3]

The original protocol of βhCG monitoring to medically treat ectopic pregnancies proposed by Stovall et al.[2] remains in widespread use and was validated in 2007 by Kirk et al.[4] who reported that a ≥15% drop between days 4 and 7 had a positive predictive value (PPV) of 93% in predicting eventual success. However, inherent to the Stovall et al. monitoring protocol is the fact that both clinician and patient must wait 7 days before obtaining the first indication of whether the methotrexate injection has been effective. An earlier indicator of treatment outcome may provide earlier reassurance. It may directly impact on clinical decision making in some situations where there is consideration to opt out of medical management and proceed to surgery.

Recently, Nguyen et al.[5] reported the novel observation that women whose serum βhCG fell between days 0 and 4

after a single dose of methotrexate for an ectopic pregnancy had a remarkable 100% treatment success rate, without need for any further treatment. In contrast, those whose serum βhCG rose between days 0 and 4 only had a 62% rate of treatment success. Their study suggests highly reliable prognostic information is available as early as day 4 after treatment, a finding that may be of keen interest to clinicians and patients. Importantly, there is no need to add extra tests to current protocols. If verified, it could be used clinically, providing early prognostic information for patients and clinicians.

However, the Nguyen et al.[5] report was a small retrospective study of 30 patients. Thus, before clinicians could consider relying on serum βhCG trends between days 0 and 4 as an early prognostic indicator, this preliminary report requires independent validation. Therefore, we examined the rates of success of methotrexate treatment for ectopic pregnancies in patients treated at our centre over a 10-year period, grouped according to whether the serum βhCG rose or fell between days 0 and 4.

Methods

We undertook a retrospective study of ectopic pregnancies managed medically with methotrexate at Monash Medical Centre, a large academic tertiary hospital in Victoria, Australia. The purpose of the study was to examine whether a rise or fall in serum βhCG from days 0 to 4 confers prognostic value as to the likelihood of success of the medical therapy. We obtained permission from Southern Health Human Research Ethics Committee before we commenced the study.

We first identified admissions coded electronically for ectopic pregnancy managed with methotrexate between January 2000 and January 2010. Patients' files were then reviewed. We included women with an ectopic pregnancy treated medically that met the following inclusion criteria, suggesting that they were suitable for medical management: no clinical suspicion of active intra-abdominal bleeding at the time of presentation; documented ultrasound diagnosis of ectopic pregnancy with no evidence of fetal heartbeat; gestational sac size of <3 cm; and serum βhCG of <3000 iu/L.

We obtained baseline clinical information (Table S1), then split our cohort into two groups according to whether serum βhCG rose or fell between days 0 and 4, and determined the percentage of women who had treatment success. A fall was defined as a drop in serum βhCG between days 0 and 4, of any value (i.e. of 1 iu or more).

Stovall et al., who developed the methotrexate protocol that is in common use today, considered the day upon which methotrexate was administered as day 1.[1,3] However, we have noted variation both in the literature and within our institution, whereby some clinicians considered the day of treatment as day 0. They therefore timed the 'day 4' venepuncture a day later relative to the original protocol. Thus, we allowed the definition of 'day 4' to include cases where serum βhCG was sampled either 4 or 5 days after methotrexate injection.

Treatment success was defined as the complete resolution of the ectopic pregnancy after a single injection of methotrexate without recourse to surgery. Unlike the original monitoring protocol proposed by Stovall et al.,[2] we defined additional doses of methotrexate as treatment failure in our primary analysis. We chose this stricter definition for three reasons. First, it provides clean and mutually exclusive outcomes. Patients in which serum βhCG did not fall by ≥15% between days 4 and 7 represent a heterogeneous cohort, where some patients will persist with medical management (second dose of methotrexate) and others will opt for surgery, even though a second dose may have succeeded. In our study, we have grouped this entire cohort as 'failure'. Secondly, we felt that the generation of an early test, able to reliably predict the resolution of the ectopic pregnancy after just one dose of methotrexate, would be of particular clinical interest. Lastly, our definition of treatment failure is the same as that used by Nyugen et al.,[5] and we thought the best way to verify their preliminary results was to closely replicate their methods.

Statistical analysis was performed using PRISM (GraphPad, La Jolla, CA, USA) with a Student's *t*-test for continuous variables and a Fisher's exact test for categorical variables. Statistical significance was defined as $P \leq 0.05$.

Results

In total, 162 patients were treated with single-dose methotrexate for a tubal ectopic pregnancy at our institution from January 2000 to January 2010. The following cases were excluded: 37 lost to follow-up (followed-up externally); ten with a starting serum βhCG >3000 iu/L; and 70 who did not have serum βhCG measured on either day 1 and/or day 4. Thus, 45 were eligible for inclusion in this study.

The overall treatment success rate with a single dose of methotrexate was 76% (34/45). Of 11 patients classed as having 'failed treatment', five required surgical management (11%) and 6 (13%) required an additional dose to achieve resolution, but did not require surgery.

We then split the entire cohort into two subcohorts, grouped according to whether serum βhCG between days 0 and 4 rose or fell. We found that 73% (33/45) of women had a fall in their serum βhCG between days 0 and 4 (subcohort 1). In subcohort 1, 88% (29/33) went on to have treatment success with a single injection of methotrexate alone. Of the subcohort of 12 women who had a rise in serum βhCG between days 0 and 4 (subcohort 2), only 42% (5/12) had treatment success.

The test performance of a decline in serum βhCG between days 0 and 4 as a predictor of treatment success was as follows: sensitivity 85%; specificity 64%; positive predictive value 88%; and negative predictive value 58%. This suggests a decline in serum βhCG is a good biomarker test for predicting treatment success (good sensitivity and positive predictive value), whereas a rise is less reliable in predicting the outcome (modest specificity and negative predictive value).

Figure 1 graphs the trajectory of serum βhCG over time for both subcohorts. The differences in rates of treatment success between the two subcohorts was statistically significant ($P = 0.03$, chi-square test comparing numbers who had successful treatment between the two cohorts). There were no major demographic or ultrasonographic differences between the two subcohorts (Table S1).

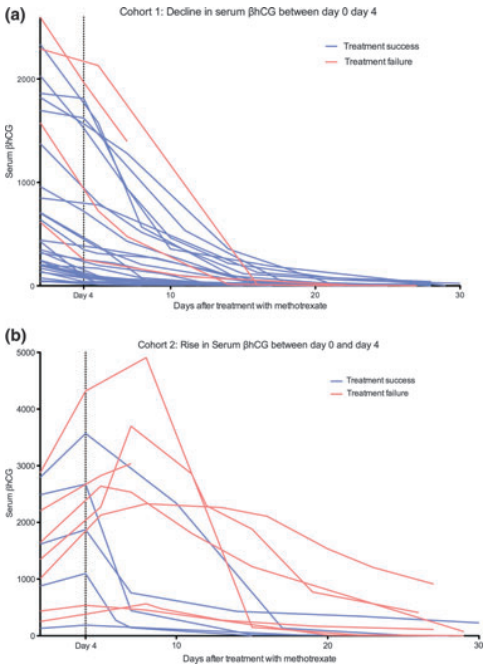

Figure 1. Serum βhCG levels plotted against days after methotrexate injection: (A) subcohort 1, where βhCG levels declined between days 0 and 4; (B) subcohort 2, where βhCG levels increased between days 0 and 4. Blue: 'treatment success' is defined as the complete resolution of the ectopic pregnancy without the need for further treatment after a single injection of methotrexate. Orange: 'treatment failure' is defined as needing further treatment to resolve the ectopic pregnancy after the initial methotrexate injection, such as surgery or a further dose of methotrexate.

Discussion

We undertook a retrospective study to determine whether a rise or fall in serum βhCG between days 0 and 4 could reliably predict the probability of the success of a single injection of methotrexate. We found a decline was associated with an 88% probability of success, without the need of a further dose of methotrexate or surgery. In contrast, those who had a rise only had a 42% probability of treatment success. Thus, a decline in serum βhCG by day 4 after methotrexate appears to be highly reassuring, and a rise in serum βhCG is less so.

Our study broadly agrees with the findings by Nguyen et al.,[5] who reported in a study of 30 participants that all 12 patients who had a decline in βhCG between days 0 and 4 had treatment success (100% success rate). Although we did not have such a high success rate, we still conclude that an early serum βhCG decline is associated with a very high probability of eventual treatment success. However, given that we found an 88% success rate, not 100%, we suggest it is prudent to continue the serial monitoring of serum βhCG until the complete resolution of the ectopic pregnancy.

The overall success rate of treatment in our entire study group was 76%, which is essentially the same as that reported by Nguyen et al.[5] (77%), suggesting that our study populations may be quite similar. However, they found a rise in serum βhCG was still associated with a 62% probability of success,[5] somewhat higher than our finding of 42%.

In further support of the contention that a decline in serum βhCG between days 0 and 4 is a highly reassuring finding is a report by Agostini et al.,[6] who concluded that there was a 97% probability of treatment success if the rate of βhCG decline between days 1 and 4 was >20%. However, that report is not directly comparable with ours because their definition of treatment success included those who required multiple doses of methotrexate. Thus, it is not surprising that they report a higher rate of successful treatment among the group with a decline in serum βhCG of >20%. Furthermore, we believe a simple algorithm of a rise or decline in serum βhCG may be more user friendly to clinicians, compared with the approach proposed by Agostini et al., which requires a (albeit simple) calculation to determine the degree of percentage drop.

With current protocols, there is no indication whether the methotrexate is working until at least day 7. We believe that a predictive test providing an early indication of likely treatment success by day 4 is clinically useful. Importantly, it provides early reassurance to anxious patients who are patiently awaiting the resolution of a potentially life-threatening condition. Secondly, an early indicator of likely resolution may allow the field to develop new protocols to improve the medical treatment of ectopic pregnancy. Those with a fall in βhCG between days 0 and 4 could be

observed with weekly measurements. In contrast, those who experience a rise in serum βhCG between days 0 and 4 (and have a 58% chance of treatment failure) could be offered a second dose of methotrexate sooner than day 7, perhaps with a second dose of methotrexate (or a short multi-dose methotrexate protocol with folinic acid rescue) around day 5. We speculate that such an approach could potentially enhance the success rates of medical treatment and/or reduce the number of days for ectopic pregnancies to resolve, although this possibility certainly requires assessment in clinical trials.

In conclusion, we have found that a decline in serum βhCG between days 0 and 4 is associated with an 88% likelihood that the single methotrexate injection will successfully resolve the ectopic pregnancy. It appears to be a reliable early clinical indicator of the likely success of the medical treatment.

Disclosure of interests
None.

Contribution to authorship
ST, EW and MS conceived the study. MS and JL collected the data. MS and ST performed the data analysis. ST and MS drafted the paper. All authors critically reviewed the manuscript and approved the final version.

Details of ethics approval
This application was approved by The Southern Health Human Research Ethics Committee (Melbourne, Victoria) on 7 April 2010 (project number 10088Q).

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Acknowledgement
None.

Supporting information

The following supplementary materials are available for this article:

Table S1. Patient demographics of the cohort grouped according to whether serum βhCG rose or fell.

Additional supporting information may be found in the online version of this article.

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting information supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author. ■

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RESEARCH ARTICLE Open Access

Using a decline in serum hCG between days 0–4 to predict ectopic pregnancy treatment success after single-dose methotrexate: a retrospective cohort study

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Abstract

Background: The current measure of treatment efficacy of single-dose methotrexate for ectopic pregnancy, is a fall in serum hCG of ≥15% between days 4–7 of treatment, which has a positive predictive value of 93% for treatment success. Two small studies have proposed a fall in serum hCG between days 0–4 after treatment confers similar, earlier prognostic information, with positive predictive values of 100% and 88% for treatment success. We sought to validate this in a large, independent cohort because of the potentially significant clinical implications.

Methods: We conducted a retrospective study of women (n=206) treated with single-dose methotrexate for ectopic pregnancy (pre-treatment serum hCG levels ≤3000 IU/L) at Scottish hospitals between 2006–2011. Women were divided into two cohorts based on whether their serum hCG levels rose or fell between days 0–4 after methotrexate. Treatment outcomes of women in each cohort were compared, and the test performance characteristics calculated. This methodology was repeated for the current measure (≥15% fall in serum hCG between days 4–7 of treatment) and an alternate early measure (>20% fall in serum hCG between days 0–4 of treatment), and all three measures were compared for their ability to predict medical treatment success.

Results: In our cohort, the positive predictive value of the current clinical measure was 89% (95% CI 84-94%) (121/136). A falling serum hCG between days 0–4 predicted treatment success in 85% (95% CI 79-92%) of cases (94/110) and a >20% fall in serum hCG between days 0–4 predicted treatment success in 94% (95% CI 88-100%) of cases (59/63). There was no significant difference in the ability of these tests to predict medical treatment success.

Conclusions: We have verified that a decline in serum hCG between days 0–4 after methotrexate treatment for ectopic pregnancies, with pre-treatment serum hCG levels ≤3000 IU/L, provides an early indication of likelihood of treatment success, and performs just as well as the existing measure, which only provides prognostic information on day 7.

Keywords: Ectopic pregnancy, Human chorionic gonadotrophin, Medical management, Methotrexate, Positive predictive value, Treatment success

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Background

Ectopic pregnancies occur in 1-2% of pregnancies [1]. Although potentially life threatening, the ability to non-invasively detect ectopic pregnancies before they rupture with ultrasound affords some women the option of medical management. Stovall *et al.* [2] first demonstrated the safety and efficacy of outpatient methotrexate to treat women with ectopic pregnancies in 1989, and today, approximately 25-30% of women presenting with this condition are eligible for such treatment [3,4].

Quantification of serum hCG provides a sensitive biomarker of viable trophoblastic tissue and is used in the medical treatment of ectopic pregnancy to monitor treatment response. In the single-dose methotrexate protocol developed by Stovall *et al.* [2,5], treatment efficacy is determined by a ≥15% fall in serum hCG between days 4 and 7 of treatment; if there has been an insufficient fall in serum hCG at this time, further doses of methotrexate and/or surgery are indicated. This measure has been validated by Kirk *et al.* [6] and shown to have a positive predictive value of 93% for treatment success. By definition, however, the first indication of treatment efficacy can be ascertained no earlier than day 7.

In 2010, Nguyen *et al.* [7] reported that a fall in serum hCG between days 0–4 after methotrexate injection predicted treatment success with no further intervention in, remarkably, 100% of cases (n=30). Investigating a cohort of 45 women in a follow-up study, we reported that a fall in serum hCG between days 0–4 predicted treatment success after single-dose methotrexate in 88% of cases [8]. Furthermore, an earlier study by Agostini *et al.* [9] of 129 cases of ectopic pregnancy reported that a ≥20% fall in serum hCG between days 1–4 after medical treatment with a single dose methotrexate had a positive predictive value of 97% for treatment success.

The fact that serum hCG trends may be able to accurately predict treatment outcomes for many women as early as day 4 has potentially important clinical implications. Firstly, it significantly reduces the duration of anxious uncertainty patients (and clinical staff) must endure before obtaining an indication of whether or not the treatment is working. Secondly, it raises the possibility of changing protocols in medical management of ectopic pregnancy and intervening with a second dose of methotrexate earlier in cases where serum hCG levels have not fallen between days 0–4, to potentially improve overall methotrexate treatment success rates and/or decrease the length of time required to achieve a successful resolution of the ectopic pregnancy.

However, before proposing a fall in day 0–4 serum hCG should be used clinically as an early prognostic indicator for medical treatment success in management of ectopic pregnancies, we felt it important to perform a further validation using a large, independent cohort, especially given that the two previous studies featured small numbers of women (n=30 and 45, respectively) [7,8]. Therefore, we examined the ability of a fall in serum hCG between days 0–4 to predict treatment success after single-dose methotrexate treatment for ectopic pregnancy in a cohort of 206 Scottish women.

Methods

Objective

A retrospective cohort study was performed to assess the prognostic value of a fall in serum hCG between days 0–4 after medical treatment of ectopic pregnancy with single-dose methotrexate.

Participants

The South East Scotland Research Ethics Service deemed that this study did not require formal NHS ethical review, as the project was audit based and used only data obtained as part of routine care.

Data was collected from electronic and linked records for women treated with single-dose methotrexate for ectopic pregnancy in early pregnancy units in Scotland between 2006 and 2011. Data for the year 2006 was available for women treated at all 11 Scottish early pregnancy units (n=210) and for treatment episodes between 2007 and 2011, records were collected from the Royal Infirmary of Edinburgh's Pregnancy Support Centre (n=187). Data collected included baseline demographics, treatment dates and outcomes and serial serum hCG measurements.

Available patient data was classified and only included if it fulfilled the ectopic pregnancy diagnostic criteria published in the consensus statement of early pregnancy outcomes [10].

Scottish protocols stipulate that to be eligible for outpatient medical management of ectopic pregnancy, a woman must be haemodynamically stable and reliable for follow up, the pre-treatment serum hCG should be <3000IU/L and the diagnostic ultrasound should show a gestational sac size no greater than 4cm in largest diameter, with little or no pelvic free fluid. Eligible women were treated as outpatients with a single dose of intramuscular methotrexate at 50 mg/m^2.

Furthermore, to be included in our analysis, participants needed to have a recorded serum hCG on day 0 or 1, day 4 and day 7 of treatment, as well as a documented treatment outcome i.e. successful medical management with or without further doses of methotrexate, or failed medical management requiring surgery.

We have previously noted confusion both clinically and in the literature relating to the day of treatment, with both day 0 and day 1 being used to denote the day of methotrexate injection [8]. The original protocol proposed by Stovall *et al.* [5] considers the day of treatment to be day

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1, however, the study by Nguyen *et al.* [7] designated day 0 as the day of treatment. We have thus included women with either a day 0 or day 1 serum hCG recorded.

Description of analysis

In order to achieve a dichotomous outcome (i.e. success/failure), we have for the purposes of this analysis defined treatment success as a complete resolution of serum hCG to <15IU/L after a single dose of methotrexate with no further intervention, medical or surgical. Thus, cases requiring repeat doses of methotrexate or surgical management were classified under treatment failure in this study.

Participants were divided into two cohorts based on whether their serum hCG rose or fell between days 0–4 after single-dose methotrexate for ectopic pregnancy (day 0/1 serum hCG - day 4 serum hCG). The positive predictive value, negative predictive value, sensitivity and specificity were calculated.

We used this method to assess the prognostic value of other measures of treatment efficacy: 1) a ≥15% fall in serum hCG between days 4–7 of medical treatment ((day 4 – day 7 serum hCG/ day 4 serum hCG) x 100) and 2) a ≥20% fall in serum hCG between days 0–4 of treatment ((day0/1 - day 4 serum hCG/day 0/1 serum hCG) x 100), as proposed by Agostini *et al.* [9].

Statistical analysis

The ability of each measure to predict single-dose methotrexate treatment success in ectopic pregnancy was compared using Fisher's exact test. Baseline clinical demographics were compared using a Student's *t* test for continuous variables and a Fisher's exact test for categorical variables. For each measure, we calculated sensitivities, specificities, positive and negative predictive values. Statistical analysis was performed using PRISM (GraphPad, La Jolla, CA, USA).

Results

Participants

Records were available for a total of 397 women treated with single-dose methotrexate for ectopic pregnancy between 2006–2011, using local protocols based on the RCOG guideline [11]. Of these: 2 were lost to follow up or had no recorded serum hCG levels, 38 had no recorded day 0 or 1 serum hCG, 104 women had no recorded day 4 serum hCG and 32 had no recorded day 7 serum hCG. A further 13 women were excluded for having a pre-treatment serum hCG of >3000IU/L and 2 had no documented treatment outcome. This left 206 women suitable for analysis.

The baseline characteristics of the cohort include a mean day 0 (or day 1) hCG level of 778.2 IU/L (SEM ± 49.25), a mean age of 30.8 (SEM ± 0.4) years, a previous ectopic pregnancy rate of 14% (28/206), previous recorded chlamydial infection in 18% (36/206) and 24% of the cohort were current or past smokers 49/206). All participants demonstrated a gestational sac size of <4cm and up to moderate amounts of pelvic free fluid on ultrasound.

Treatment outcomes

The treatment success rate achieved with a single dose of methotrexate i.e. without need for a subsequent dose of methotrexate and/or surgery in the overall cohort was 71% (147/206). 40 women (19%) required further doses, so that the overall medical treatment success rate (allowing for additional doses of methotrexate) was 90% (186/206). 20 women (10%) required surgical management.

Falling hCG between days 0–4 to predict medical treatment success

110/206 women demonstrated a falling serum hCG between days 0–4 of treatment (mean hCG fall −161 IU/L (SEM ± 21.5)); of these, 94/110 women experienced treatment success with a single dose of methotrexate (no repeat doses), giving this measure a positive predictive value of 85% (95% CI 79-92%). The sensitivity and specificity for a falling hCG between days 0–4 to predict single-dose methotrexate treatment success were 64% and 73%, respectively, and the negative predictive value 46%.

Of the remaining 96/206 women who had a rising serum hCG between days 0–4 (mean hCG rise 317 IU/L (SEM ± 32.2)), 53/96 (55%) experienced medical treatment success (i.e. negative predictive value of 45%).

We examined the pre-treatment serum hCG trends of participants with a falling serum hCG between days 0–4, to show that the early falling hCG levels were an effect of treatment and not just a pre-existing trend. The pre-treatment serum hCG trend was available for 98/110 of women with a falling serum hCG betweens days 0–4. Observation of pre-treatment hCG levels ranged from 1–32 days prior to methotrexate administration. A rising hCG trend was noted in 61/98 (62%) women in this cohort prior to treatment, and of these, 52/61 (85%) were successfully treated with one dose of methotrexate; 9/61 (15%) required either additional doses of methotrexate or surgical management. A falling pre-treatment hCG trend was noted in 37/98 women in this cohort, with 31/37 (84%) of these successfully treated and 6/37 (16%) requiring additional doses of methotrexate and/or surgery.

≥15% Fall in hCG between days 4–7 to predict medical treatment success

Using the current test of medical treatment efficacy in ectopic pregnancy, 136/206 women demonstrated a ≥15% fall in serum hCG between days 4–7. Of these, 121/136 women experienced treatment success resulting

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in a positive predictive value of 89% (95% CI 84-94%). Of the 70/206 women whose serum hCG did not fall by ≥15% between days 4–7, 25/70 (36%) experienced treatment success (negative predictive value of 64%). The sensitivity of this measure to predict treatment success was 82% and specificity 75%.

≥20% Fall in hCG between days 0–4 to predict medical treatment success

63/206 women had a ≥20% fall in their serum hCG concentrations between days 0–4. Of these, 59/63 experienced treatment success providing a positive predictive value of 94% (95% CI 88-100%). Of the 143/206 women where serum hCG did not fall ≥20% between days 0–4, 87/143 (61%) experienced treatment success (i.e. negative predictive value 39%). The sensitivity of this measure to accurately predict medical treatment success was 40% and the specificity 93%. The test characteristics of all 3 measures are summarised in Table 1.

Comparison of the predictive value of the 3 measures

There was no significant difference between the ability of any of the tests to accurately predict medical treatment success with a single dose of methotrexate when comparing them individually using Fisher's exact test (p=≥0.13).

Table 1 Summary test characteristics of each of the 3 measures analysed

TEST 1: Falling hCG between days 0-4					
	Treatment Success	**Treatment Failure**	**Total**		
Falling hCG	94	16	110	**PPV:**	85% (95% CI 79-92%)
Rising hCG	52	44	96	**NPV:**	46%
Total	146	60	206		
	Sensitivity:	**Specificity:**			
	64%	73%			
TEST 2: ≥15% Fall in hCG between days 4-7					
	Treatment Success	**Treatment Failure**	**Total**		
Falling hCG	121	15	136	**PPV:**	89% (95% CI 84-94%)
Rising hCG	25	45	70	**NPV:**	64%
Total	146	60	206		
	Sensitivity:	**Specificity:**			
	83%	75%			
TEST 3: >20% Fall in hCG between days 0-4					
	Treatment Success	**Treatment Failure**	**Total**		
Falling hCG	59	4	63	**PPV:**	94% (95% CI 88-100%)
Rising hCG	87	56	143	**NPV:**	39%
Total	146	60	206		
	Sensitivity:	**Specificity:**			
	40%	93%			

Discussion

This study shows that a fall in serum hCG between days 0–4 of treatment represents an 85% likelihood of treatment success with no further intervention, medical or surgical, for single-dose methotrexate treatment of ectopic pregnancy. We analysed the early serum hCG trends of 206 women treated with single-dose methotrexate for their ectopic pregnancies in a range of treatment centres, and these are numbers far greater than previously reported (n=30, n=45 and n=129) [7-9].

A large number of women were excluded from our analysis due to inaccurate timing of (or missed) serum hCG level measurements, in contrast to the requirements of the single-dose methotrexate treatment protocol. Specifically, the analyses performed in this study required serum hCG levels to be available for days 0 or 1, day 4 and day 7 after methotrexate treatment. While this raises the possibility of a selection bias, it is not immediately clear how this may have affected our results. Certainly attendance at such numerous and specific time points for serum sampling requires significant patient (and physician) compliance, but non-compliance is unlikely to have any bearing on treatment outcome, which was the primary outcome assessed in this study. Indeed, the results obtained are consistent with those of previous studies in other populations [7-9]. This study therefore, strengthens the validity of the prognostic value of a falling serum hCG between days 0–4 after single-dose methotrexate treatment for ectopic pregnancy.

Treatment success for the purposes of this study was defined more strictly than in the current protocol and clinical practice, in that it did not allow for any additional doses of methotrexate. This was for the purposes of rigorously testing the prognostic value of a falling serum hCG between days 0–4 for an actual single (rather than a variable) dose methotrexate treatment course. Allowing for additional doses of methotrexate is likely to only improve the positive predictive value of this measure.

The application of a quantified ≥20% fall in serum hCG between days 0–4 of medical treatment improves the positive predictive value from 85% to 94%. This is likely explained by the fact that the greater the fall in serum hCG between days 0–4 of treatment, the more likely the patient is to experience treatment success. There is a clear trade-off, however, between increasing accuracy of prediction with such a cut-off (specificity) and clinical applicability of this measure to a

greater number of women (sensitivity). The sensitivity of a falling serum hCG between days 0–4 fell substantially from 64% to 40% when a ≥20% cut-off was applied, so that the test could only provide meaningful prognostic information to 29% (59/206) of women. In contrast, prognostic information was available for 53% (110/206) of women if *any* fall in serum hCG between days 0–4 was used as the cut-off for a measure of treatment efficacy, with no difference in prognostic accuracy.

It is possible that women with a falling serum hCG between days 0–4 after single-dose methotrexate for ectopic pregnancies may have had already failing pregnancies and did not require treatment. This was not the case, however, as inspection of the pre-treatment serum hCG trends for women with early falling serum hCG levels after methotrexate indicated that the majority (62%) in fact had rising serum hCG levels prior to treatment. Interestingly, regardless of a rising or falling pre-treatment hCG trend, a falling serum hCG level between days 0–4 predicted single-dose methotrexate treatment success with equal measure in this sub-cohort of women (85% and 84%, respectively).

This study examined ectopic pregnancies with pre-treatment serum hCG levels of ≤3000 IU/L, and the results may not apply to medically treated ectopic pregnancies with pre-treatment serum hCG levels >3000 IU/L. Furthermore, given that a fall in serum hCG between days 0–4 is not 100% predictive of treatment success, it is still prudent to continue monitoring hCG levels until normalisation.

Conclusions

We have found that a fall in serum hCG between days 0–4 after single-dose methotrexate treatment of ectopic pregnancy (where the pre-treatment serum hCG is ≤3000 IU/L), predicts treatment success in 85% of cases, with no further intervention required (medical or surgical).

With this measure, prognostic information is obtained three days earlier than from the current, standard clinical measure, and with comparable accuracy. This raises the possibility of altering existing methotrexate protocols, with the aim of increasing medical treatment success rates, by intervening with an earlier repeat dose where early serum hCG levels are still rising; this would, however, require further investigation.

We believe that clinical staff caring for women with ectopic pregnancies, treated with single-dose methotrexate, can confidently use a falling serum hCG between days 0–4 of treatment to provide earlier prognostic information and reassurance to patients with this potentially life-threatening condition.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
ST, AH, CD, MS and PD conceived the study. PD collected the data. ST and MS performed the data analysis and drafted the manuscript. All authors critically reviewed the manuscript and approved the final version.

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PART B: Declaration for Thesis Chapter 4

Monash University

Declaration by Candidate:

In the case of Chapter 4, the nature and extent of my contribution to the work was the following:

Section	Nature of Contribution	Contribution (%)
4.3.2	Conceived the study, performed the experiments, performed the data analysis and drafted the paper.	50%
4.3.4	Conceived the study, performed the experiments, performed the data analysis and drafted the paper.	55%

The following co-authors contributed to the work. If co-authors are students at Monash University, the extent of their contribution in percentage terms must be stated:

Name	Nature of Contribution	Contribution (%)
Jeremy Brown	Collected and prepared the specimens, performed the data analysis.	
Stephen Tong	Conceived and supervised the study, performed the data analysis, drafted the paper and provided critical input.	
Tu'uhevaha Kaitu'u-Lino	Performed the experiments and data analysis.	
Andrew Horne	Supervised the study, drafted the paper and provided critical input.	

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-author's contributions to this work:

Candidate's Signature:  Date: 21/05/2014

Main Supervisor's Signature:  Date: 21/05/2014

Chapter 4: Potential Biomarkers of Ectopic Pregnancy

4.1 Introduction

A serum biomarker is a naturally occurring molecule specific to a pathological or physiological process, whose relative levels in the blood can help diagnose conditions, or predict their occurrence (Soanes and Stevenson, 2005). Biomarkers aid clinicians in diagnosing disease processes before they are clinically apparent, or when they are clinically indistinguishable from related conditions. Human chorionic gonadotrophin (hCG) is one of the first and most specific biomarkers in common clinical use. It is almost always associated with pregnancy, except in very rare cases of germ cell tumours and some bladder cancers (Cole, 2010). When a woman presents in early pregnancy with complications such as abdominal pain and vaginal bleeding, hCG trends can furthermore help to distinguish between a viable intrauterine pregnancy (IUP) and a miscarriage (Kirk et al., 2014). Serum hCG level and trends cannot, however, determine the location of a pregnancy.

The development of transvaginal ultrasound (TVUS) in the latter part of the 20th century has provided clinicians with the ability to non-invasively locate a pregnancy, and evaluate its viability. The importance of this most significantly relates to the ability to diagnose an ectopic pregnancy (Lewis, 2007). A minimum serum hCG level has been established above which an IUP should be visible on TVUS; this so-called 'discriminatory zone' is defined as a serum hCG level of between 1500-2000 IU/L, which equates to a gestation of approximately 5 weeks (Cartwright et al., 2009). Ectopic pregnancies may not develop and grow as would a gestationally-matched IUP, and therefore may not be visible on TVUS even when the serum hCG level is above the discriminatory zone. There is therefore a gap in the ability of clinicians to diagnose or exclude ectopic pregnancy in women with early pregnancy complications and a serum hCG level of <2000 IU/L.

If pregnancy cannot be visualised by TVUS, it is classified as a pregnancy of unknown location (PUL). Between 8-10% of women presenting with early pregnancy complications are given the interim diagnosis of PUL after serum hCG testing and TVUS; of these, 6-20% ultimately prove to have an ectopic pregnancy (Kirk et al., 2014). It is these women who would benefit most from a biomarker of ectopic pregnancy, minimising their risk of complications in the time between first presentation and ultimate diagnosis. Currently women with a PUL are followed up with serial hCG measurement and repeat TVUS after the pregnancy has grown sufficiently to be identified, self-resolves without ever having being visualised or represents with serious clinical complications necessitating surgical management (Kirk et al., 2014).

4.2 The Current State of Play

Research into a potential biomarker of ectopic pregnancy in the pre-genomic era has relied on investigation of molecules known to be associated with various aspects of normal pregnancy development. These molecules can be grouped by their biological function, which include: implantation and trophoblast function, corpus luteal function, endometrial function, Fallopian tube angiogenesis and smooth muscle injury, as well as markers of inflammation and peritoneal irritation (see Table 2.2). None of the molecules investigated to date have demonstrated sufficient discriminatory ability to diagnose ectopic pregnancy (Cartwright et al., 2009, Rausch and Barnhart, 2012). Combinations of pregnancy biomarkers with differing biological function and differential expression in ectopic pregnancy compared to IUP have shown more promise, with improved sensitivity and specificity in diagnosing ectopic pregnancy (Rausch and Barnhart, 2012). Nevertheless, this has not yet been sufficiently validated for clinical use, and there is a continued need for the ability to confidently diagnose (or exclude) ectopic pregnancy in women with early pregnancy complications where TVUS has not been able to locate the pregnancy (PUL).

4.3 Novel Potential Biomarkers of Ectopic Pregnancy

From recent publications in the early pregnancy literature, we identified two novel potential biomarkers of ectopic pregnancy.

4.3.1 Adrenomedullin and the Pathophysiology of Ectopic Pregnancy

A group from The University of Hong Kong have published a series of papers relating to the role of the vasoactive peptide adrenomedullin (ADM) in human reproduction, and in particular, in the pathophysiology of ectopic pregnancy. They have shown plasma ADM levels to be significantly lower in women with ectopic pregnancy compared to women with viable IUPs, and similarly, Fallopian tube explant ADM mRNA levels to be significantly lower compared to those measured in pregnancy-simulated controls; lowered ADM expression appeared to be related to reduced Fallopian tube cilial beat frequency and smooth muscle contractility (Liao et al., 2012). The authors thus conclude that reduced ADM levels may affect embryo-tubal transport in women with ectopic pregnancy.

The identification of a novel molecule implicated in the pathophysiology of ectopic pregnancy prompted us to investigate its potential as a biomarker of the condition. Furthermore, we wanted to determine if there was an association between serum ADM levels, Fallopian tube explant ADM mRNA expression and the two main risk factors of ectopic pregnancy, namely exposure to *C. trachomatis* and cigarette smoke. Such an association would strengthen the hypothesis that ADM is implicated in the pathophysiology of ectopic pregnancy and further characterise a possible causal mechanism.

The study in section 4.3.2 details the first investigation of ADM as a potential biomarker of ectopic pregnancy and the association between serum and tubal ADM levels and exposure to *C. trachomatis* and cigarette smoke. Serum ADM levels were measured in 120 samples from women with early pregnancy complications whose ultimate pregnancy outcomes were prospectively

documented; Fallopian tube explants were collected at the time of surgery for either salpingectomy (ectopic pregnancy) or other surgery for treatment of benign gynaecological conditions (non-pregnant controls). The findings were published as a Letter to the Editor in *The Journal of Clinical Endocrinology and Metabolism*, commenting on the differences in findings between this work and the earlier published findings relating to the possible role of ADM in the pathophysiology of ectopic pregnancy (Liao et al., 2012).

4.3.2 Adrenomedullin expression is not significantly altered in ectopic pregnancy

Dear Editors of *The Journal of Clinical Endocrinology and Metabolism*,

Adrenomedullin is a vasoactive peptide hormone increasingly being shown to play a role in human reproduction. Recently, Liao and colleagues proposed that adrenomedullin expression in the oviducts of women with tubal ectopic pregnancies (tEPs) is decreased relative to that of controls, and that this correlates with decreased ciliary beat frequency, lower oviductal smooth muscle tone and contraction frequency, thus contributing to the pathogenesis of tEP (Liao et al., 2012). They also demonstrated that median (interquartile) plasma adrenomedullin levels in women with tEP (n=14) were 79.2 (72.1-94.5 pg/ml), significantly lower than in women with normal pregnancies (123.2 (111.5-163.1 pg/ml) n=14; P <0.001). Collectively, this work raises the exciting possibility that adrenomedullin may play a significant pathophysiological role in tEPs and that circulating adrenomedullin may be a biomarker of ectopic pregnancy. However, recent work by our group somewhat contradicts these findings.

We first investigated the potential of adrenomedullin as a biomarker for diagnosing tEP. We measured serum adrenomedullin levels (Adrenomedullin (Human) – Fluorescent EIA Kit, Phoenix Pharmaceuticals Inc., Burlingame, USA) in samples from 120 women presenting with symptoms of abdominal pain and/or vaginal bleeding in early pregnancy, with a pregnancy of unknown location

(PUL) after transvaginal ultrasonography (previously described) (Horne et al., 2012). Results were classified by final pregnancy outcome according to a recent PUL consensus statement (Barnhart et al., 2011). We similarly found that adrenomedullin levels were significantly reduced in non-pregnant women (n=11) compared to women with a viable intrauterine pregnancy (VIUP; n=28; $P<0.05$). However, there was no significant difference in the median serum adrenomedullin expression between women with a definite ectopic pregnancy (dEP) (n=17) compared to women with a VIUP (n=28; median adrenomedullin levels 0.97 vs. 1.26 ng/mL, $P=0.69$). Serum adrenomedullin as a single biomarker was not able to distinguish between dEP and other pregnancy outcomes (AUC 0.53; P=0.68). Hence we were unable to confirm that circulating adrenomedullin is a useful biomarker, although it is possible that the use of serum versus plasma may account for the contradictory findings.

We also performed q-RT-PCR analysis of adrenomedullin expression in oviducts from non-pregnant women (n=41) compared to women with tEPs (n=18). We found no difference in oviductal adrenomedullin expression across the menstrual cycle and critically, we failed to find a difference between tEP and non-pregnant controls (one-way analysis of variance, $P=0.11$). This again contrasts with the findings of Liao et al (2012).

Finally, we investigated the effect that past exposure to genital *Chlamydia trachomatis* infection (through measurement of PgP3 antibody titres (Shaw et al., 2011)) and cigarette smoke exposure (CSE; through measurement of serum cotinine levels) had on oviductal adrenomedullin expression, given these are important risk factors in the pathogenesis of tEP. Relative adrenomedullin expression was not affected by exposure to either (*C. trachomatis* and CSE negative controls [n=18], *C. trachomatis* positive and CSE negative [n=8]; and CSE positive and *C. trachomatis* negative (n=13); One-way analysis of variance, $P=0.20$).

Thus, whilst it appears that adrenomedullin regulates ciliary beating in the oviduct (Chiu et al., 2010), we were unable to confirm any change in adrenomedullin expression in association with tEP or its main risk factors.

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4.3.3 Macrophage Inhibitory Cytokine-1 as a Biomarker of Ectopic Pregnancy

Serum macrophage inhibitory cytokine-1 (MIC-1) has recently been shown to be significantly reduced in women who go on to miscarry, before they experience any early pregnancy complications and whilst the pregnancy is still demonstrably viable on TVUS (Tong et al., 2012). The role of MIC-1 in the establishment and maintenance of normal pregnancy is unconfirmed, however, it is thought to contribute to maternal immunotolerance of pregnancy by suppressing the pro-inflammatory cytokine response within the uterus (Moore et al., 2000). Because of its powerful predictive ability in identifying early pregnancy failure, we hypothesised that MIC-1 could be differentially expressed by ectopic pregnancies and that this could make it a useful clinical biomarker of ectopic pregnancy.

In section 4.3.4 I present the findings of our research and analysis regarding the potential of MIC-1 to serve as a biomarker of ectopic pregnancy. This is the first known study to investigate the clinical aptitude of MIC-1 to discriminate between ectopic pregnancy and other pregnancy outcomes. The manuscript, published in *PLoS One* (Skubisz et al., 2013), shows that MIC-1 was able to exclude ectopic pregnancy above a certain threshold in our cohort, and that it may serve as a useful adjunct in clinical management algorithms for PUL; more importantly, MIC-1 could form part of a multiple biomarker test for ectopic pregnancy.

4.3.4 Manuscript

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Maternal Serum Macrophage Inhibitory Cytokine-1 as a Biomarker for Ectopic Pregnancy in Women with a Pregnancy of Unknown Location

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Abstract

Background: Ectopic pregnancy (EP) occurs in 1–2% of pregnancies, but is over-represented as a leading cause of maternal death in early pregnancy. It remains a challenge to diagnose early and accurately. Women often present in early pregnancy with a 'pregnancy of unknown location' (PUL) and the diagnosis and exclusion of EP is difficult due to a lack of reliable biomarkers. A serum biomarker able to clearly distinguish between EP and other pregnancy outcomes would greatly assist clinicians in diagnosing and safely managing PULs. This study evaluates the ability of maternal serum macrophage inhibitory cytokine-1 (MIC-1) levels to differentiate between EP and other pregnancy outcomes in women with a PUL.

Methods: Sera were collected from 120 women with a PUL at first clinical presentation and assayed for MIC-1 by ELISA. Results were classified according to ultimate pregnancy outcome and the discriminatory ability of MIC-1 to diagnose EP was assessed.

Results: Serum MIC-1 levels were lower in women with histologically confirmed (definite) EP (dEP) (median 552 ng/mL; interquartile range (IQR) 414–693 ng/mL) compared to women with definite viable intra-uterine pregnancies (dVIUPs) (722 ng/mL; IQR 412–1122 ng/mL), and higher when compared to women with definite non-viable intra-uterine pregnancies (dNVIUPs) (465 ng/mL; IQR 341–675 ng/mL). MIC-1 levels were significantly higher in women with dEP compared to women whose PULs resolved without medical intervention (srPUL) (401 ng/mL; IQR 315–475 ng/mL) ($p<0.003$). There were no women with an ectopic pregnancy where serum MIC-1>1000 ng/mL.

Conclusion: Serum MIC-1 levels in PUL were not able to categorically diagnose EP, however, MIC-1 could distinguish women with an EP that required medical intervention and those women whose PULs spontaneously resolved. A single serum MIC-1 measurement also excluded EP at levels above 1000 ng/mL. MIC-1 may play a role in the development of a combined assay of biomarkers for the diagnosis of EP.

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Introduction

Ectopic pregnancy (EP) occurs in 1–2% of pregnancies and remains a leading cause of maternal death in early pregnancy [1,2]. The most common symptoms of EP are abdominal pain and/or vaginal bleeding, however, these are not specific to EP and can be consistent with a range of pregnancy outcomes, including on-going (definite) viable intrauterine pregnancies (dVIUPs) and miscarriages (definite non-viable intrauterine pregnancies – dNVIUPs). The challenge for the clinician is to be able to accurately distinguish between pregnancy outcomes in order to safely manage any woman presenting with these symptoms.

The diagnosis of EP relies on transvaginal ultrasonography (TVUS), and three quarters of EPs will be diagnosed at the initial scan (sensitivity of 73.9% and a specificity of 98.3%) [3]. When neither an intrauterine nor an EP can be visualized by TVUS, the woman is classified as having a pregnancy of unknown location (PUL) [4]. This requires further time and resource intensive follow-up, including serial serum human chorionic gonadotrophin (hCG) levels and repeat TVUS to determine pregnancy location and appropriate management [5,6]. Between 7–20% of women with PUL are ultimately diagnosed with EP [7].

Recent research endeavour has focused on the potential for a serum biomarker to be able to accurately diagnose EP at the time of first presentation for women with PUL. A number of candidate molecules have been proposed, but as single markers, none are sufficiently discriminatory for diagnosing EP [8]. Some groups have attempted combining serum biomarkers in tests with

improved diagnostic accuracy, ranging from 52.9–98% sensitivity and 92.4–100% specificity, however, these still require further validation before potentially being used in a diagnostic algorithm for EP [8].

Serum macrophage inhibitory cytokine-1 (MIC-1) is a divergent member of the TGF-ß superfamily of cytokines, originally shown to inhibit macrophages [9]. MIC-1 has been localized to the syncytiotrophoblast and shown to increase in serum across the first trimester of pregnancy [10], and is thought to increase the population of tolerogenic dendritic cells in the decidua [11]. We previously identified maternal serum concentrations of MIC-1 as a potential predictive biomarker of miscarriage [12]. We then validated this in a subsequent prospective cohort study of 782 women, which showed serum MIC-1 in early pregnancy to be significantly lower in women who went on to miscarry [multiples of the median (MOM) 0.63 (IQR 0.33–0.88) compared to women with successful pregnancy outcomes (MOM 1.00 (IQR 0.76–1.29) ($p = <0.001$), with an area under the curve (AUC) of 0.73 (95% CI 0.63–0.84) as a single biomarker predictor of miscarriage [13]].

In this prospective cohort study, we assessed the ability of serum MIC-1 levels to diagnose EP amongst women with PULs who presented with abdominal pain and/or vaginal bleeding in early pregnancy.

Results

We recruited 120 Caucasian women aged between 18–45 years and diagnosed with a PUL after presentation with abdominal pain and/or vaginal bleeding in early pregnancy. The outcomes of these pregnancies were classified according to the recent PUL consensus statement [4]. Table 1 details the definition and final breakdown of pregnancy outcomes for the cohort, with no differences demonstrated in their baseline characteristics (one-way ANOVA).

Serum MIC-1 levels were lower in women with histologically confirmed (definite) EP (dEP) (median 552 ng/mL; IQR 414–693 ng/mL) compared to women with definite viable intrauterine pregnancies (dVIUP) (722 ng/mL; IQR 412–1122 ng/mL), and higher compared to women with definite non-viable intrauterine pregnancies (dNVIUP) (465 ng/mL; IQR 341–675 ng/mL) and treated PULs (tPUL) (400 ng/mL; IQR 388–473 ng/mL). Levels were also higher in women with dEP than in women who were medically managed for probable EPs (pEP) (434 ng/mL; IQR 315–541 ng/mL). Additionally, MIC-1 levels were significantly higher in women with dEP compared to women with a PUL that resolved spontaneously (i.e. without medical intervention) (srPUL) (401 ng/mL; IQR 315–475 ng/mL) ($p<0.003$). Non-pregnant (NP) women were included as a control and their serum MIC-1 levels were significantly lower than in women with dEP (330 ng/mL; IQR 298–463 ng/mL) ($p = 0.001$) (Figure 1). Interestingly, a correlation was observed between serum MIC-1 and serum hCG levels in women with dVIUPs and dNVIUPs (Spearman; $P<0.04$ and $P<0.03$, respectively), but not in any other pregnancy outcome categories ($P>0.35$).

ROC curve analysis suggests MIC-1 would have limited use in the diagnosis of EP as a single biomarker ($AUC = 0.5547$; $p>0.4$) (Figure 2a). Removal of ambiguous PUL outcomes (srPUL, tPUL and pEP) did not improve the performance of MIC-1 as a biomarker of EP ($AUC = 0.5335$; $p>0.6$) (Figure 2b), nor did grouping pregnancy outcomes by those that required treatment (dEP, pEP and tPUL) compared to those that did not (dNVIUP/dVIUP/NP/srPUL) ($AUC = 0.5363$; $p>0.5$) (Figure 2c). A single serum MIC-1 measurement, however, excluded EP at levels above 1000 ng/mL (Figure 1) (positive predictive value (PPV) 100%,

Table 1. Participant categorisation and baseline characteristics according to final pregnancy outcome (median ± SEM).

Group	Indusion criteria	hCG (mU/mL)	PgP3 ELISA absorbance (450 nm)	Age (years)	Weight (kg)	BMI	n
dVIUP	Definite viable intrauterine pregnancy: TVUSS confirmation of intrauterine gestational sac with yolk sac and embryo with cardiac activity.	4022±1904	0.53±0.18	32±1	74±3	27±1	17
dNVIUP	Definite nonviable intrauterine pregnancy: USS confirmation of intrauterine gestational sac with yolk sac and/or embryo without cardiac activity seen prior to uterine evacuation.	6844±2017	0.34±0.15	28±1	70±4	26±2	8
dEP	Definite ectopic pregnancy: intervention prompted by adnexal mass on TVUSS or by abnormal rise in serum hCG levels and confirmed at surgery and by histopathology.	1151±238	0.92±0.34	29±1	70±4	25±1	28
NP	Not pregnant: positive home pregnancy test result subsequently not confirmed by serum hCG measurement.	<5	0.72±0.42	26±2	70±8	27±3	26
srPUL	Spontaneously resolving PUL: PUL with spontaneous resolution of serum hCG levels.	428±114	0.70±0.17	32±1	74±4	28±1	27
tPUL	Treated persistent PUL: abnormal rise in serum hCG levels but no adnexal mass or IU sac seen on TVUSS after monitoring, managed medically with methotrexate.	400±188	0.73±0.73	32±4	83±15	28±5	3
pEP	Probable ectopic pregnancy: heterogenous adnexal mass or extra-uterine sac-like structure on TVUSS managed medically with methotrexate.	597±200	0.43±0.34	33±1	63±4	25±1	11

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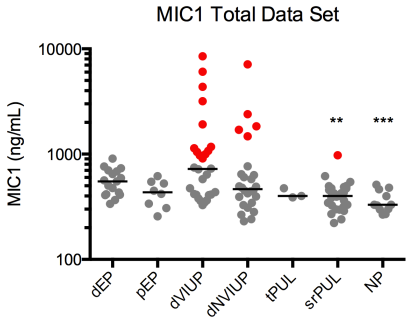

Figure 1. Serum MIC-1 levels in women at first presentation with a PUL, categorised according to final pregnancy outcome. Serum MIC-1 levels >1000 ng/mL exclude EP.
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negative predictive value (NPV) 24%, sensitivity 17% and specificity 100%; $P<0.05$).

Discussion

In this study, we demonstrated that serum MIC-1 measurement has some potential as a diagnostic biomarker of EP in women with a PUL. We prospectively recruited 120 women with an interim diagnosis of PUL, who presented with symptoms of abdominal pain and/or vaginal bleeding after a positive home pregnancy test. Importantly, serum samples were collected at the time of first presentation and not at the time of diagnosis, as would reflect true clinical practice. We followed these women until complete resolution of their pregnancies and classified their outcomes according to the PUL consensus statement [4]. Whilst the number of women in each pregnancy outcome category was relatively small, the full range of PUL outcomes were represented in the cohort. We additionally verified that previous exposure to C. *trachomatis* did not influence MIC-1 levels in the sera of participants. Hence this study robustly tests the potential of serum MIC-1 levels as a biomarker of EP.

Although this study showed that a single serum MIC-1 level is not sufficiently discriminatory to *diagnose* EP, serum MIC-1 level measurement may help to *exclude* EP. No woman participating in this study had an EP where her serum MIC-1 level >1000 ng/mL. Hence, clinicians could use a serum MIC-1 cut-off, above which a woman is very unlikely to have an EP, to help determine which women with PULs can be safely followed up as outpatients. Furthermore, serum MIC-1 levels were significantly higher in women with dEPs compared to women with srPULs. Therefore clinicians could potentially use serum MIC-1 levels in PUL to help determine which women are likely to require treatment. Both these approaches, however, would require further validation before being used in a clinical management algorithm for PUL.

The inability of any single biomarker being able to sensitively and specifically diagnose EP has led some groups to combine serum biomarkers in an attempt to improve diagnostic accuracy. In particular, Rausch notes that biomarkers with varied biological actions seem to perform best together [8]. This reflects an attempt to identify numerous deviations in physiological processes common to both intrauterine and ectopic pregnancy, the sum of which may allow a combined biomarker panel to predict location. To date, the best combination of biomarkers (progesterone, vascular endothelial growth factor (VEGF), inhibin A and activin A) has

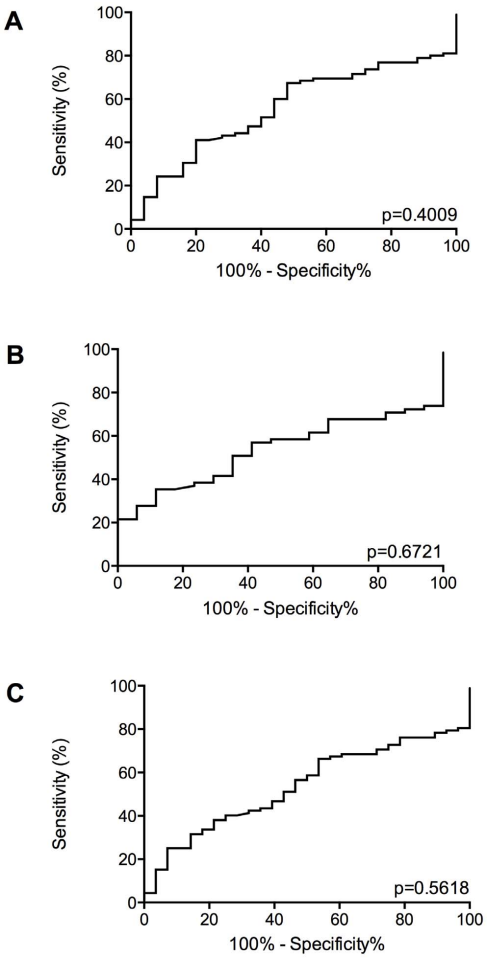

Figure 2. ROC Curve analyses of MIC-1 as a biomarker of EP. A) Comparing MIC-1 levels in women with EP (dEP and pEP) compared to all other pregnancy outcomes. B) Comparing MIC-1 levels in women with dEP compared to women with a definite non-EP outcome (dVIUP, dNVIUP, NP), excluding women with ambiguous pregnancy outcomes (srPUL, tPUL and pEP). C) Comparing MIC-1 levels in women requiring treatment (dEP, pEP and tPUL) compared to women who did not (dVIUP, dNVIUP, srPUL and NP).
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achieved a sensitivity of 98% and a specificity of 100%, but only in 42% of the cohort that was able to be characterised [14]. This panel of biomarkers still requires external validation in a prospective cohort to determine its clinical viability.

MIC-1, Placental Growth Factor (PlGF) and fms-related tyrosine kinase 1 (flt1) levels are all lower in EP compared to dVIUP and higher compared to dNVIUP, [15,16] however, their reported test performance as single biomarkers for the diagnosis of EP are difficult to compare due to different cut-offs and outcome

groupings used in their assessment [8]. What is apparent though, is that MIC-1, PlGF and flt1 all correlate with pregnancy viability. VEGF in comparison, is elevated in EP compared to dVIUP [14,17], and in terms of biomarker performance, can distinguish between EP and dVIUP and EP and dNVIUP with 100% specificity at cut-offs of 200 pg/mL and 174.5 pg/mL, respectively [18,19]. As MIC-1>1000 ng/mL excludes EP and VEGF >200 pg/mL diagnoses EP, both with 100% specificity, the use of these two biomarkers in a diagnostic algorithm may prove clinically useful, provided their physiological relationship is not simply diametrically opposed. This would require further investigation.

Previous studies in early pregnancy show a correlation between low maternal serum MIC-1 levels and non-viable pregnancies [12,13]. In this study, serum MIC-1 levels were lower in women with dEP compared to women with dVIUP, consistent with the likely insufficient trophoblastic invasion of an EP and its limited viability within the Fallopian tube environment. In contrast, serum MIC-1 levels were significantly higher in women with dEP compared to women with a srPUL, suggesting that serum MIC-1 levels can discriminate between a pregnancy that continues to grow and requires treatment compared to one that is demising.

Furthermore, a correlation was noted between serum MIC-1 and serum hCG levels in women with dVIUPs and dNVIUPS, but not in any other pregnancy outcome categories. MIC-1 and hCG were both lower in women with dNVIUPs compared to women with dVIUPs ($P = 0.1$ and $P < 0.02$, respectively). This suggests MIC-1 may be up-regulated in viable pregnancy, to help mediate an appropriate immune response within the uterine environment. Hence serum MIC-1 levels in this study continue to correlate with relative pregnancy viability.

In conclusion, as a single biomarker, a serum MIC-1 level at the time of TVUS diagnosis of a PUL is not sufficiently discriminatory to diagnose EP. Serum MIC-1 levels do, however, correlate with pregnancy viability and may be used as a cut-off for exclusion of EP and for determination of which women with a PUL will require treatment. Serum MIC-1 levels may therefore be useful as part of a management algorithm for PUL and could likely form part of a multiplex biomarker assay, to assist clinicians in diagnosing EP.

Materials and Methods

Patient Samples

This study was approved by the Lothian Research Ethics Committee (LREC 04/S1103/20 and 09/S1103/39). Written, informed consent was obtained from all participants. Women presenting with abdominal pain and/or vaginal bleeding after a positive home pregnancy test and in whom a TVUS was not able to locate the pregnancy (i.e. PUL) were recruited. Whole blood was collected from participants and after clotting for 2 hrs at room temperature, the sera were collected and stored and −80°C in multiple aliquots. Participants were followed up and their ultimate pregnancy outcomes classified according to the recent PUL consensus statement [4].

Ultrasound Assessments

TVUSs were performed using the Toshiba Aplio XG machine by a team of trained, qualified and experienced sonographers.

MIC-1 ELISA

Sera were assayed using the GDF-15 (MIC-1) Duoset ELISA kit (R&D systems, Abingdon, UK) according to the manufacturer's instructions. Further details and performance parameters are available from the manufacturer (www.rndsystems.com/pdf/DY957). The ELISA was performed in one run, with samples scrambled throughout the plate. The scientist performing the ELISA was blinded to clinical groupings of samples.

Because current or past infection with Chlamydia *trachomatis* is strongly correlated with risk of EP, and MIC-1 is a cytokine whose expression may be influenced by other inflammatory and/or infective processes in the body, we also measured participant's serum PgP3 antibody titres as a marker of previous C. *trachomatis* exposure by ELISA, as previously described [20].

Statistical Analysis

Statistical analysis and ROC curves were generated using Prism 5.0 (GraphPad Software, La Jolla, USA).

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Author Contributions

Conceived and designed the experiments: AWH ST TK MS. Performed the experiments: TK MS. Analyzed the data: MS JKB. Contributed reagents/materials/analysis tools: AWH ST. Wrote the paper: MS AWH ST TK JKB.

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PART B: Declaration for Thesis Chapter 5

Monash University

Declaration by Candidate:

In the case of Chapter 5, the nature and extent of my contribution to the work was the following:

Section	Nature of Contribution	Contribution (%)
5.2	Conceived and designed the study, obtained ethics approval, recruited and treated participants, performed the data analysis and drafted the paper.	55%

The following co-authors contributed to the work. If co-authors are students at Monash University, the extent of their contribution in percentage terms must be stated:

Name	Nature of Contribution	Contribution (%)
Andrew Horne	Helped design and supervise the study, obtained ethics approval, recruited and treated participants, drafted the paper and provided critical input.	
Terrance Johns	Supervised the study and provided critical input.	
Ulrika Nilsson	Provided critical input.	
Colin Duncan	Supervised the study, recruited and treated participants and provided critical input.	
Euan Wallace	Supervised the study, recruited and treated participants and provided critical input.	
Hilary Critchley	Provided critical input.	
Stephen Tong	Conceived, designed and supervised the study, obtained ethics approval, recruited and treated participants, drafted the paper and provided critical input.	

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-author's contributions to this work:

Candidate's Signature:  Date: 21/05/2014

Main Supervisor's Signature:  Date: 21/05/2014

Chapter 5: Combination Gefitinib and Methotrexate to Treat Ectopic Pregnancy: A Phase I Clinical Study

5.1 Introduction

Although ectopic pregnancies only account for 1-2% of pregnancies, they are significantly over-represented as a cause for maternal death in early pregnancy (Farquhar, 2005). Medical advances of the 20th century have seen the mortality rate for this condition plummet and the therapeutic intent shift to preservation of fertility; nevertheless, surgical excision and the associated reduction or loss of fertility potential remain the only treatment option for some 60% of women diagnosed with ectopic pregnancy (Jurkovic and Wilkinson, 2011). This is because the only medical treatment option – systemic methotrexate – has limited efficacy, recommended only for women with ectopic pregnancies with pre-treatment serum human chorionic gonadotrophin (hCG) levels of <5000 IU/L, who are haemodynamically stable and who demonstrate no embryonic cardiac motion on transvaginal ultrasonography (TVUS) (Farquhar, 2005, 2008). Furthermore, The Royal College of Obstetricians and Gynaecologists' guidelines recommend a serum hCG cut-off of <3000 IU/L for medical treatment of ectopic pregnancy with single-dose methotrexate, citing cost-effectiveness studies that show at serum hCG levels above 1500 IU/L, the laparoscopic salpingectomy is more cost effective (RCOG, 2010).

Given that only approximately 5-20% of women with ectopic pregnancy present as acutely unstable (Pisarska et al., 1998, Fritz, 2011), many more could be treated non-invasively if a more effective medical intervention was available. The introduction of small molecule inhibitors of the epidermal growth factor receptor (EGFR) into cancer treatment parlance has created the possibility of a novel therapeutic approach to disorders of trophoblastic tissue, where human EGFR expression is highest

(Cohen et al., 2003, Su et al., 2004). Indeed, the placenta has been shown to be highly dependent on EGFR signalling for proper development, the ability abrogate toxic and hypoxic insults and to achieve successful pregnancy outcomes (Johnstone et al., 2005, Dackor et al., 2009, Wolff et al., 2007). We therefore hypothesised that EGFR inhibition could transform the treatment paradigm of ectopic pregnancy from majority surgical to a predominantly medical approach.

Pre-clinical research showed EGFR inhibition through gefitinib treatment to have a mainly cytostatic effect on trophoblastic cell lines and primary tissues; however, my colleagues in the Translational Obstetrics Group convincingly demonstrated that combining gefitinib with relatively low doses of methotrexate produced a significantly supra-additive treatment effect in causing cell death when compared to methotrexate-only treatment. Similarly, primary trophoblastic xenografts in severe combined immunodeficient (SCID) mice were more significantly regressed with combination gefitinib and methotrexate than when treated with methotrexate alone (Nilsson et al., 2013). This data provided the foundation for applying a combination gefitinib and methotrexate treatment approach to the women with ectopic pregnancy.

The main aim of my PhD was to begin to translate the theory and pre-clinical experience of combination gefitinib and methotrexate treatment into clinical practice, through early phase clinical trials. Because combination gefitinib and methotrexate treatment in humans has not been previously reported, we designed a small, phase I single-arm open-label study recruiting 12 women with stable ectopic pregnancies who would normally be offered single-dose methotrexate treatment according to current protocols (Appendix B) (RCOG, 2010). These women received gradually increasing treatment duration with oral gefitinib in addition to single-dose methotrexate, and were strictly monitored. The primary outcome assessed was safety and toxicity of the combination in women of reproductive age, and the secondary outcome, treatment efficacy compared to a historic cohort of women treated with single-dose methotrexate only.

We achieved our recruitment target and maximum intended treatment dose, with no undue toxicity observed from combination treatment. Importantly, combination gefitinib and methotrexate appeared to resolve ectopic pregnancies more effectively than single-dose methotrexate when comparing rates of serum hCG decline and time (in days) to resolution. These results were published in *Obstetrics & Gynecology* (see section 5.2).

5.2 Manuscript

Combination Gefitinib and Methotrexate Compared With Methotrexate Alone to Treat Ectopic Pregnancy

Monika M. Skubisz, MBBS, Andrew W. Horne, PhD, Terrance G. Johns, PhD, Ulrika W. Nilsson, PhD, W. Colin Duncan, MD, Euan M. Wallace, MD, Hilary O. D. Critchley, MD, and Stephen Tong, PhD

OBJECTIVE: To determine the safety, tolerability, and efficacy of combination gefitinib and methotrexate to treat ectopic pregnancy.

METHODS: We performed a phase I, single-arm (non-randomized), open-label study. Twelve women with ectopic pregnancies were administered methotrexate (50 mg/m^2, intramuscular) and 250 mg oral gefitinib in a dose-escalation protocol: one dose (day 1) n=3; three doses (days 1–3) n=3; seven doses (days 1–7) n=6. Efficacy was examined by comparing human chorionic gonadotrophin (hCG) decline and time to resolution with historic controls administered methotrexate only.

See related editorial on page 733 and article on page 737.

From the Translational Obstetrics Group, Department of Obstetrics and Gynaecology, University of Melbourne, Mercy Hospital for Women, Heidelberg, and the Ritchie Centre and the Centre for Cancer Research, Monash Institute of Medical Research, Monash University, Clayton, Victoria, Australia; and the MRC Centre for Reproductive Health, the Queen's Medical Research Institute, Edinburgh, United Kingdom.

Supported by National Health and Medical Research Council of Australia (NHMRC) Project Grants #606611 (S.T. and T.G.J.) and #1008276 (S.T., E.M.W., and T.G.J.); NHMRC Career Development Fellowship #1050765 (S.T.); The Monash Institute of Medical Research Flagship Grant (S.T., T.G.J., E.M.W.); Medical Research Council Clinician Science Fellowship (A.W.H.); Medical Research Council Centenary Award #G0802808 (A.W.H.); The Helen MacPherson Trust (S.T.); and The Victorian Government's Operational Infrastructure Support Program (M.S., T.G.J., and E.M.W.).

The authors thank Ms. Ann Doust for recruiting participants in Edinburgh and Professors Susan Walker and Peter Rogers for input into trial management.

Presented at The Society for Gynecological Investigation annual meeting, March 22–24, 2012, San Diego, California.

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Financial Disclosure
Drs. Johns, Nilsson, and Tong are joint holders of patents that relate to the use of Epidermal Growth Factor Receptor inhibition in treating ectopic pregnancies. The other authors did not report any potential conflicts of interest.

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RESULTS: Common side effects were transient acneiform rash in 67% (8/12) and diarrhea in 42% (5/12) of participants. There was no clinical or biochemical evidence of serious pulmonary, renal, hepatic, or hematologic toxicity. Of six participants with a pretreatment serum hCG level between 1,000 and 3,000 international units/L, hCG levels declined significantly faster than in the control group. Median serum hCG levels by day 7 after treatment were less than one fifth of levels observed among 71 historic controls treated with methotrexate alone (median [interquartile range] hCG in participants 261 [55–1,445] international units/L compared with controls 1,426 [940–2,573]; *P*=.008). Median time for the ectopic pregnancies to resolve with combination therapy was 34% shorter compared with methotrexate alone (21 days compared with 32 days; *P*=.018).

CONCLUSION: Combination gefitinib and methotrexate has potential as a treatment for ectopic pregnancy but is commonly associated with minor side effects such as transient rash and diarrhea. The treatment requires validation of safety and efficacy in a larger trial.

CLINICAL TRIAL REGISTRATION: Australian New Zealand Clinical Trials Registry, www.anzctr.org, AC'TRN12610000684022.

(Obstet Gynecol 2013;122:745–51)
DOI: 10.1097/AOG.0b013e3182a14cfb

LEVEL OF EVIDENCE: II

Ectopic pregnancies represent 1–2% of all pregnancies (100,000 per year in the United States[1] and 12,000 per year in the United Kingdom[2]) and are a leading cause of maternal morbidity and mortality in the first trimester, responsible for 3–8% of all pregnancy-related deaths.[2,3] Ectopic pregnancies can be treated surgically or medically by administering intramuscular methotrexate. A systematic meta-analysis concluded the cost-effectiveness of medical treatment with methotrexate drops significantly with higher pretreatment

human chorionic gonadotrophin (hCG) levels (greater than 1,500 international units/L) and that laparoscopic excision remains the most effective treatment for ectopic pregnancy.[3] Hence, many are treated surgically[4] and there exists a need for more effective medical therapies to reduce operative intervention (and its inherent risks) in women diagnosed with ectopic pregnancy.

Human placenta expresses very high levels of the epidermal growth factor (EGF) receptor, a cellular signaling pathway that activates a potent cell survival response.[5] The placenta has, by far, the highest expression of EGF receptor compared with all nonmalignant tissues in the body. A bioinformatics search on BioGPS suggests EGF receptor expression in human placenta is more than 30 times higher than average tissue expression,[6,7] and there is good evidence that the placenta heavily relies on EGF receptor signaling for successful pregnancy outcomes.[8–11] Therefore, EGF receptor inhibition may be a molecularly targeted approach to treat ectopic pregnancy.

Gefitinib is a small-molecule tyrosine kinase inhibitor that blocks EGF receptor signaling and is approved to treat non–small-cell lung cancer, in which 250 mg is taken daily on a continuing basis. It has good oral bioavailability and often causes transient diarrhea and skin rash.[12,13] In preclinical studies,[14] we have found gefitinib supra-additively augments methotrexate-induced regression of placental tissue. Given the encouraging results obtained from the preclinical studies,[14] we translated our finding to humans, conducting a phase I trial to assess the safety, tolerability, and efficacy of combination gefitinib (once-daily 250 mg oral gefitinib taken up to 7 days) and intramuscular methotrexate to treat ectopic pregnancy.

MATERIALS AND METHODS

We performed a phase I single-arm (nonrandomized) open-label dose-escalation study, recruiting 12 women with an ultrasonographic diagnosis of a tubal ectopic pregnancy (trial registration: Australian New Zealand Clinical Trials Registry, ACTRN12610000684022). Women were recruited between October 2010 and October 2011 at two academic teaching hospitals: Monash Medical Centre (Melbourne, Australia) and The Royal Infirmary of Edinburgh (Edinburgh, United Kingdom). This was an investigator-initiated study with no funding from pharmaceutical companies. All drugs were purchased from the hospital pharmacy and none was provided from the manufacturer. Participants were not paid for their participation.

We recruited participants for the case group who were eligible for single-agent methotrexate treatment according to our local institutional protocols: hemodynamically stable, serum hCG less than 3,000 international units/L, minimal free fluid in the pelvis, gestational sac size of less than 4 cm and no fetal cardiac activity on ultrasonogram, and normal baseline (day 1) liver, renal, and hematologic indices. The upper cutoff of less than 3,000 international units/L was chosen because it is the current cutoff for eligibility for medical management at both enrolling institutions. We could not ethically propose a higher cutoff for this phase I study. We did not recruit women in whom there was a diagnosis or suspicion of a ruptured ectopic pregnancy.

The clinical definition of an ectopic pregnancy for this trial was the presence of a positive serum hCG and ultrasonographic findings of 1) a definite tubal ectopic pregnancy (extrauterine gestational sac with yolk sac, embryo, or both with or without cardiac activity and an empty uterus); or 2) a highly probable tubal ectopic pregnancy, defined as an inhomogeneous adnexal mass or extrauterine sac-like structure and an empty uterus.

Given the side effect profile of gefitinib,[12] we excluded women with a history of significant respiratory, gastrointestinal, or dermatologic illnesses. We also excluded women of Japanese ethnicity because the incidence of interstitial lung disease has been reported to be higher among Japanese people. We initially excluded smokers when we started the study, but amended our protocol study to include them (amendment approved by our ethics committees). Written informed consent was obtained from all participants and human research ethics approvals were obtained at both sites before recruitment started (Monash Health Human Research Ethics Committee B, Monash Medical Centre, Melbourne, Australia, project number 10142B; and Scotland A Research Ethics Committee, Edinburgh, Scotland, project number 11/MRE00/2).

We administered 50 mg/m^2 intramuscular methotrexate on day 1 and daily oral 250 mg gefitinib using a dose-escalation protocol. The first three participants received 250 mg gefitinib on day 1, the next three received 250 mg per day of gefitinib for 3 days, and the last six participants received 250 mg per day of gefitinib for 7 days.

Our primary outcome was safety and tolerability. Women were assessed clinically (history and examination) and biochemically (full blood count [hemoglobin, white cell count, and platelet count], renal function test [serum electrolytes, urea, and creatinine], and liver enzymes) on days 4, 7, and 11, then weekly until the ectopic pregnancy resolved (hCG$<$5 international units/L). All adverse events were documented and classified according to the Common Terminology Criteria for Adverse Events version 4.03 (National Cancer Institute, National Institutes of Health, U.S. Department of

Health and Human Services, June 14, 2010). Participants were contacted at 3 months and 6 months posttreatment to document the return of menstrual cycles and any subsequent pregnancies. We offered screening for chlamydia and gonorrhea by polymerase chain reaction of the urine sample at the time of recruitment.

To monitor treatment response, we followed protocols commonly used for single-dose methotrexate. Serum hCG levels were measured on days 4, 7, and 11 then weekly until serum hCG levels resolved (<5 international units/L). No further treatment was administered if serum hCG fell 15% or more between days 4 and 7 of treatment and continued to decline thereafter. If the serum hCG did not fall 15% or more between days 4 and 7, we administered a second dose of methotrexate. If ectopic pregnancy ruptured or uncontrolled internal bleeding was suspected, we offered prompt surgical management.

We performed a planned subgroup analysis in which pretreatment hCG levels were between 1,000 and 3,000 international units/L, comparing participants successfully treated and women treated at our institutions with single-agent methotrexate (historic controls). These historic controls were women presenting to our institutions over the past 5 years and treated with methotrexate in which there was strict adherence to the treatment protocol, including a record of all serum hCGs until resolution of the ectopic pregnancy (including a record of serum hCG levels at days 1, 4, and 7). We compared pretreatment serum hCG levels and at days 4 and 7 and time to resolution (in days) between trial participants and historic controls.

The treatment protocol used for the historic controls (including inclusion criteria, dose of methotrexate, and hCG monitoring) was the same as that used for trial participants, except no gefitinib was administered and biochemical tests of organ function (full blood count, renal function tests, and liver enzymes) were performed only on days 1 and 7.

We used descriptive statistics to describe our data. The Mann-Whitney *U* test was used to compare serum hCG levels between participants and historic controls at days 1, 4, and 7 and time taken for the ectopic pregnancies to resolve. We used parametric tests or Fisher's exact test to compare baseline clinical characteristics between participants and those in the control group. Statistical analysis was performed using GraphPad Prism Version 5 (La Jolla, CA).

RESULTS

One hundred eighty-four women were diagnosed with ectopic pregnancies at the two recruiting institutions during the period of this study. Fifty-seven of these were referred for medical treatment and potentially suitable for recruitment (ie, fulfilled the protocol criteria for medical management with methotrexate at our institutions, the same criteria we used for possible recruitment to our study).

Participation depended on whether any of the three investigators involved in recruiting were available for both recruitment and the anticipated length of follow-up. Of the 57 patients referred for medical management, an investigator was available to approach 25 women (32 were not approached). Of these 25, six declined, seven were excluded, and 12 were recruited. We therefore recruited 12 out of 44 possible participants (27%). Figure 1 provides further details regarding the reasons for exclusions and nonrecruitment.

The mean (±standard deviation) age of participants was 31.5 (5.9) years, and the mean (±standard deviation) body mass index (calculated as weight (kg)/[height (m)]^2) was 26.7 (6.3). Nine (75%) had previously been pregnant, two (17%) had previously been treated for an ectopic pregnancy, and two (17%) were smokers. All conceptions were spontaneous and no participants reported a history of endometriosis or sexually transmitted infections. Chlamydia and gonorrhea screening performed during the study was negative for all participants.

Combination treatment did not cause significant toxicities (Table 1). The most common side effects were transient acneiform rash in 67% (8/12) and diarrhea in 42% (5/12), known side effects of gefitinib. There was no clinical or biochemical evidence of serious pulmonary, renal, hepatic, or hematologic toxicity. All participants promptly resumed their menstrual cycles.

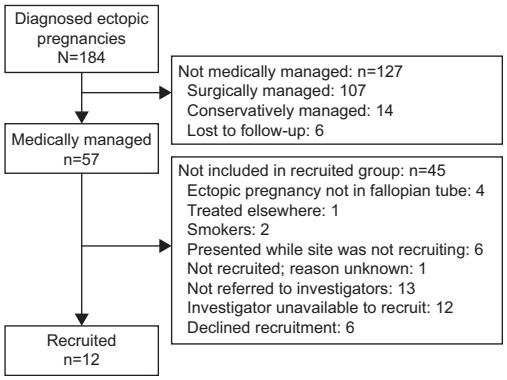

Fig. 1. Study recruitment.

Skubisz. Gefitinib and Methotrexate for Ectopic Pregnancy. Obstet Gynecol 2013.

Combination gefitinib and methotrexate resolved the ectopic pregnancy in 10 of 12 (83%) participants (Table 2). Of the two participants with abdominal pain and offered laparoscopic salpingectomy for possible tubal rupture, one was confirmed as ruptured at operation and on histopathology. Only one of the 10 women successfully treated with the combination treatment required a second dose of methotrexate.

In a planned subgroup analysis, we compared treatment efficacy between trial participants whose pretreatment serum hCG levels were between 1,000 and 3,000 international units/L (and successfully treated without requiring surgery, n=6) and 71 women presenting to our institutions with similar pretreatment serum hCGs levels (1,000–3,000 international units/L) and successfully managed with single-agent methotrexate without requiring surgery (historic controls).

There were no significant differences in baseline characteristics between the subgroup of six participants and the 71 historic controls. The groups were similar in maternal age (mean±standard error of the mean 32.8±3.0 years in participants compared with 30.7±0.7 years in participants in the control group; *P*=.5), parity (33% primigravid women among participants compared with 56% among participants in the control group; *P*=.4), previous ectopic pregnancy (33% among participants compared with 16% participants in the control group; *P*=.3), history of pelvic inflammatory disease (0% among participants compared with 18% participants in the control group; *P*=.6), and smoking status (17% among participants compared with 28% participants in the control group; *P*=1.0).

The serum hCG levels over time are shown in Figure 2. There were no differences in pretreatment or day 1 serum hCG levels (median [interquartile range] hCG in participants was 2,190 [1,811–2,507] international units/L compared with 1,646 [1,357–2,212] international units/L for historic controls; *P*=.17). Median hCG levels among participants dropped by almost one third between days 1 and 4. In contrast, hCG levels among historic controls rose between days 1 and 4. At day 4, median hCG levels among participants were significantly lower compared with participants in the control group (median [interquartile range] hCG in participants 785 [204–2,047] international units/L compared with participants in the control group 1,838 [1,500–2,649] international units/L; *P*=.017). By day 7, median serum hCGs were less than one fifth of levels observed among participants in the control group (median [interquartile range] hCG in participants 261 [55–1,445] international units/L compared with participants in the control group 1,426 [940–2,573]; *P*=.008).

The median time for the ectopic pregnancies to resolve (serum hCG<5 international units/L) was 11 days shorter (34% shorter) among trial participants compared with participants in the control group (participants 21 days (interquartile range 17–27 days) compared with participants in the control group 32 days (interquartile range 25–49 days, *P*=.018). One participant successfully treated for an ectopic pregnancy in her only remaining fallopian tube (previous unilateral salpingectomy for ruptured ectopic pregnancy) later conceived spontaneously and delivered a healthy neonate at term.

DISCUSSION

Here we report a phase I study, administering the combination of gefitinib and methotrexate to 12 women with ectopic pregnancies. The combination commonly caused transient diarrhea and rash but did not cause significant biochemical abnormalities to the liver, hematologic, and renal systems. Notably, one participant with an ectopic pregnancy in her only remaining fallopian tube was successfully treated and subsequently conceived a spontaneous intrauterine pregnancy. This provides encouraging evidence that fallopian tubes affected by ectopic pregnancy and exposed to combination gefitinib and methotrexate treatment remain functional. If so, it compares favorably with surgical excision where often, the entire tube is removed (salpingectomy).

Furthermore, we have obtained encouraging efficacy data suggesting the addition of oral gefitinib to methotrexate may considerably enhance its efficacy. In a planned subgroup analysis, trial participants with pretreatment serum hCG levels between 1,000 and 3,000 international units/L had a significantly more rapid decline in hCG levels and were cured significantly faster (34% less time to cure) than a historic cohort receiving methotrexate alone.

Our analysis of efficacy in this trial is a preliminary finding based on small numbers and was compared with a historic control group rather than a randomized comparison trial arm. As such, it requires validation. However, we believe our preliminary observations on potential efficacy are considerably strengthened when considered together with our preclinical data.[14] We have generated laboratory data, both in vitro and in animal models, suggesting adding gefitinib to methotrexate enhances its ability to induce placental cell death. Therefore, the possibility that gefitinib may enhance the ability of methotrexate to resolve ectopic pregnancy is biologically plausible. We have started a phase II trial to further investigate efficacy where we are including ectopic pregnancies of larger size that

Table 1. Adverse Events

Participant Number	Event Description	Severity (Common Terminology Criteria for Adverse Events Grade)	Related to Trial Drug	Admission to Hospital	Treatment	Withdrawal From Follow-Up
Nonserious adverse events						
1	Abdominal pain	Moderate (grade 2)	No	No	Nonprescription analgesia	No
1	Rash	Mild (grade 1)	Possibly	No	Nil	No
1	Acne	Mild (grade 1)	Possibly	No	Nil	No
2	Abdominal pain	Severe (grade 3)	No	Yes	Laparoscopic salpingectomy	No
2	Elevated bilirubin	Mild (grade 1)	Possibly	No	Monitoring	No
2	Elevated alanine aminotransferase	Mild (grade 1)	Possibly	No	Monitoring	No
3	Lethargy	Mild (grade 1)	Possibly	No	Nil	No
3	Malaise	Mild (grade 1)	Possibly	No	Nil	No
3	Dizziness	Mild (grade 1)	Possibly	No	Nil	No
3	Bloating	Mild (grade 1)	Possibly	No	Nil	No
3	Anorexia	Mild (grade 1)	Possibly	No	Nil	No
4	Elevated creatinine	Mild (grade 1)	Possibly	No	Hydration	No
5	Rash	Mild (grade 1)	Possibly	No	Nil	No
6	Acne	Mild (grade 1)	Possibly	No	Nil	No
7	Abdominal pain	Mild (grade 1)	No	No	Nonprescription analgesia	No
7	Dizziness	Mild (grade 1)	Possibly	No	Nil	No
7	Diarrhea	Mild (grade 1)	Possibly	No	Nil	No
7	Palpitations	Mild (grade 1)	Possibly	No	Nil	No
7	Vaginal infection (bacterial vaginosis)	Mild (grade 1)	No	No	Antibiotics	No
8	Abdominal pain	Moderate (grade 2)	No	No	Nonprescription analgesia	No
8	Diarrhea	Mild (grade 1)	Possibly	No	Nil	No
8	Bloating	Mild (grade 1)	Possibly	No	Simethicone	No
8	Acne	Mild (grade 1)	Possibly	No	Nil	No
8	Scalp pain	Mild (grade 1)	Possibly	No	Nil	No
9	Diarrhea	Mild (grade 1)	Possibly	No	Nil	No
10	Rash	Mild (grade 1)	Possibly	No	Antihistamine	No
10	Diarrhea	Mild (grade 1)	Possibly	No	Nil	No
10	Abdominal pain	Severe (grade 3)	No	Yes	Laparoscopic salpingectomy	No
11	Sore throat	Mild (grade 1)	No	No	Nil	No
11	Acne	Mild (grade 1)	Possibly	No	Nil	No
12	Diarrhea	Mild (grade 1)	Possibly	No	Nil	No
12	Nausea	Mild (grade 1)	Possibly	No	Nil	No
12	Abdominal pain	Moderate (grade 2)	No	Yes	Analgesia and observation	No
Serious adverse events						
4	Renal colic	Severe (grade 3)	No	Yes	Analgesia	No
4	Renal calculus	Severe (grade 3)	No	Yes	Intravenous hydration, tamsulosin	No

Table 2. Treatment Outcomes

Participant	Oral 250 mg Gefitinib (d)	Day 1 hCG (international units/L)	Day 4 hCG (international units/L)	Day 7 hCG (international units/L)	15% or More Fall in Serum hCG Between Days 4 and 7	Treatment Outcome	Time to Resolution (d)
1	1	2,495	917	153	Yes	Success	21
2	1	1,724	NA	NA	NA	Surgery (day 5)	NA
3	1	38	Not done	4	NA	Success	5
4	3	2,101	211	48	Yes	Success	21
5	3	2,541	183	57	Yes	Success	14
6	3	497	675	644	No	Success (2 doses of methotrexate)	31
7	7	2,278	2,027	1,423	Yes	Success	41
8	7	2,071	2,105	1,510	Yes	Success	32
9	7	405	177	110	Yes	Success	18
10	7	1,462	1,713	1,454	Yes	Surgery (day 17)	NA
11	7	162	175	89	Yes	Success	18
12	7	1,029	653	369	Yes	Success	18

hCG, human chorionic gonadotropin; NA, not applicable.

are currently managed surgically (Australian Trials Registration No. 12611001056987).

If verified in future trials, a significantly faster time to cure with gefitinib and methotrexate (compared with methotrexate alone) may not only be clinically beneficial, but could make medical treatment of ectopic pregnancies the preferred option economically. In a systematic meta-analysis, Mol et al[3] found when compared with laparoscopic surgery, the single-dose methotrexate protocol resulted in significant cost savings (of approximately $518 per patient) if only one dose was required. These savings resulted from both reduced operating room use and hospital stay. However, savings for the medical option were lost if the pretreatment serum hCG levels were higher than 1,500 international units/L as a result of the need for prolonged follow-up and a higher rate of surgical reinterventions.

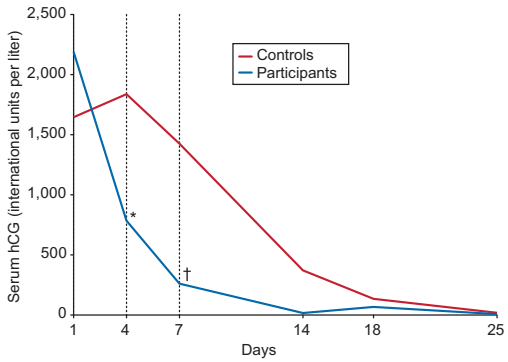

Fig. 2. Serum human chorionic gonadotropin (hCG) levels in trial participants treated with gefitinib and methotrexate compared with historic controls given methotrexate alone. Subanalysis of six participants and 71 historic controls in which pretreatment serum hCG levels were between 1,000 and 3,000 international units per liter in both groups. Comparisons of hCG levels were made between the groups at days 1, 4, and 7. At day 1, serum hCG levels between both cohorts were not significantly different. *$P<.05$. †$P<.01$.

Skubisz. Gefitinib and Methotrexate for Ectopic Pregnancy. Obstet Gynecol 2013.

It is well known that transient diarrhea and skin rash are common side effects of gefitinib.[15,16] These side effects were commonly observed among our cohort and probably occur more often than treatment with single-agent methotrexate. These side effects were transient and were responsive to treatments for symptomatic relief such as topical skin emollients or antidiarrheal therapies. It is possible the inconvenience of enduring these side effects may be acceptable to patients if it were confirmed in future trials that combination therapy provides a faster time to cure and is more efficacious in resolving larger ectopic pregnancies compared with single-agent methotrexate. Because these minor side effects occurred commonly, future randomized studies should include evaluations of participant satisfaction.

Gefitinib is associated with a rare but serious side effect of interstitial lung disease, a thickening of the lung parenchyma. Postmarketing surveillance data of 31,045 patients in the United States reported to the Food and Drug Administration suggests the incidence

of interstitial lung disease among those taking gefitinib indefinitely is 0.3%.[12] Interestingly, there appears to be a 1% incidence of interstitial lung disease among Japanese people, and we therefore excluded women of this ethnic background.[12] Of note, the median time to develop interstitial lung disease while taking gefitinib is 42 days, and risk factors for developing interstitial lung disease include male sex and age older than 55 years.[12] Importantly, it is possible coexistent lung pathology needs to be present for gefitinib to induce interstitial lung disease. This premise is supported by the fact trials examining cetuximab treatment (monoclonal antibody that blocks the EGF receptor) for colon cancer did not report the occurrence of interstitial lung disease.[17] For these reasons, we believe the administration of a short course of gefitinib to women (non-Japanese descent) of reproductive age without coexisting lung pathology avoids these five risk factors and may be safe. Although we did not observe adverse effects in major organs (lungs, kidney, liver, and hematologic systems), we caution that we have not conclusively shown this regimen is safe in this small trial. In a phase II trial (in progress), we are continuing to collect data on safety and tolerability.

Combination gefitinib and methotrexate may have other pregnancy-related indications such as improving the treatment of persistent gestational trophoblastic disease,[18] aiding in the conservative management of placenta accreta (placenta pathologically adherent to the myometrium), and in the treatment of ectopic pregnancies occurring at sites other than the fallopian tube (eg, the cesarean delivery scar in the uterus, the cervix, or the uterine cornua). These are particularly dangerous situations and notoriously difficult to manage even surgically, often requiring hysterectomy.[19]

In conclusion, we have undertaken a phase I trial suggesting the combination of gefitinib and methotrexate is potentially more effective in resolving ectopic pregnancies than methotrexate alone. The combination could prove to be a potent therapy that not only improves the medical treatment of ectopic pregnancy, but also other disorders of trophoblastic growth. However, this treatment should not be used until its efficacy and safety is confirmed in larger trials.

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PART B: Declaration for Thesis Chapter 6

Monash University

Declaration by Candidate:

In the case of Chapter 6, the nature and extent of my contribution to the work was the following:

Section	Nature of Contribution	Contribution (%)
6.2	Conceived and designed the study, obtained ethics approval, recruited and treated participants, performed the data analysis and drafted the paper.	55%

The following co-authors contributed to the work. If co-authors are students at Monash University, the extent of their contribution in percentage terms must be stated:

Name	Nature of Contribution	Contribution (%)
Andrew Horne	Helped design the study, obtained ethics approval, recruited and treated participants, drafted the paper and provided critical input.	
Stephen Tong	Conceived, designed and supervised the study, obtained ethics approval, drafted the paper and provided critical input.	
Colin Duncan	Supervised the study, recruited and treated participants and provided critical input.	
Peter Neil	Recruited and treated participants and provided critical input.	
Euan Wallace	Supervised the study, recruited and treated participants and provided critical input.	
Terrance Johns	Conceived and supervised the study, provided critical input.	

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-author's contributions to this work:

Candidate's Signature:  Date: 21/05/2014

Main Supervisor's Signature:  Date: 21/05/2014

Chapter 6: Combination Gefitinib and Methotrexate to Treat Non-Tubal Ectopic Pregnancies: A Case Series

6.1 Introduction

Ectopic pregnancy is a diagnosis applied to pregnancies implanted in a range of intra- and extra-uterine sites. The vast majority occur in the Fallopian tube (over 95% of cases) (Bouyer et al., 2002), however, an ectopic pregnancy can implant anywhere in the abdomino-pelvic cavity (Figure 2.1). Collectively, non-tubal ectopic pregnancies are significantly rarer than tubal ectopic pregnancies, and present particular diagnostic and treatment challenges, both medical and surgical. Consequent to this, non-tubal pregnancies are associated with significantly higher morbidity and mortality rates than tubal ectopic pregnancies (Jurkovic and Wilkinson, 2011).

Because of their rarity, the literature regarding best management of non-tubal pregnancies is limited to case-series and reports, usually grouped by implantation site because of the unique challenges presented by each. Medical treatment with systemic methotrexate often utilises higher doses and more adjunctive treatments compared to use in tubal ectopic pregnancy, to counter the higher pre-treatment serum human chorionic gonadotrophin (hCG) levels and other negative predictors of medical treatment success, such as larger gestational sac size and the presence of embryonic cardiac motion on transvaginal ultrasound (TVUS), features often associated with non-tubal ectopic pregnancy; this is usually a result of advanced gestation at diagnosis due to delayed onset of symptoms or difficult TVUS diagnosis, or both (Fylstra, 2012). Surgical management too poses greater risk to subsequent fertility, particularly where uterine interstitial, caesarean section scar and ovarian resection or cervical curettage is required.

Perhaps even more so than in the treatment of tubal ectopic pregnancy then, treatment of non-tubal ectopic pregnancy would greatly benefit from a more potent and effective medical treatment option. Because of this, we decided to recruit women with non-tubal ectopic pregnancies to a case series study, treating them with combination gefitinib and methotrexate. We recruited 8 women in total, 5 with interstitial and 3 with caesarean section scar ectopic pregnancies, and achieved a 100% successful, non-invasive treatment success rate with pre-treatment serum hCGs as high as 48,550 IU/L, and despite embryonic cardiac motion being detectable on TVUS in 2 participants. These results have been published in *Human Reproduction* (see section 6.2) and present the most exciting and significant application of combination gefitinib and methotrexate treatment to date.

6.2 Manuscript

Hum. Reprod. Advance Access published May 7, 2014
Human Reproduction, Vol.0, No.0 pp. 1–5, 2014
doi:10.1093/humrep/deu091

human reproduction
CASE REPORT Early pregnancy

Combination gefitinib and methotrexate treatment for non-tubal ectopic pregnancies: a case series

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ABSTRACT: Non-tubal ectopic pregnancies are a rare subgroup of ectopic pregnancies implanted at sites other than the Fallopian tube. Mortality from non-tubal ectopic pregnancies is higher compared with that for tubal ectopic pregnancies, and they are becoming more common, partly due to the rising incidence of Caesarean sections and use of assisted reproductive technologies. Non-tubal ectopic pregnancies can be especially difficult to treat. Surgical treatment is complex, and follow-up after medical treatment is usually protracted. There is therefore a need for more effective medical therapies to resolve non-tubal ectopic pregnancies and reduce operative intervention. We have recently reported successful use of combination gefitinib (an orally available epidermal growth factor receptor inhibitor) and methotrexate for treatment of tubal pregnancies. To our knowledge, this combination has not been used to treat non-tubal pregnancies. Here we report the use of combination gefitinib and methotrexate to treat eight women with stable, non-tubal ectopic pregnancies at two tertiary academic teaching hospitals (Edinburgh, UK and Melbourne, Australia); five interstitial and three Caesarean section scar ectopic pregnancies. Pretreatment serum hCG levels ranged from 2458 to 48 550 IU/l, and six women had pretreatment hCG levels >5000 IU/l. The women were co-administered 1–2 doses of i.m. methotrexate (50 mg/m^2 on Day 1, ± Day 4 or Day 7) with seven once daily doses of oral gefitinib (250 mg). The women were monitored until complete resolution of the ectopic pregnancy, defined as a serum hCG <15 IU/l. Time to resolution (days from first methotrexate dose until serum hCG <15 IU/l), safety and tolerability, complication rates and subsequent fertility outcomes were also recorded. All eight women were successfully treated with combination gefitinib and methotrexate. The most common side effects were transient acne/rash and diarrhoea, known side effects of gefitinib. All women promptly resumed menstruation and importantly, three women subsequently conceived spontaneously. Two have delivered a healthy infant at term and the third is currently in her second trimester of pregnancy. Hence, our case series supports a future clinical trial to determine the efficacy of combination gefitinib and methotrexate to treat non-tubal ectopic pregnancies.

Key words: ectopic pregnancy / epidermal growth factor receptor / gefitinib / methotrexate / non-tubal

Introduction

Ectopic pregnancies (EPs) have an incidence of ~1–2% of all pregnancies. They occur when a fertilized ovum implants away from the endometrial cavity, most commonly (>95%) in one of the Fallopian tubes (Jurkovic and Wilkinson, 2011; Fylstra, 2012). EPs can, however, implant in more unusual locations such as within a Caesarean section scar, within the interstitial portion of the Fallopian tubes, in the cervix, on the ovary and potentially anywhere in the abdominal cavity. Mortality from non-tubal ectopic pregnancies is higher than ectopic pregnancies generally, and they are becoming more common due to assisted reproductive technologies, and possibly due to increasing Caesarean section rates (Chetty and Elson, 2009; Verma *et al.*, 2011). Non-tubal ectopic pregnancies are generally difficult to treat and often require a combination of surgical and medical methods.

The literature around management of non-tubal EPs is limited to case reports and series, describing a range of minimally invasive surgical,

† Denotes equal contribution.

radiological and medical interventions including laparoscopic and hysteroscopic resection, uterine artery embolization, ultrasound guided injections of the gestational sac with potassium chloride and/or methotrexate, and systemic treatment with drugs such as methotrexate, mifepristone and misoprostil (Verma *et al.*, 2011; Fylstra, 2012). Treatment choice depends on the site, size and pretreatment serum hCG level of the non-tubal EP. In particular, interstitial, Caesarean section scar and cervical EPs often still require surgical resection and/or instrumentation of the uterus, with potential risks to the woman's subsequent reproductive capacity. Because of their rarity as a clinical entity, the best management of non-tubal EPs has been difficult to establish.

In preclinical studies and a phase I single arm, open label study, we demonstrated that co-administering gefitinib (an epidermal growth factor receptor inhibitor) with methotrexate to treat ectopic pregnancies appeared safe. Furthermore, we obtained preliminary data suggesting this combination may have a time to resolution which is 34% faster compared with treatment using methotrexate alone (Nilsson *et al.*, 2013; Skubisz *et al.*, 2013). This suggested adding gefitinib to methotrexate may improve on its efficacy in medically resolving ectopic pregnancies. We therefore wondered whether this combination could be potentially used to treat non-tubal ectopic pregnancies more effectively. Here we report a case series of eight women with non-tubal EPs treated with gefitinib and methotrexate.

Methods

Institutional human research ethics approval was sought and obtained at both participating sites (Southern Health Human Research Ethics Committee B, 11180B, and Scotland A Research Ethics Committee, 11/AL/0350) to allow administration of combination gefitinib and methotrexate to eight women with non-tubal EPs, and written informed consent was obtained from each participant. The diagnosis of non-tubal EP was made according to set ultrasound diagnostic criteria (Jurkovic *et al.*, 2003; Jurkovic, 2007) in combination with quantitative serum hCG measurement. Inclusion criteria required the women to be assessed as haemodynamically stable (with no pallor, postural change in blood pressure, syncope or pre-syncope, severe abdominal pain or signs of abdominal peritonism, as well as requiring a normal serum haemoglobin and haematocrit) and to have normal baseline white cell count, renal and hepatic indices. Exclusion criteria included severe dermatological, gastrointestinal and pulmonary comorbidities (systems most likely to be affected by combination treatment), allergy to gefitinib and/or methotrexate and Japanese ethnicity (the latter being an increased risk factor for gefitinib-associated interstitial lung disease).

Participants were treated with daily oral gefitinib 250 mg for 7 days in addition to 50 mg/m^2 of i.m. methotrexate on Day 1. Quantitative serum hCG measurement was repeated on Day 4 and Day 7 of treatment, and initial treatment success was defined as a $\geq$15% fall in serum hCG between these two measurements. Additional doses of methotrexate at 50 mg/m^2 were administered where this did not occur, or where there was a significant rise in the serum hCG between Day 1 and Day 4. Serum hCG was then measured weekly until there was complete resolution of the EP, defined as a serum hCG of $\leq$15 IU/l. Haematological, renal and hepatic blood indices were monitored at each visit.

Treatment outcome parameters recorded included time to resolution (days from first methotrexate dose until serum hCG $<$15 IU/l), safety, tolerability and complication rates. Side effects and symptoms were classified according to the Common Terminology and Criteria for Adverse Events (CTCAE) version 4.03 (National Cancer Institute, National Institutes of Health, U.S. Department of Health and Human Services, June 14, 2010). Participants were contacted at 3, 6 and 12 months post-treatment to document return of menstrual cycles and any subsequent fertility outcomes.

Results

We recruited eight women with stable non-tubal EPs: five women with interstitial EPs and three women with Caesarean section scar EPs. The range of pretreatment serum hCG levels of participants was between 2458 and 48550 IU/l.

All women were successfully treated with combination gefitinib and methotrexate—none of the women required surgical and/or invasive intervention to achieve cure and furthermore, none of the participants experienced blood loss requiring transfusion. A second dose of i.m. methotrexate was administered to 5/8 of participants, in 3/5 participants because of a significant rise in serum hCG between Days 1 and 4 of treatment and in the remaining 2/5, because the serum hCG had not fallen $\leq$15% between Days 4 and 7 of treatment. Duration of follow-up ranged from 25 to 196 days. Table I summarizes the non-tubal pregnancy characteristics, treatment and outcomes of each participant. Supplementary Table I

Table I Participant ectopic pregnancy (EP) and treatment details.

Participant	EP type	Day 1 hCG (IU/l)	Day 4 hCG (IU/l)	Day 7 hCG (IU/l)	Fetal heart on ultrasound?	2nd dose of MTX given?	Time to resolution[a] (days)
1	Interstitial	2458	2049	2350	No	Yes (Day 7)	31
2	Interstitial	6528	6163	6502	Yes	Yes (Day 7)	38
3	Caesarean scar	8716	13 836	9906	No	Yes (Day 4)	48
4	Interstitial	8575	6125	4810	No	No	67
5	Caesarean scar	48 558	54 747	47 551	Yes	Yes (Day 4)	196
6	Interstitial	9730	11 966	12 484	No	Yes (Day 4)	63
7	Interstitial	2649	3662	3497	No	No	25
8	Caesarean scar	8707	5981	3041	No	No	53

MTX, methotrexate.
[a]Resolution defined as serum hCG $<$15 IU/l.

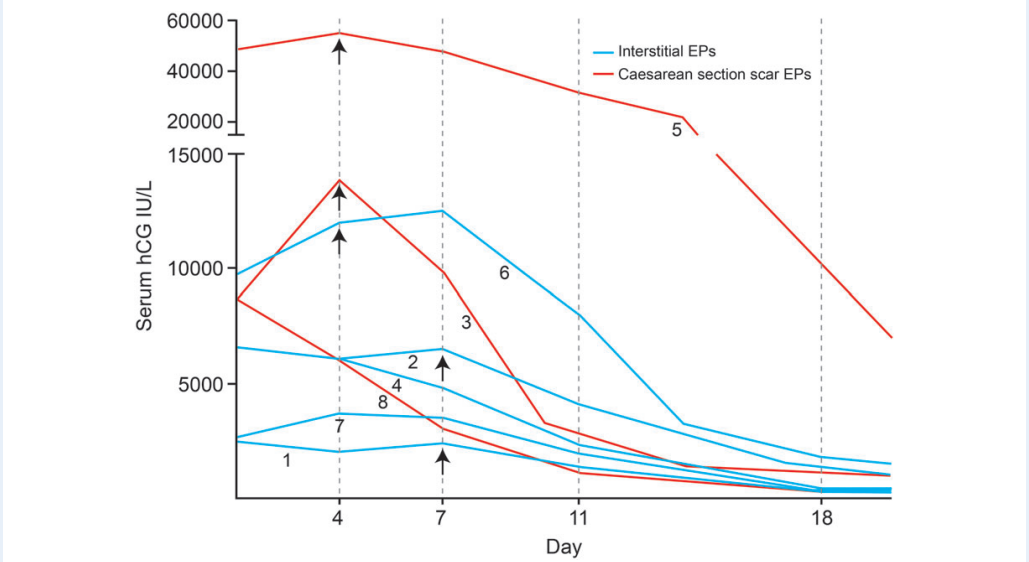

Figure 1 The serum hCG courses of participants. Interstitial ectopic pregnancies shown in blue and Caesarean section scar ectopic pregnancies shown in red. Serum hCGs were measured on Days 4, 7 and 11 of treatment and weekly thereafter until resolution (hCG, 15 IU/l), with the protocol commencing again after a repeat dose of methotrexate on Day 4 or Day 7 in 5/8 participants. Numbers on the diagonal correlate to the participant and arrows indicate additional doses of methotrexate.

provides participant baseline demographic data and additional ultrasound characteristics of the non-tubal pregnancies.

The hCG courses of the five interstitial EPs were quite varied (Fig. 1). Two participants demonstrated an adequate fall in hCG between Day 4 and Day 7 (participants 4 and 7; see Table I). Participants 1 and 2 experienced an initial fall in serum hCG between Day 1 and Day 4, but this curiously rose to pretreatment levels at Day 7 in both cases. This was despite ultrasound evidence of treatment efficacy in participant 2, where a fetal heart seen pretreatment was not detected on re-scanning at Day 4. In contrast, the hCG courses of the Caesarean section scar EPs all demonstrated a fall in hCG between Days 4 and 7 of treatment, including one with an extremely high pretreatment hCG level of 48 558 IU/l and a fetal heart seen on ultrasound prior to treatment.

The combination of oral gefitinib and systemic methotrexate was well tolerated. The most commonly reported adverse events were a papulopustular rash (Fig. 2), diarrhoea and dizziness, consistent with the known side effect profile of gefitinib. All adverse events were classified as either grade 1 (mild) or grade 2 (moderate) according to the CTCAE, and all women were able to continue with employment and/or family responsibilities after discharge from hospital and during follow-up. All reported adverse events resolved spontaneously after completion of treatment, with only occasional symptomatic treatment required. Importantly, there were no complications of treatment and in particular, none of the participants experienced haemorrhage requiring blood transfusion.

All participants promptly resumed their menstrual cycles (i.e. within 6 weeks of cure), and 3/8 so far have achieved a subsequent spontaneous intrauterine pregnancy. Two of these pregnancies have resulted in the successful births of a healthy infant at term, with a third woman in the second trimester of an uncomplicated pregnancy.

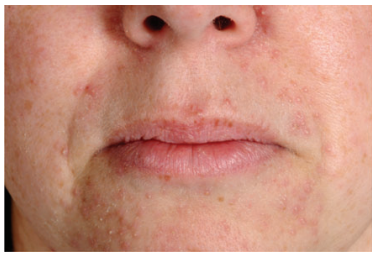

Figure 2 An example of the papulopustular (acneiform) rash experienced by some participants in response to treatment with oral gefitinib. The rash is most prominent in areas exposed to UV light, i.e. the face, neck and décolletage.

Discussion

The results of this case series suggest that combination gefitinib and methotrexate therapy could be a safe and effective treatment for non-

tubal ectopic pregnancies. The combination treatment was successful in resolving the pregnancies without recourse to surgery or more invasive medical treatments in all cases. Six of the eight women had serum hCG levels of >5000 IU/l, levels where previous studies would suggest the single-dose methotrexate protocol (which includes a second dose if required) may be less effective (Menon *et al.*, 2007).

Non-tubal EPs are uncommon, and consequently, their optimal management has not been firmly established. Additionally, each type of non-tubal EP presents different management challenges (Chetty and Elson, 2009). Advances in ultrasound have enabled earlier and more accurate diagnosis, and the use of minimally invasive techniques has significantly improved the outcomes of women diagnosed with non-tubal EPs (Chetty and Elson, 2009). Nevertheless, non-invasive management remains key to minimizing any risk to subsequent pregnancies.

Non-invasive treatment of non-tubal and indeed all EPs is almost exclusively limited to systemic methotrexate (Hajenius *et al.*, 2000). The effectiveness of methotrexate in the treatment of EP is limited by the pretreatment serum hCG, with ectopic pregnancies with levels >5000 IU/l significantly less likely to be treated successfully (Menon *et al.*, 2007). Because of difficulty accurately characterizing their location with ultrasound, non-tubal EPs are still diagnosed at more advanced gestations with higher pretreatment serum hCG levels, thus limiting the usefulness of this non-invasive approach (Chetty and Elson, 2009). We have demonstrated effective management of women with non-tubal EPs and pretreatment hCGs as high as 48 558 IU/l by combining minimal doses of methotrexate with a short course of gefitinib. This co-treatment approach can achieve better treatment outcomes with lower overall drug exposure (Nilsson *et al.*, 2013).

Another clinical factor negatively associated with methotrexate treatment success is the presence of a fetal heart motion on ultrasound (Bachman and Barnhart, 2012). In this case series of eight women, we successfully treated two non-tubal EPs with embryonic cardiac activity, including one participant who at repeat scanning on Day 4 of treatment, showed the fetal heart motion to have already resolved. These cases provide further encouraging preliminary evidence that this combination treatment is efficacious.

The main goal of non-invasive treatment is preservation of reproductive potential. Importantly, all participants promptly resumed their menstrual cycles after resolution of their non-tubal EPs with combination gefitinib and methotrexate (within 6 weeks). Three participants have subsequently conceived spontaneous intrauterine pregnancies, with two women delivering a healthy infant at term (both normal vaginal deliveries) and a third woman being in her second trimester of an uncomplicated pregnancy.

Our continued experience with combination gefitinib and methotrexate treatment of women with ectopic pregnancies is that it appears safe and well tolerated. There were no serious adverse events recorded during the treatment of these eight women. Non-serious adverse events, predominantly gastrointestinal and mucocutaneous, were consistent with the known side effect profiles of both gefitinib and methotrexate. Furthermore, all side effects were transient, requiring only occasional symptomatic management and completely resolving after discontinuation of treatment.

Treatment of non-small cell lung cancer with gefitinib is associated with interstitial lung disease (ILD) in ~1% of white and 5% of Japanese patients, and is fatal in up to one-third of cases (Cataldo *et al.*, 2011). We have had no occurrences of ILD in any of our participants from this and other studies, cumulatively 70 women treated with ectopic pregnancies and persistent gestational trophoblastic disease from published and unpublished data (Skubisz *et al.*, 2013). We have screened for and excluded women with significant pulmonary comorbidities and Japanese ethnicity, and in addition to a short and limited 7-day course of gefitinib, believe to risk of ILD in women of reproductive age to be unlikely.

In conclusion, we believe that combination gefitinib and methotrexate is a promising new treatment approach for non-tubal EPs. Whilst we understand that it requires assessment of efficacy in a large clinical trial before it can be introduced into clinical practice, we believe that it has the potential to reduce the need for surgical intervention, improve future reproductive outcomes and minimize the burden of treatment to both health services and women.

Supplementary data

Supplementary data are available at http://humrep.oxfordjournals.org/.

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Authors' roles

A.W.H., M.M.S., W.C.D., E.M.W., P.N. and S.T. recruited and treated the participants. A.W.H, M.M.S, S.T. and T.G.J. collated and analysed the data. A.W.H., M.M.S. and S.T. drafted the manuscript. E.M.W. and T.G.J. provided clinical and intellectual oversight. All authors critically reviewed the manuscript and approved the final version.

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Conflict of interest

T.G.J., and S.T. are joint holders of patents that relate to the use of EGFR inhibition in treating ectopic pregnancies.

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Chapter 7: Combination Gefitinib and Methotrexate to Treat Ectopic Pregnancy: A Phase II Clinical Study

7.1 Abstract

Chapter 7 presents the findings of a phase II single-arm, open-label clinical study treating ectopic pregnancy with combination gefitinib and methotrexate. We aimed to recruit 28 women with stable ectopic pregnancies and pre-treatment serum human chorionic gonadotrophin (hCG) levels of between 1000-10,000 IU/L, with or without evidence of embryonic cardiac motion on transvaginal ultrasound (TVUS). Women were recruited from 4 sites: 3 in Melbourne, Australia and 1 in Edinburgh, Scotland. All participants were treated with our proposed combination protocol: a single dose of 50mg/m^2 of intramuscular methotrexate on day 1 and a 7 day course of once-daily 250mg oral gefitinib commencing on day 1. Progress was monitored with clinical reviews and serial serum hCG levels on days 1, 4, 7 and weekly until resolution (defined as a serum hCG ≤15 IU/L). Treatment was considered successful if a fall of serum hCG ≥15% was noted between day 4 and day 7 and hCG resolved without further intervention.

The primary outcome assessed by this study was the success rate of combination gefitinib and methotrexate treatment to resolve tubal ectopic pregnancies of any size, without recourse to surgery. In order to progress to a phase III study, we would need to reject the null hypothesis that the true efficacy of combination gefitinib and methotrexate is ≤70%. Secondary outcome measures included time to resolution (in days) as a surrogate marker of efficacy, and safety and toxicity profiling. Treatment success and time to resolution were compared to a contemporaneous cohort of potentially eligible women treated at our recruitment sites with single-dose methotrexate only for ectopic pregnancies during the conduct of this study.

Altogether, 30 women were recruited and 2 women withdrew prior to completion of the treatment protocol (n=28). Combination gefitinib and methotrexate successfully treated 24/28 (86%) of women, compared to 13/24 (54%) of controls (p=<0.02). Time to resolution was 30 days for participants and 37 days for controls, however, this was not statistically significant (p=0.09). Combination gefitinib and methotrexate was safe and well tolerated, with no undue toxicities reported.

This study shows that combination gefitinib and methotrexate is safe and effective in treating larger ectopic pregnancies with higher pre-treatment serum hCG levels, where single-dose methotrexate is known to be less effective. It provides sufficient proof to progress to a larger, randomised controlled study to validate this treatment approach for clinical use, to ultimately allow a greater proportion of women with ectopic pregnancy to avoid surgical management and concomitant loss of reproductive potential.

7.2 Introduction

In chapter 5 we presented the findings of a phase I study investigating the potential of gefitinib and methotrexate to improve medical treatment of ectopic pregnancy. We recruited women with ectopic pregnancies currently eligible for medical treatment with single-dose methotrexate, according to institutional and published protocols. In particular, we recruited haemodynamically stable women with ectopic pregnancies and pre-treatment serum hCG levels of <3000 IU/L, where ultrasound demonstrated no significant signs of rupture, no embryonic cardiac motion and a gestational sac size of <3cm (Appendix B) (RCOG, 2010).

In our phase I study, combination gefitinib and methotrexate administered to 12 women with an ectopic pregnancy appeared safe and well tolerated. The study suggested a possibility of improved treatment efficacy compared to single-agent methotrexate treatment at serum hCG levels between 1000-3000 IU/L, with a more rapid rate of hCG decline and shorter time to resolution observed in participants compared to historic controls (Skubisz et al., 2013). The overall combination gefitinib

and methotrexate treatment success rate in phase I participants was 83% (10/12 participants), comparable to reported success rates of around 90% for single-dose methotrexate treatment of ectopic pregnancy (Barnhart et al., 2003, Lipscomb et al., 2005).

The literature regarding the use of single-dose methotrexate to treat ectopic pregnancy has consistently shown that the strongest predictor of treatment outcome is the pre-treatment serum hCG level (Lipscomb et al., 1999, Menon et al., 2007, Mol et al., 2008). Menon *et al.* (2007) have shown that where the serum hCG level >5000 IU/L, the risk of single-dose methotrexate treatment failure is significantly increased (OR 5.45 (95% CI 3.04-9.78); p <0.01). Furthermore, health economic evaluation has shown that single-dose methotrexate treatment of ectopic pregnancy is only more cost-effective than laparoscopic salpingectomy when the pre-treatment serum hCG is <1500 IU/L (Mol et al., 2008). Hence, only an estimated 25-30% of women diagnosed with an ectopic pregnancy are eligible for consideration of medical treatment (Jurkovic and Wilkinson, 2011), a minority of women diagnosed with the condition. In order to improve the medical treatment of ectopic pregnancy therefore, a novel intervention must not only achieve an equivalent or greater treatment success rate compared to single-dose methotrexate, but also be a viable treatment option for a greater number of women diagnosed with the condition.

In this chapter we present the findings of a phase II single-arm, open-label, multi-centre study further investigating the potential of combination gefitinib and methotrexate to improve medical treatment of ectopic pregnancy. It builds on the previous phase I study by accumulating additional evidence of the safety and efficacy of combination gefitinib and methotrexate to treat ectopic pregnancy, but also extends the inclusion criteria for medical treatment to include women with higher pre-treatment serum hCG levels, larger gestational sac sizes and with embryonic cardiac motion detectable on TVUS. In fact, this current study did not recruit women with a pre-treatment serum hCG of <1000 IU/L, as we intended to recruit ectopic pregnancies of a relatively larger size. Thus, this trial explores the possibility that combination gefitinib and methotrexate is not only more

effective than single-dose methotrexate in the treatment of ectopic pregnancy within current medical treatment parameters, but also examines whether medical treatment could be administered to women who would currently be recommended surgical management.

7.3 Methods

We designed a single-arm, open-label phase II study combining gefitinib and methotrexate to treat ectopic pregnancy. Compared to our phase I study, we sought to recruit a larger cohort of haemodynamically stable women with ectopic pregnancies of any gestational sac size and significantly higher pre-treatment hCG levels, including women with evidence of embryonic cardiac activity on TVUS.

We renewed our collaboration with colleagues at the Royal Infirmary of Edinburgh, Scotland and expanded our recruitment sites in Melbourne, Australia to include Monash Medical Centre and Dandenong Hospital (Monash Health network), as well as the Mercy Hospital for Women, giving a total of 4 recruitment centres. HREC approval was sought and obtained for each site (Scotland A Research Ethics Committee (MREC 11/AL/0350), Monash Health Human Research Ethics Committee (MH HREC 11180B) and the Mercy Health Human Research Ethics Committee (R12/25).

We aimed to recruit 28 women to the study. The sample size was calculated using A'Hern's formula for phase II one-stage designs (A'Hern, 2001). With 80% power and a 5% level of significance, 28 patients were required to determine if the true efficacy of combination gefitinib and methotrexate is ≤70% or ≥90%. If 24 participants were to be treated successfully, this would enable us to reject the hypothesis that the true efficacy of combination treatment is ≤70% and progress to a phase III study. Potentially eligible women had to be diagnosed with a definite ectopic pregnancy (dEP) or probable ectopic pregnancy (pEP), defined on TVUS as having: 1) an extra-uterine gestational sac with yolk sac and/or embryo, with or without cardiac activity (dEP); or 2) an inhomogeneous adnexal mass or

extra-uterine sac-like structure (pEP) (Barnhart et al., 2011). Participants had to have a rising or static pre-treatment serum hCG level of between 1000-10,000 IU/L and be between 18-45 years of age. Other inclusion criteria required an assessment of haemodynamic stability (no clinical evidence of intra-abdominal bleeding; no pallor; no guarding/rigidity on abdominal examination; stable blood pressure and heart rate) as well as normal baseline haematological, renal and hepatic indices.

Exclusion criteria were based on methotrexate treatment safety protocols and a harm minimization strategy relating to the known side-effect profile of gefitinib. We therefore excluded women with any significant pre-existing pulmonary, gastrointestinal or dermatological disease, abnormal liver, renal or haematological indices on baseline blood tests, as well as women of Japanese ethnicity (Skubisz et al., 2013). This latter exclusion criterion is based on the significantly increased incidence of interstitial lung disease (ILD) in Japanese people compared to white people in post-marketing surveillance data published for gefitinib treatment of non-small cell lung cancer (NSCLC) (Cataldo et al., 2011, Cohen et al., 2004). Women with a pregnancy of unknown location (PUL) who were recommended for medical treatment were not eligible to participate in the study, and we excluded women with any clinical signs and symptoms of haemodynamic instability, or with complex or echogenic free fluid on TVUS above the level of the uterine body.

Potentially eligible women were identified and referred to trial investigators by colleagues in our emergency departments and/or outpatient clinics. A copy of the patient information and consent form was given to women prior to review by an investigator. Each woman was then screened and offered time for questions and private consultation. If eligible for participation, written, informed consent was obtained. Participating women were treated with a single-dose of intramuscular methotrexate at 50mg/m^2 on day 1, as well as 7-day course of once-daily oral 250mg gefitinib, commencing on the same day as methotrexate administration.

To monitor treatment response, we followed current clinical protocols for medical management of ectopic pregnancy with single-dose methotrexate, including serum hCG level measurement on days

1, 4 and 7 of treatment, and then weekly until serum hCG levels dropped to non-pregnant levels (<15 IU/L). We did not offer a second dose of methotrexate.

Treatment was considered effective if a ≥15% fall in serum hCG level was observed between day 4 and day 7 of treatment. Medical management was discontinued and surgical management offered for women who did not demonstrate and adequate response to treatment, who developed toxicity or who demonstrated signs of tubal ectopic pregnancy rupture, such as severe abdominal pain and/or collapse, increased free fluid on TVUS, unstable vital signs such as low blood pressure and tachycardia, or a significant fall in haemoglobin.

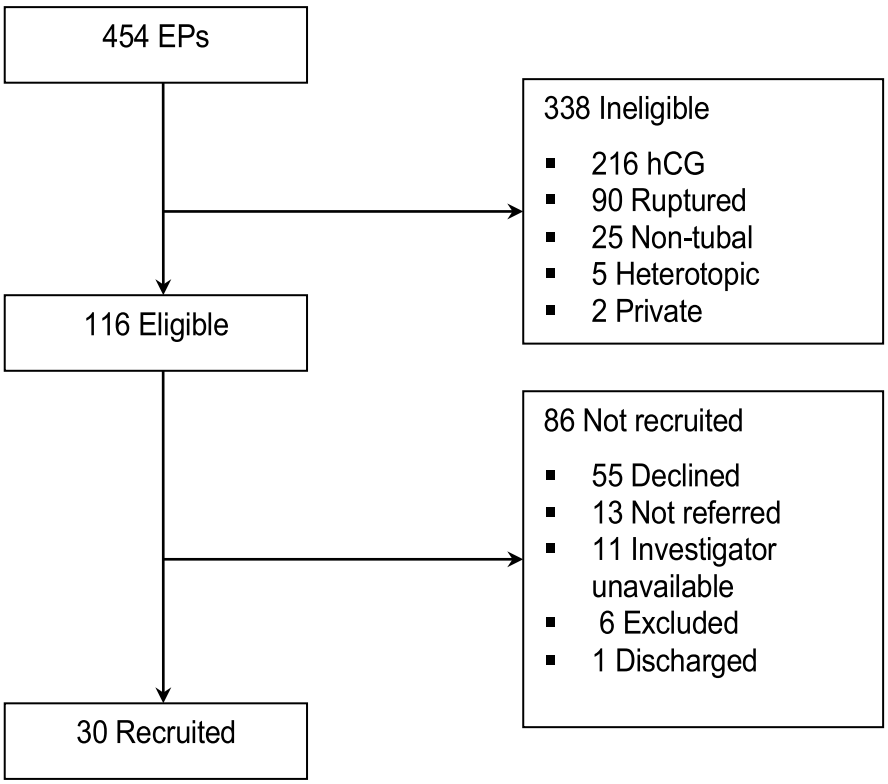
The primary outcome assessed by this study was resolution of ectopic pregnancy with combination gefitinib and methotrexate treatment, without additional doses of methotrexate or recourse to surgery. Resolution was defined as a serum hCG of <15IU/L (non-pregnant on urine pregnancy tests). Secondary outcomes assessed included: 1) time to resolution (in days) compared to a contemporaneous cohort of women treated with single-dose methotrexate (a surrogate maker of efficacy); and 2) safety and tolerability as assessed clinically and biochemically with haematological, renal and hepatic indices measured at each clinical review (or if abnormal, until normalised). All adverse events were documented and graded according to the Common Terminology Criteria for Adverse Events (CTCAE) version 4.03, published by the National Cancer Institute, National Institutes of Health, U.S Department of Health and Human Services June 14, 2010 (Appendix D). Participants were contacted at 3 and 6 months post-treatment to document return of menstrual cycles and any subsequent pregnancies. This protocol has been published in *BMJ Open* (Appendix C) (Horne et al., 2013).

7.4 Results

We recruited a total of 30 women with stable ectopic pregnancies and pre-treatment serum hCG levels between 1000-10000 IU/L between the 16th of January 2012 and the 9th of April 2014.

Altogether, 454 women diagnosed with ectopic pregnancies presented to our institutions during this time, of which 116 were potentially eligible for recruitment. Of the 86 potentially eligible but not recruited women, 55 declined participation, 13 were not referred to investigators by treating clinicians, 11 presented when investigators were unavailable, 6 were excluded at screening and 1 was discharged from hospital prior to making contact with an investigator. Of the 338 ineligible women, 216 had serum hCG levels outwith the protocol, 90 were considered to have signs or symptoms consistent with tubal rupture and/or haemodynamic instability, 25 women had non-tubal ectopic pregnancies, 5 women were diagnosed with heterotopic pregnancies and 2 women opted to be treated privately (Figure 7.1).

Figure 7.1: Phase II recruitment flowchart.



Two women withdrew from the study. One woman requested surgical management on day 4 when her serum hCG level had significantly risen, despite not meeting any study exit criteria, and the other withdrew on day 7 with an appropriately falling serum hCG level, stating she was no longer able to comply with follow up. Both women were referred back to our gynaecology units for consideration of surgical management. We collected treatment and outcome data from the records of potentially eligible women treated with single-dose methotrexate for ectopic pregnancy at our institutions during study recruitment. We identified 29 such women, however, complete outcome data was only available for 24, and these women form our contemporaneous control.

The success rate of combination gefitinib and methotrexate to treat women with stable ectopic pregnancies and pre-treatment serum hCG levels of between 1000-10,000 IU/L was 86% (24/28), compared to 54%(13/24) in controls treated with a single dose of methotrexate (p<0.02). Four participants and 6 controls were presumed to have ruptured during medical treatment and were subsequently treated surgically. A further 5 controls were successfully treated with additional doses of methotrexate when their serum hCG did not fall appropriately between day 4 and day 7 of treatment. Two women were recruited with evidence of embryonic cardiac motion on TVUS, however, both of these represented with severe abdominal pain and were subsequently treated surgically. Gestational sac sizes on TVUS ranged from 0.5-5cm in maximum diameter, with a median of 2cm. The median pre-treatment serum hCG level of participants was 2039 IU/L, compared to 2255 IU/L in our contemporaneous control (p=0.82); 8 participants and 6 controls had serum hCG levels of >3000 IU/L, the upper limits of which were 8575 and 4985 IU/L, respectively. The median time (in days) to resolution (defined as a serum hCG level of <15 IU/L) was 30 days for participants and 37 days for controls (p=0.09). There was no difference in baseline characteristics between study participants and contemporaneous controls (Table 7.1).

Table 7.1: Baseline characteristics of participants and controls.

Characteristic		Participants (n=28)	Controls (n=24)	*p* Value	Test
Age (Mean ± SEM)		30.0 ± 1.0	30.3 ± 1.2	0.739	t Test
Pre-treatment hCG (IU/L) (Mean ±SEM)		2580 ± 336.1	2327 ± 186.9	0.817	t Test
Parity	**Nulliparous**	16	14	1.000	Fisher's exact
	Multiparous	12	10		
BMI (weight kg/height m^2) (Mean ±SEM)		27.3 ± 1.1	25.7 ± 1.3	0.223	t Test
Previous EP		6	1	0.107	Fisher's exact
History of PID		4	2	0.674	Fisher's exact
History of Smoking		14	6	0.089	Fisher's exact

Safety and tolerability data was only collected for participants receiving combination gefitinib and methotrexate. There were no serious adverse events relating to the study intervention. The only unscheduled hospital presentations and/or admissions related to abdominal pain from the pre-existing condition of ectopic pregnancy (CTCAE grade III), presumed to be rupturing in 4 participants who were subsequently managed surgically. Abdominal pain was also the most commonly reported non-serious adverse event, with 24/28 participants reporting this at some stage during treatment. If requiring symptomatic relief, abdominal pain was managed with paracetamol and/or judicious use of ibuprofen.

Rash was the next most common non-serious adverse event. Nineteen participants developed a rash during treatment, most commonly involving the face, neck and upper torso, areas most commonly exposed to UV light. The rash was papulopustular or acneiform, and frequently associated with dry skin and pruritus (CTCAE grades I-II). It appeared in participants between day 1 and day 11 of treatment, with a median onset on day 7. It resolved spontaneously or with

symptomatic treatment in all cases between 3 and 57 days after appearance, with a median resolution after 17 days.

Other common side effects in order of occurrence were nausea (17/28) and diarrhoea (16/28). Vomiting only accompanied nausea for 2 participants and was treated with anti-emetics. Diarrhoea experienced by participants predominantly related to loose stools rather than increased frequency of motions and required no treatment. Lethargy was a feature for 11/28 participants, transient dizziness for 10/28 and mildly deranged liver function tests for 6/28. Liver function test abnormalities all resolved prior to normalisation of hCG for affected participants.

All participants had resumed regular menstrual cycling when questioned at 6 months of follow-up. Fertility outcomes for participants include 6 spontaneous pregnancies. At the time of writing, 1 has resulted in a live birth, 1 was a subsequent ectopic pregnancy in the ipsilateral tube (treated surgically) and 4 are ongoing pregnancies at various gestations.

7.4 Discussion

This phase II study was primarily designed to evaluate the efficacy of combination gefitinib and methotrexate to treat an extended range of stable ectopic pregnancies. We recruited women with pre-treatment serum hCG levels of between 1000-10000 IU/L and included women with evidence of embryonic cardiac motion and any gestational sac size, as visualised on TVUS. The secondary outcomes of this study sought further evidence of the safety and tolerability of combination gefitinib and methotrexate to treat ectopic pregnancy in women of reproductive age.

We successfully treated 86% (24/26) recruited women. Our success rate is comparable to the reported efficacy of 'single-dose' methotrexate treatment for ectopic pregnancy of around 90%, bearing in mind that up to 20% of these women required additional doses of methotrexate to resolve their pregnancies (Barnhart et al., 2003, Lipscomb et al., 2005). In an analysis comparing participants (n=28) with contemporaneous controls treated with single-agent methotrexate (n=24),

combination treatment was significantly more likely to result in treatment success, without recourse to additional doses of methotrexate and/or surgical intervention.

The time taken (in days) to achieve resolution (defined as a serum hCG of <15 IU/L) was assessed as a secondary outcome and surrogate marker of efficacy. This was not statistically different between participants (30 days) and contemporaneous controls (35 days). The study was not powered to show a difference in this outcome measure, and the relative numbers of participants and controls were likely too small to do so. Nevertheless, even a non-statistical difference in duration of follow-up may have significant implications for health care costs and economic evaluations of different treatment strategies. Single-dose methotrexate has been shown to only be cost-effective compared to laparoscopic salpingectomy at pre-treatment serum hCG levels of <1500 IU/L, with much of the initial savings in direct costs (theatre usage and hospital stay) lost to prolonged follow-up and surgical re-intervention (indirect costs) (Mol et al., 2008). Despite the initial increase in direct costs due to the cost of gefitinib, combination treatment could improve the cost-effectiveness of non-surgical treatment of ectopic pregnancy by improving the success rates and potentially decreasing the length of follow up required for women with pre-treatment serum hCG levels >1500 IU/L.

Importantly, 8/28 participants and 6/24 controls had a pre-treatment serum hCG of >3000 IU/L, the upper limits of which were 8575 and 4985 IU/L, respectively; knowing that the higher the pre-treatment serum hCG level, the less likely a woman is to achieve medical treatment success, it is probable that the selection of study participants well outside of hospital protocols produced a biased sample with which to compare to contemporaneous controls. Hence a subsequent randomised controlled trial is necessary to truly establish whether or not treatment success and time to resolution are improved with combination gefitinib and methotrexate treatment, compared to treatment with methotrexate alone. Additionally, the presence of embryonic cardiac motion would

still appear to be a relative contraindication to medical treatment, as both these participants were ultimately treated surgically.

This phase II study provides further substantial evidence of the safety and tolerability of combination gefitinib and methotrexate to treat women with tubal ectopic pregnancies. There were no undue toxicities observed from combining the two drugs in women of reproductive age. The most commonly reported side effects were consistent with the known side-effect profile for both drugs, namely rash and diarrhoea associated with gefitinib therapy and mild, clustered gastrointestinal symptoms attributable to methotrexate treatment. These side effects occasionally required short-term symptomatic management including administration of analgesia, anti-emetics and topical creams. All side effects resolved upon completion of treatment with no long-term sequelae reported at follow-up. The only serious adverse events occurring during study treatment all consisted of severe abdominal pain and hospital re-presentation related to possible rupture of the pre-existing condition, ectopic pregnancy.

All women participating in this study had resumed regular menstrual cycling at follow-up. This was critical as a marker of ongoing fertility after treatment with a new class of drugs (EGFR inhibitors) in this age group (18-45). A number of subsequent spontaneous conceptions, including one live birth and 4 on-going pregnancies at various gestations, provide additional evidence for the safety of combination treatment, and gefitinib therapy in particular, to women of reproductive age.

In summary, this phase II study provides further evidence for the safety and efficacy of combination gefitinib and methotrexate to treat ectopic pregnancy. It suggests that that combination of gefitinib and methotrexate is significantly more effective than single-dose methotrexate to treat ectopic pregnancy when compared to a contemporaneous cohort of women, and rejects the *a priori* hypothesis that the true efficacy of combination gefitinib and methotrexate is ≤70%. The use of combination gefitinib and methotrexate merits further investigation and progression to a larger,

randomised-controlled study directly comparing combination treatment with single-agent methotrexate for the treatment of ectopic pregnancy.

Chapter 8: Combination Gefitinib and Methotrexate to Treat Persistent Gestational Trophoblastic Disease: A Phase I Clinical Study

8.1 Abstract

In Chapter 8 I present the findings of a phase I single-arm open-label dose-escalation toxicity study using combination gefitinib and methotrexate to treat persistent gestational trophoblastic disease (pGTD). The study sought to assess the safety of combination treatment and potentially improve treatment efficacy through more rapid resolution of pGTD (as evidenced by more rapidly falling serum human chorionic gonadotrophin (hCG) levels) and reduced number of chemotherapy cycles required to achieve cure.

We aimed to recruit 12 women with low-risk pGTD recommended for single-agent chemotherapy through a tertiary referral centre in Melbourne, Victoria. Women were treated with a standard 8-day multi-dose mg/kg methotrexate protocol, and increasing duration of oral 250mg gefitinib co-treatment, such that the first 3 women recruited (cohort 1) received just one dose of gefitinib on day 1, the next 3 women recruited (cohort 2) received once-daily oral gefitinib on days 1-3 and the last 6 women recruited (cohort 3) received once-daily oral gefitinib for 7 days, commencing on day 1. Women were reviewed clinically second daily during active treatment and again at day 14, prior to commencement of subsequent methotrexate cycles. Safety and toxicity was also monitored biochemically on days 1, 5 and 12. Treatment success was defined as serum hCG of <2 IU/L.

The primary outcome measure was safety and toxicity profiling of combination gefitinib and multi-dose methotrexate treatment. All adverse events were documented and graded according to the Common Terminology Criteria for Adverse Events (CTCAE) version 4.03, published by the National

Cancer Institute, National Institutes of Health, U.S Department of Health and Human Services June 14, 2010 (Appendix D). Secondary outcome measures pertaining to efficacy included number of participants treated successfully with combination gefitinib and methotrexate and the number of chemotherapy cycles required to achieve cure; this was compared to a contemporaneous cohort of potentially eligible women treated with single-agent methotrexate at the recruitment site, who for various reasons were not recruited to the study.

We recruited 6 women with pGTD during the conduct of this study. The study was terminated without reaching the recruitment target and therefore without administering our maximum intended treatment dose. Combination gefitinib and methotrexate treatment was nevertheless successful in treating 5/6 (83%) of participants; 1 woman developed resistant disease and was subsequently treated with an established combination chemotherapy protocol. There was no difference in treatment outcomes and number of chemotherapy cycles between participants and controls. The study reported no undue toxicity from treating women with combination gefitinib and multi-dose methotrexate.

This study is the first to apply combination gefitinib and methotrexate to the treatment of low-risk pGTD. It suggests the combination is safe and well tolerated in a small number of women administered up to 3 doses of oral gefitinib. The study provides limited proof-of-principle of the concept in humans, and proposes that combination gefitinib and multi-dose methotrexate for treatment of pGTD merits further investigation in a larger, randomised controlled trial.

8.2 Introduction

In 1956, methotrexate effected the first cure of a solid tumour in humans when it was used to treat a terminally ill 24 year old woman with metastatic choriocarcinoma (Hertz et al., 1956). The condition was previously fatal in more than 90% of women in whom it was diagnosed (Yarris and Hunter, 2003). Since then, methotrexate has revolutionised the treatment of gestational trophoblastic

neoplasias (GTNs), such that they remain among the most curable cancers in medicine (Berkowitz and Goldstein, 1996, Seckl et al., 2013).

GTNs comprise a group of malignancies, all trophoblastic (fetal) in origin. GTNs most commonly occur after a molar pregnancy, where hCG surveillance is used to detect persistent and/or invasive gestational trophoblastic disease (pGTD), however, other forms of GTN can arise after any pregnancy event (Berkowitz and Goldstein, 1996). The incidence of molar pregnancy in Australia is reported as 1:1500 pregnancies by Cancer Australia, with similar incidences reported in Europe and North America (Steigrad, 2003, Seckl et al., 2013). There are two types of molar pregnancy or hydatidiform mole, classified according to genotype; 1) partial hydatidiform moles (PHMs), which have a triploid genotype including genetic material from an ovum fertilised by two (80%) or a diploid sperm (20%); and 2) complete hydatidiform moles (CHMs), which occur when maternally derived genetic material is lost or extruded from the ovum, resulting in a completely paternally derived or androgenetic diploid genotype (see Figure 2.3) (Seckl et al., 2013). These molar pregnancies further differ in their likelihood of persisting, with remaining viable and indeed invasive trophoblastic tissue detected by a plateauing or rising serum hCG level. Approximately 0.5-1% of PHMs and 15% of CHMs progress to pGTD and require treatment (Seckl et al., 2013). The remaining and significantly rarer GTNs consist of choriocarcinoma (approximately 1 in 50,000 deliveries in the UK), placental site trophoblastic tumour (PSTT) (approximately 0.2% of gestational trophoblastic disease) and epithelioid trophoblastic tumour (ETT) (Seckl et al., 2010).

Unlike any other cancer, treatment of GTN is based upon a prognostic scoring system rather than anatomical staging. The International Federation of Gynecology and Obstetrics' (FIGO) Prognostic Score Index (PSI) for classification of GTN uses surrogate markers such as age, time elapsed since the index pregnancy, serum hCG, site, size and location of metastases, as well as prior chemotherapy resistance, to determine a woman's likelihood to respond to single-agent chemotherapy with either methotrexate or actinomycin D (FIGO, 2009). A PSI of 0-6 is classified as

low-risk and amenable to single-agent treatment, whereas a high-risk PSI of ≥7 is associated with a poor likelihood of cure with single-agent treatment, indicating that the woman should be treated with combination chemotherapy from the outset (Seckl et al., 2013). For approximately 95% of women developing pGTD after a molar pregnancy, the PSI is low-risk (Seckl et al., 2013).

Despite overall cure rates of women with low-risk GTN approaching 100% (Seckl et al., 2013), a large, retrospective review of 618 low-risk women treated with single agent methotrexate chemotherapy revealed that 75% of women with a PSI between 0-1 and only 31% with a PSI of 6 achieved cure with this regimen (Sita-Lumsden et al., 2012). Consequently, a large proportion of women with even low-risk disease will develop resistance to methotrexate and need alternative treatment with actinomycin D, exposing them to a concomitant increased side effect profile (Soper, 2006). Ultimately, if both single-agent regimens fail to achieve cure, even low-risk women may require combination chemotherapy for treatment of their resistant GTN.

Combination chemotherapy for high-risk and resistant GTN most commonly consists of EMA-CO – etoposide, methotrexate and actinomycin D, alternating weekly with cyclophosphamide and vincristine (Oncovin™, Eli Lilly and Company). The reported 5-year overall survival rates with use of this regimen for treatment of high-risk GTN are between 75-90% (Seckl et al., 2013). Use of EMA-CO for treatment of women with GTN, however, is associated with significantly greater toxicity and long-term sequelae compared to single-agent regimens, including alopecia, neutropenia, earlier menopause by 3 years and importantly, an increased risk of secondary tumours (RR 1.5, 95%CI 1.1-2.1; $p<0.011$) (Rustin et al., 1996). This is in contrast to the 2.3% of women treated with single-agent methotrexate, who may experience minor mucosal and serosal complaints such as mouth ulcers, dry eyes and pleuritic chest pain (McNeish et al., 2002).

Ultimately, there is significant scope to improve the treatment of pGTD with a safe and effective protocol that maximises cure rates whilst minimising resistance, duration of treatment and long-term toxicity. pGTD parallels ectopic pregnancy in terms of its trophoblastic origin and response to

treatment with methotrexate. Importantly, the literature also confirms the abundance of the epidermal growth factor receptor (EGFR) in gestational trophoblastic disease tissues, with some evidence of greater or altered expression compared to healthy placental tissues (Tuncer et al., 2000, Sebire and Seckl, 2010, Jacob and Balaram, 2012). In this chapter's work therefore, we sought to apply the principle of combination gefitinib and methotrexate treatment for ectopic pregnancy to a novel but highly related condition - pGTD. Specifically, we hypothesised that by adding gefitinib to methotrexate, more women with low-risk pGTD would be successfully treated without recourse to actinomycin D and/or EMA-CO, and that the duration of treatment and follow up might be significantly reduced, allowing women to conceive again, sooner.

8.3 Methods

We designed a single-arm, open-label phase I dose-escalation study aiming to recruit 12 women with low-risk pGTD, who would normally be recommended for single-agent methotrexate chemotherapy.

Because of its rarity as a clinical entity and the requirement for long-term follow-up, best practice management guidelines for gestational trophoblastic disease (GTD) recommend that each case of molar pregnancy and/or GTN be referred to a specialist regional centre, which in turn should maintain a register of affected women (RCOG, 2010, Seckl et al., 2010). This allows for the most experienced clinicians to provide up-to-date, evidence-based care for women with GTD, and minimises loss to follow-up. The Hydatidiform Molar Registry and specialist treatment centre for GTD in Victoria, Australia, is located at the Royal Women's Hospital (RWH) in Melbourne. The centre treats an average of 12 women a year with pGTD. My supervisor and I formed a collaboration with senior clinical, allied health and clerical staff at the centre in order to conduct this study. We prepared and submitted an application to the RWH Human Research Ethics Committee (HREC) to conduct the study at the site (approval number 11/12) and prospectively registered the

study with the Australian and New Zealand Clinical Trials Registry (ANZCTR) (study number ACTRN12611000104954).

Potentially eligible women were aged between 18-45 and identified at a weekly review of all registered patients with GTD undertaking hCG surveillance. Women were deemed to require further treatment if their serum/urinary hCG level had either plateaued or risen, as defined by FIGO; 1) hCG level plateau: plus or minus 10% of baseline hCG recorded over a 3-week duration (days 1, 7, 14, 21) or 2) hCG level rise: greater than 10% above baseline recorded over a 2-week duration (days 1, 7, 14) (Ngan et al., 2003). Once identified, potential participants were contacted by phone, advised of their results and invited to attend the centre for specialist imaging, review and treatment. A plain language statement describing the study and inviting women to participate was either posted or emailed to potential participants prior to attendance. Once a senior specialist decision had been made to progress with methotrexate single-agent treatment, potential participants were reviewed and screened by an investigator, and informed written consent was obtained if the woman was willing to participate.

Our exclusion criteria were largely based on the known side-effect profile of gefitinib with the express purpose of minimising risk of significant toxicity, in addition to methotrexate administration safety practice points. Exclusion criteria included abnormal liver, renal or haematological indices noted on baseline blood tests, a history of any significant pre-existing dermatological, gastrointestinal or pulmonary disease, especially interstitial lung disease (ILD) and lung cancer, and Japanese ethnicity. As relates to the last two exclusion criteria, post-marketing surveillance data for gefitinib suggests that ILD occurs in less than 1% of white patients and in 5% of Japanese patients (Cataldo et al., 2011). ILD was fatal in a third of cases associated with gefitinib therapy (Cohen et al., 2004), and although the association with Japanese race is not fully characterised, for the purposes of this study we felt it best to minimise the risk to women of this ethnicity.

The study treatment protocol included a 14-day multi-dose methotrexate treatment cycle with

intramuscular administration of 1mg/kg doses on days 1, 3, 5 and 7, with 7.5mg folinic acid (leucovorin) rescue orally on days 2, 4, 6 and 8. This was followed by a 6-day break with clinical review on day 14. In addition to this, oral 250mg gefitinib was co-administered in a stepwise fashion such that the first three women recruited (cohort one) received gefitinib on day 1, the next three women recruited (cohort two) received gefitinib daily for the first 3 days, and the last six women to be recruited (cohort three) were to receive gefitinib once-daily for 7 seven days; this co-treatment pertained only to the first methotrexate treatment cycle.

Observation and monitoring for adverse events consisted of clinical review second daily to day 8 (on presentation for administration of methotrexate), as well as on day 14, prior to commencement of the next methotrexate chemotherapy cycle. Blood tests monitoring haematological, renal and hepatic indices were performed at baseline on day 1 and repeated on day 5 and day 12 of treatment. Participants were also called on alternate days to clinical presentation to document any adverse events, which were classed and graded according to the Common Terminology Criteria for Adverse Events (CTCAE) version 4.03, published by the National Cancer Institute, National Institutes of Health, U.S Department of Health and Human Services June 14, 2010 (Appendix D).

Treatment efficacy was measured by the number of women achieving remission with combination treatment, as well as the number of treatment cycles required to achieve cure, which was defined as a sustained serum hCG level of <2 IU/L. Women were required to have 2 consolidation cycles after achieving a hCG of <2 IU/L, so the minimum number of treatment cycles was 3. If participants demonstrated resistant disease despite treatment, signified by a plateauing or rising serum hCG level, they were withdrawn from the study and referred for specialist review to consider subsequent treatment with either actinomycin D or EMA-CO.

Study data was compared to that of a contemporaneous cohort of women treated with single-agent methotrexate at the RWH during recruitment, who declined participation or for other reasons were not recruited to the study. Long-term follow-up of women was conducted by phone for up to 18

months after completion of treatment, to document return of regular menstrual cycles and subsequent fertility outcomes. Statistical analysis, including t test and Fisher's exact test analyses of baseline characteristics and treatment outcomes, was performed using Prism 6.0 (GraphPad, La Jolla CA, USA).

8.4 Results

The study commenced on the 1st of September 2011 and we recruited 6 eligible women with pGTD before the study was terminated on the 31st May 2013. We did not attain our planned recruitment of 12 participants. During the 20 months that the study was open for recruitment, 15 women were referred to and treated at the RWH for pGTD. Of the 9 potentially eligible women who presented during this period but were not recruited to the study, 5 declined participation, 2 lived in rural Victoria and completed their treatment in regional centres (in consultation with specialists at the RWH), one woman did not speak English and one further woman was excluded by senior staff due to behavioural issues encountered at initial consultation. Hence, only 11 women with pGTD suitable for recruitment to study were referred to the RWH during the conduct of this study.

All of the 6 recruited women completed their treatment protocols and 5/6 (83%) were treated successfully with combination gefitinib and multi-dose methotrexate. One participant required combination chemotherapy for resistant disease as demonstrated by a plateauing serum hCG level. Treatment and outcome data for the 7 non-recruited women treated with methotrexate only at the RWH was also collected (contemporaneous control group), all of whom were successfully treated. Table 8.1 compares the baseline characteristics and treatment outcomes of participants and contemporaneous controls treated for pGTD.

Table 8.1: Comparison of baseline characteristics, disease severity and treatment outcomes between participants and contemporaneous controls.

	Participants (n=6)	**Controls** (n=7)	***p* Value**	**Test**
Age (±SEM)	36.4 (±3.9)	36.4 (±3.9)	t Test	0.911
Ethnicity: Caucasian	5	3	0.265	Fisher's exact
SE Asian	1	4		
Gravidity (Median)	2.5	2	0.818	t Test
Parity (Median)	0.5	1	0.615	t Test
Previous GTD	1	0	1.000	Fisher's exact
Gestation (Median)	8	8*	0.267	t Test
hCG (IU/L) Median (Range)	5888 (74-25,267)	1393 (40-13,496)	0.713	t Test
PSI (Median)	1.5	0.5#	0.297	t Test
Cycle no. Median (Range)	7 (4-10)	6 (4-6)	0.695	t Test
Successful treatment (%)	5 (83%)	7 (100%)	0.462	Fisher's exact

* 3 data points missing
# 1 data point missing

There were no differences in baseline characteristics or treatment outcomes between participants and the contemporaneous control group. There was no difference in the severity of disease treated, with both groups demonstrating similar (low-risk) PSIs. The median pre-treatment hCG of participants (n=6) was higher at 5888 IU/L compared to that of contemporaneous controls 1393 IU/L (n=7), however, this was not statistically significant. The study did not demonstrate a difference in treatment outcomes or duration of treatment when comparing combination gefitinib and methotrexate and single-agent methotrexate treatment for pGTD, with women requiring a median of 7 and 6 cycles of treatment, respectively.

Safety and toxicity data was only available for recruited participants. Two women made 3 unscheduled presentations to hospital during treatment, all relating to severe pleuritic chest pain (CTCAE grade III). None of these presentations resulted in admission, and the women were

discharged home with analgesia after assessment. The most common side effects related to gastrointestinal discomfort, with 5/6 (83%) participants experiencing nausea and/or vomiting, 4/6 (67%) experiencing at least one episode of diarrhoea and 3/6 (50%) experiencing abdominal pain (CTCAE grades I-II). A papulopustular rash was a feature for 4/6 (67%) participants during treatment (CTCAE grades I-II). Other documented side effects with an incidence of at least 2/6 (33%) included dizziness, lethargy, dry eyes and mouth ulcers (CTCAE grades I-II). All adverse events resolved upon completion of treatment and occasionally required symptomatic treatment with analgesia, anti-emetics, topical anaesthetic, eye drops and/or moisturiser.

8.5 Discussion

In this study, we sought to apply the theory and pre-clinical experience of combination gefitinib and methotrexate treatment to a novel disorder of trophoblastic tissue – pGTD. The study required us to successfully collaborate with a multi-disciplinary team, prepare and receive approval for our HREC application, and conduct a clinical trial in accordance with good clinical practice. We successfully treated 5 women with pGTD using the study protocol, and completed up to 18 months of follow-up. The study demonstrated that a 1-3 day course of oral 250mg gefitinib in combination with the first multi-dose mg/kg methotrexate chemotherapy cycle for treatment of pGTD causes no untoward toxicity.

GTD is rare, with a reported incidence of 1:1500 pregnancies in Australia (Steigrad, 2003). In the 5 years prior to commencement of the study, the RWH treated an average of 12 women a year with pGTD, ranging from 7 women in 2006 to 23 women in 2010 (unpublished data). At the time HREC approval was first sought in January 2011, we had designed a phase I dose-escalation protocol aiming to recruit 12 women with pGTD over 2.5 years. We anticipated needing a 20-70% recruitment rate based on these figures. We ultimately received HREC approval in May 2011 and the study was opened for recruitment in September 2011, with further delays attributable to contract

negotiations, Therapeutic Goods Administration of Australia notification and leave. During the conduct of the study we achieved a recruitment rate of 55% of referred women (6/11), and 40% of all women treated for pGTD in Victoria between September 2011 and May 2013 (6/15).

Owing to the small number of women recruited to the study, we did not achieve our maximum intended dose of 7 days of oral 250mg gefitinib in combination with the first cycle of multi-dose mg/kg methotrexate chemotherapy. In designing the study, we had decided on a subsequent phase I dose-escalation protocol combining gefitinib and methotrexate for 2 reasons: 1) the primary phase I study combining gefitinib and methotrexate to treat ectopic pregnancy (Chapter 5) was still in progress, with results pertaining to safety and toxicity therefore inconclusive, and 2) because methotrexate treatment of pGTD involves a multi-dose, repeated protocol with significantly greater exposure to the grandfather drug, therefore increasing the risk of any interactive toxicity with gefitinib. We therefore felt it prudent to again gradually increase the amount of co-administered gefitinib in this protocol. Ultimately, however, given that up to 10 cycles of methotrexate were administered to participants and up to 16 cycles to controls, co-administering gefitinib in only the first cycle was likely of marginal clinical significance. The optimal timing of gefitinib administration in relation to methotrexate and folinic acid dosing to maximise the supra-additive treatment effect seen pre-clinically, is also something that merits further investigation.

This is the first known attempt to combine gefitinib and methotrexate for the treatment of low-risk pGTD. This small proof-of-principle study does not provide sufficient evidence to show that combination treatment is more effective than single-agent methotrexate chemotherapy as a treatment for pGTD, but it does suggest that the combination is safe and causes no undue toxicity in women with the condition. In addition to the likely under-dosing of gefitinib mentioned previously, an important contributor to the lack of significant findings was the very small absolute number of participants and controls being compared. Further investigation of the potential of gefitinib to enhance the efficacy of multi-dose methotrexate chemotherapy to improve treatment of low-risk

GTN is merited, with a larger, preferably randomised clinical study in a much larger centre, with capacity to continue the study for the necessary time period to achieve sufficient recruitment targets of this rare but important condition.

Chapter 9: Discussion

Ectopic pregnancy remains an important cause of maternal morbidity and mortality (Cantwell et al., 2011, Sullivan et al., 2007). Significant medical advances of the 20th century including non-invasive diagnosis with transvaginal ultrasound (TVUS), improved peri-operative care and surgical techniques, as well as the development of a successful medical treatment option, all served to shift the therapeutic intent from preservation of life to preservation of fertility (Lurie, 1992). Despite this, women still regularly succumb to the condition, particularly in developing countries with poor access to resources, but also in developed countries with the highest standards of medical care (Leke et al., 2004, Cantwell et al., 2011). In light of this, there is still significant scope to improve the diagnosis and treatment of ectopic pregnancy, and this has been the central tenet of my PhD.

The trophoblastic invasion and implantation of pregnancy shares many parallels with cancer growth, not only in terms of behaviour, but also through common molecular pathways (Ferretti et al., 2007). One of the most important of these is the signalling cascade of the epidermal growth factor receptor (EGFR), which when activated or constitutively expressed, causes downstream cellular proliferation, angiogenesis and induction of anti-apoptotic cell mechanisms (Cataldo et al., 2011). EGFR is highly expressed in human placental tissues, as evidenced by data that can be mined from publically available mRNA microarray repositories such as BioGPS (www.biogps.org) (Su et al., 2004, Wu et al., 2009). Furthermore, EGFR activation has been shown to be critical for normal placental development and the placenta's ability to resist toxic and hypoxic insults (LaMarca et al., 2008, Wolff et al., 2007, Johnstone et al., 2005). Hence, we hypothesised that the placenta would be an attractive drug target for EGFR inhibition in conditions of disordered trophoblastic tissue growth.

Placental development is normally a tightly regulated and limited process, however, there are instances when this goes awry: in ectopic pregnancy, implantation occurs away from the endometrial cavity of the uterus and places the woman at significant risk of life-threatening

haemorrhage; in gestational trophoblastic neoplasia (GTN), trophoblastic tissues have lost their regulatory mechanisms and take on a truly malignant phenotype; and in the spectrum of disease that is placenta accreta/increta/percreta, an otherwise normal placenta loses its cell-contact inhibition and invades beyond the usual tissue boundaries of pregnancy. These are all conditions in gynaecology that require prompt and effective treatment, and could potentially benefit from EGFR inhibition.

The main aim of my PhD has been to begin translating the theory of EGFR inhibition for the treatment of trophoblastic tissue disorders into clinical practice, by way of early-phase human clinical trials. Previous laboratory work performed by my colleagues in the Translational Obstetrics Group has demonstrated the supra-additive treatment effect associated with combined gefitinib and methotrexate administration to trophoblastic cell lines and primary trophoblastic tissue, both *in vitro* and *in vivo* (Nilsson et al., 2013). We decided to focus the investigation of clinical combination therapy on the most common disorder of trophoblastic tissue, ectopic pregnancy.

I have investigated ways of improving the diagnosis and treatment of ectopic pregnancy in a number of ways. In chapter 3 I reviewed the current clinical monitoring protocol for medical treatment of ectopic pregnancy, to ascertain if the serum hCG trend between day 1 and day 4 could provide earlier prognostic information about the likelihood of medical treatment success. In chapter 4, I examined the potential of two molecules present in maternal serum – adrenomedullin (ADM) and macrophage inhibitory cytokine-1 (MIC-1) – to serve as novel biomarkers of ectopic pregnancy, to assist clinicians in more accurately and more promptly diagnosing the condition. Chapters 5, 6 and 7 present the findings of early-phase human clinical trials assessing the safety and efficacy of combination gefitinib and methotrexate in the treatment of both tubal and non-tubal ectopic pregnancies. Finally, in chapter 8, I seek to extend the possible clinical application of combination gefitinib and methotrexate to the treatment of persistent gestational trophoblastic disease (pGTD), also by way of conducting an early-phase human clinical trial. What follows is a discussion of each

of these areas of investigation and how the findings of my PhD thesis have contributed to the fields of ectopic pregnancy and GTN research, and the clinical management of ectopic pregnancy and pGTD.

9.1 Early Prediction of Medical Treatment Success in Ectopic Pregnancy

The current monitoring protocol for the medical treatment of ectopic pregnancy was proposed concurrently with the first outpatient use of methotrexate for the treatment of this condition; Stovall *et al.* (1991) proposed a baseline serum hCG level on day 1 of treatment, followed by serial hCG sampling on day 4, day 7 and weekly thereafter until resolution of the ectopic pregnancy (serum hCG of <15 IU/L). A ≥15% fall in serum hCG between day 4 and day 7 was taken to indicate successful treatment (Stovall et al., 1991). This test of single-dose methotrexate treatment success in ectopic pregnancy has been independently verified and shown to have a positive predictive value (PPV) of 93% (Kirk et al., 2007). Nevertheless, the protocol requires an interval of 1 week between treatment administration and any indication of treatment efficacy, a long period of uncertainty for both patient and clinician in the context of managing a potentially life-threatening condition.

Recent examination of the predictive value of an early falling serum hCG level between day 1 and day 4 of treatment suggested this to be 100% predictive of medical treatment success in 30 women administered single-dose methotrexate for ectopic pregnancy (Nguyen et al., 2010). This was an exciting potential improvement in the medical management of ectopic pregnancy, utilising the day 4 serum hCG at the time of its collection and providing meaningful clinical information 3 days earlier than is currently possible. This study required independent validation, especially given the very small sample size on which the results were based.

In two separate clinical studies I was able to show that a falling serum hCG level between day 1 and day 4 is between 85-89% predictive of single-dose methotrexate treatment success in ectopic pregnancy (Skubisz et al., 2011, Skubisz et al., 2013b). This work ultimately represents data

collected from 251 women and provides a strong body of evidence on which to base clinical counselling; women with a falling serum hCG between day 1 and day 4 of their single-dose methotrexate treatment of ectopic pregnancy can be reassured that the treatment is working, and that they have an overwhelming likelihood of being cured with no further intervention, medical or surgical. Although it does not materially change the outcome of treatment, I believe this is a significant advance in the care provided to women with ectopic pregnancy, who endure an anxious and prolonged wait for advice as to whether their chosen treatment option is effective or not.

The negative predictive value (NPV) (46-58%) of a rising serum hCG level between day 1 and day 4 of single-dose methotrexate treatment of ectopic pregnancy is not clinically useful. In this case, we recommend clinicians should continue with the standard monitoring protocol to determine treatment efficacy and the need for further doses of methotrexate. This research raises the question as to whether single-dose methotrexate treatment of ectopic pregnancy success rates could be improved by earlier recognition of potentially unsuccessful treatment, and therefore earlier administration of additional doses of methotrexate to improve outcomes (i.e. on day 4, once it is apparent the serum hCG levels have risen since day 1). This hypothesis merits verification in clinical studies.

9.2 Potential Biomarkers of Ectopic Pregnancy

The discovery and characterisation the human chorionic gonadotrophin (hCG) molecule, and in particular the development of a sensitive and specific enzyme immunoassay (EIA) for hCG in the latter half of the 20th century, have produced an as yet unparalleled clinical biomarker, whose serum levels appear directly correlated to the amount of viable trophoblastic tissue (Visconti and Zite, 2012). Despite being almost exclusively specific to pregnancy, a single serum hCG level cannot distinguish between different types of pregnancy. Trends in serum hCG levels over 48 hours can potentially help distinguish between a viable and non-viable intrauterine pregnancy (IUP), with a

doubling of the hCG level expected in the former and a fall of >20% in the latter; however, even meeting these parameters does not exclude ectopic pregnancy (Fritz, 2011). Serum hCG levels also determine the sensitivity of TVUS in identifying an IUP, with a pregnancy of unknown location (PUL) at serum hCG levels >2000 IU/L highly suspicious for, but still not diagnostic of, ectopic pregnancy (Fritz, 2011).

Research into a specific biomarker of ectopic pregnancy is therefore driven by clinical need, namely the ability to diagnose or at least exclude an ectopic pregnancy in women where TVUS is non-diagnostic (PUL). Women with a PUL are currently counselled regarding the possible risk of ectopic pregnancy and its sequelae, and followed up with further hCG levels and TVUS. Ideally, the availability of a single diagnostic biomarker, or panel of biomarkers, would minimise risk to the estimated 6-20% of women with PUL who will ultimately be diagnosed with an ectopic pregnancy (Kirk et al., 2014). To date, no identified single molecule has demonstrated sufficient discriminatory ability to diagnose ectopic pregnancy. Research combining multiple biomarkers with differing biological functions has shown greater promise at identifying ectopic pregnancy, the best result so far obtained with a combination of progesterone, vascular endothelial growth factor (VEGF), inhibin A and activin A, giving a sensitivity of 98% and a sensitivity of 100%; however, only 42% of the sample (n=200) could be characterised as either ectopic or intrauterine (Rausch and Barnhart, 2012).

I identified 2 molecules, ADM and MIC-1, as hitherto untested potential biomarkers of ectopic pregnancy. ADM levels have recently been shown to be decreased in the Fallopian tube explants and plasma of women affected by ectopic pregnancy, and furthermore, to negatively affect the cilial beat frequency and smooth muscle contractions of the Fallopian tube (Liao et al., 2012). We also noted that the relationship between tubal ADM levels and the 2 main risk factors of ectopic pregnancy, infection with *C. trachomatis* and smoking, was also unexplored, a potentially important link in proving ADM's role in the pathophysiology of ectopic pregnancy.

MIC-1 was previously reported to be detectable at high levels in amniotic fluid, placental extracts and in maternal sera, where levels increase with advancing gestation; it is thought to promote fetal survival by modulating pro-inflammatory cytokine expression in the uterus (Moore et al., 2000). MIC-1 is significantly decreased in the sera of women with pregnancies that go on to miscarry, well before symptoms arise and whilst TVUS still demonstrates a viable IUP (Tong et al., 2012). I therefore investigated whether ADM and MIC-1 serum levels are altered in ectopic pregnancy compared to other pregnancy outcomes, and whether this could help to diagnose ectopic pregnancy in women with PUL.

Disappointingly, this work did not confirm that serum ADM levels are significantly decreased in women with ectopic pregnancy compared to women with viable IUPs, nor was ADM expression significantly decreased in Fallopian tube explants affected by ectopic pregnancy compared to non-pregnant controls. Correspondingly, serum ADM levels did not prove useful as a biomarker of ectopic pregnancy in our cohort of women with PULs. Furthermore, I found no relationship between ADM expression in Fallopian tube explants exposed to *C. trachomatis* or cigarette smoke, the two main risk factors for ectopic pregnancy. My research therefore, questions the validity of the association between ADM and the pathophysiology of ectopic pregnancy.

When considering these differing findings, it is worth noting the subtle differences in technique between the two bodies of work: whereas I measured serum ADM levels, previous significant differences were based on plasma levels (Liao et al., 2012). Similarly, our control (non-pregnant) Fallopian tube explants were not treated with hormones to mimic the normal pregnancy milieu, as was the case in Liao *et al.* (2012). Whether or not these method differences are sufficient to explain the subsequent differences in results is difficult to say, and the challenge of ectopic pregnancy research remains finding a suitable animal model of disease, as well as an appropriate human control.

My research relating to MIC-1 demonstrated serum levels of this molecule to be significantly lower in women with ectopic pregnancies compared to women with viable IUPs, and furthermore, significantly higher than in ectopic pregnancy compared to pregnancies that went on to miscarry. Although MIC-1 did not function well as a single biomarker of ectopic pregnancy on ROC curve analysis, it did exclude the condition at levels of >1000ng/mL with 100% specificity (Skubisz et al., 2013a). Furthermore, MIC-1 levels were significantly higher in ectopic pregnancy compared to self-resolving PULs, suggesting MIC-1 can differentiate between women requiring treatment compared to those that can safely be followed up without intervention.

MIC-1 could be incorporated into a diagnostic algorithm for the clinical management of PUL; MIC-1 could also form part of a panel of biomarkers for ectopic pregnancy with differing biological function to improve sensitivity and specificity. For example, VEGF has been shown to exclude ectopic pregnancy at levels >200pg/mL, also with 100% specificity (Daponte et al., 2005), and combined with MIC-1, could form a diagnostic test for ectopic pregnancy in women with PUL. These possible clinical applications of my findings would require further investigation and validation in clinical trials.

The diagnosis of ectopic pregnancy can still be elusive; an estimated 8-10% of women presenting with early pregnancy complications are left with an interim diagnosis of PUL after serum hCG quantification and TVUS, even in specialist centres (Kirk et al., 2014). This by definition exposes women to the risk of untreated ectopic pregnancy and its sequelae, despite best current care. Whilst there remains no single diagnostic biomarker of ectopic pregnancy, it is likely that a combination of biomarkers with separate biological functions and differential expression in ectopic pregnancy and viable IUPs will be required to form a clinically useful test, as the molecular circuitry of all pregnancies is too similar for any one molecule to distinguish between them. This work has contributed a novel biomarker of ectopic pregnancy (MIC-1) with evidence of discriminatory ability, which may prove useful in moderating risk in women with a PUL through clinical algorithms, and possibly even in diagnosing the condition as part of a panel of biomarkers.

Meanwhile, it is important for any new theory or finding in scientific endeavour to be reproducible, both in serial experiments and independent research, before being widely accepted. Unfortunately in the case of the vasoactive peptide ADM and its expression and role in the pathophysiology of ectopic pregnancy, this was not borne out by my work. Further independent research is required to ultimately adjudicate whether or not ADM is important to the pathophysiology of ectopic pregnancy, and whether it has any role as a clinical biomarker of this condition.

9.3 Combination Gefitinib and Methotrexate to Treat Ectopic Pregnancy

Whilst a medical treatment option in the form of single-dose methotrexate has formed a routine part of clinical management of ectopic pregnancy since the 1990s, the benefit of this treatment option is only available to an estimated 25-30% of all women diagnosed with the condition (Jurkovic and Wilkinson, 2011). This exposes the majority of women with ectopic pregnancy to the additional risks inherent to surgery, and potentially diminishes their fertility potential.

The reason why surgery is still the mainstay of treatment of ectopic pregnancy is that methotrexate has limited efficacy in resolving the condition; research has consistently shown that the higher the pre-treatment serum hCG level, the less likely methotrexate is to achieve cure, and particularly with levels >5000 IU/L (Menon et al., 2007, Lipscomb et al., 1999). Additional ectopic pregnancy features that are negatively associated with medical treatment success include the presence of embryonic cardiac motion and a large gestational sac size, as determined by TVUS (Farquhar, 2005). Furthermore, surgical management is mandated if a woman shows any signs of tubal rupture and/or haemodynamic instability; this represents between 5-20% of diagnosed women (Pisarska et al., 1998, Fritz, 2011). Hence candidates for medical treatment of ectopic pregnancy are a highly selected and specific subset of women.

Because the large majority of women with ectopic pregnancy are stable at the time of diagnosis (i.e. with no clinical evidence of ectopic pregnancy rupture), many more could be offered medical

treatment if a more efficacious treatment option was available. Hence, the discovery that EGFR inhibition in combination with methotrexate treatment could supra-additively regress trophoblastic tissues provided a unique opportunity to potentially extend the benefits of medical treatment to a larger percentage of women with ectopic pregnancy. This could have flow-on benefits of potentially reduced costs to health services and improved fertility outcomes for women. This ambitious hypothesis has been the main focus of my PhD thesis.

We have now cumulatively treated 48 women with ectopic pregnancies with combination gefitinib and methotrexate, 32 women receiving the complete suggested treatment protocol of 50mg/m^2 IM methotrexate and 7 days of oral 250mg gefitinib. This work has shown the combination to be safe and well tolerated, with no undue toxicity caused. The most commonly reported side effects were consistent with known post-marketing side effect profiles of both drugs, and centred on mild and transient gastrointestinal symptoms. Importantly, there was no suggestion of the development of interstitial lung disease (ILD) in any of the women treated with combination gefitinib and methotrexate, a potentially lethal side effect of gefitinib treatment in non-small cell lung cancer NSCLC.

Perhaps the most notable difference in side effects compared to methotrexate-only treatment of ectopic pregnancy, was the high incidence of an acneiform rash, likely attributable to EGFR inhibition with gefitinib. This occasionally required symptomatic treatment, but did not cause any participant to discontinue treatment and in all cases, completely resolved during follow up. The probable development of an acneiform rash disinclines some women to combination treatment, but is balanced against the risks of surgical management. We saw no correlation with presence of the rash and improved treatment efficacy as has been shown in the oncology setting, however, we likely have insufficient power to examine such an association (Potthoff et al., 2011).

As regards the efficacy of combination gefitinib and methotrexate treatment for ectopic pregnancy, our preliminary data shows it to be more effective at treating higher risk ectopic pregnancies

compared to methotrexate alone, with more rapidly falling serum hCG levels and reduced follow-up times (Skubisz et al., 2013c). A particularly promising area of clinical application of combination gefitinib and methotrexate is for the treatment of non-tubal ectopic pregnancies, which generally present later with much higher serum hCG levels, thereby posing a greater medical treatment challenge. Furthermore, their location of implantation can often make surgical intervention significantly more risky compared to salpingectomy or salpingostomy performed for tubal ectopic pregnancies, with greater fertility implications for the woman. Here we have shown in a case series of 8 cases that combination gefitinib and methotrexate was 100% successful in treating non-tubal ectopic pregnancy with pre-treatment serum hCG levels as high as 48,000 IU/L.

This research recruited women with ectopic pregnancies with evidence of embryonic cardiac motion on TVUS, a feature known to be negatively associated with medical treatment success. This work still showed that the presence of embryonic cardiac motion to be a relative risk factor for medical treatment failure, with 2/4 (50%) of such recruited women experiencing ectopic pregnancy rupture and surgical management; interestingly, both ruptures occurred in tubal ectopic pregnancies whereas both successfully treated cases were non-tubal ectopic pregnancies.

Importantly by seeking to introduce a fertility-sparing treatment in women of reproductive age, we found no impact on resumption of regular menstrual cycles as a surrogate marker of ovulation, and a high subsequent pregnancy rate in women wishing to conceive again. Nevertheless, we are unable to comment on the long-term impact of combination gefitinib and methotrexate treatment on ovarian reserve and time of menopause. This will be important to document and follow up in future clinical research combining gefitinib and methotrexate for the treatment of ectopic pregnancy, as one of the main medical treatment goals is preservation of reproductive potential.

Lastly, the feasibility of combination gefitinib and methotrexate treatment of ectopic pregnancy becoming an accepted clinical treatment option requires a sophisticated cost-benefit analysis. Single-agent methotrexate treatment of ectopic pregnancy has been consistently shown to be less

cost-effective than surgical management at pre-treatment serum hCG levels of >1500 IU/L (Westaby et al., 2012). Gefitinib is still patented by AstraZeneca in most jurisdictions worldwide, and comes at substantial off-label cost to the consumer, over AUD100 (approximately GBP70) per tablet. This must be weighed against the not insignificant costs of surgical treatment and perhaps less obviously, the costs of artificial reproductive technologies (ARTs) that may be required by women who are surgically managed. If combination gefitinib and methotrexate treatment can be shown to improve the medical treatment of ectopic pregnancies with higher serum hCG levels as has been suggested by this body of work, the cost-benefit cut-off of medical treatment too may be increased. The growing availability of gefitinib generics, however, may help to favour the economic argument for combination gefitinib and methotrexate treatment of ectopic pregnancy.

Combination gefitinib and methotrexate has been shown by this research to safe and more effective than single-agent treatment with methotrexate for the treatment of ectopic pregnancy. This PhD provides the proof-in-principle evidence that will form the basis of applications for funding of larger, randomised clinical studies to confirm the clinical benefits of combination gefitinib and methotrexate treatment for ectopic pregnancy, which if proved, will offer a viable medical treatment option to a larger percentage of women diagnosed with the condition. Our team is designing such a larger study, in collaboration with The Birmingham Trials Unit. The current favoured design (still undergoing consultation) will be a randomised controlled trial of 150 women, randomised to methotrexate, or combination methotrexate and gefitinib. The suggested upper serum hCG cut-off for this trial will be 5000 IU/L and the primary outcome will be time to resolution. Importantly, however, the percentage of women that are successfully cured with medical treatment will also be a pre-specified outcome of interest. Should this trial show combination gefitinib and methotrexate is superior to methotrexate alone, it may be sufficient data to translate this new approach as the standard of care for the medical treatment of ectopic pregnancy.

9.4 Combination Gefitinib and Methotrexate to Treat pGTD

Methotrexate has long been used to treat GTN, so exploring the potential of combination gefitinib and methotrexate to improve the treatment of low-risk pGTD was a natural progression from our investigations of its clinical application in ectopic pregnancy. Although there are no good randomised trials comparing the commonly used methotrexate regimens for treatment of low-risk GTN, they all feature significantly higher doses and duration of exposure to methotrexate than the single-dose protocol for treatment of ectopic pregnancy (Alazzam et al., 2009). It was thus prudent to prove the safety of combination gefitinib and high-dose methotrexate treatment for low-risk GTN in a subsequent phase I dose-escalation toxicity study.

Whilst single-agent treatment of low-risk GTN with either methotrexate or actinomycin D achieves cure rates approaching 100% (Seckl et al., 2013), the scope to improve treatment of this condition focuses on 1) reducing toxicity, 2) reducing drug resistance and the need for alternative therapies and 3) reducing duration of treatment and therefore follow up, during which women must refrain from conceiving again. Higher methotrexate exposure results in greater toxicity, with pleuritic chest pain, mucositis, dry eyes, nausea, alopecia and neutropenia being frequently experienced by low-risk GTN patients compared to single-dose methotrexate use in ectopic pregnancy. Some 70% of women with low-risk GTN with a prognostic score index (PSI) of 5-6 will develop single-agent drug resistance and require alternative treatment (Seckl et al., 2013). Furthermore, GTN treatment protocols require women to demonstrate a negative hCG for 2 treatment cycles, implying a minimum treatment duration of 6 weeks with at least 1 year of follow up. The reality is that many women will not conceive again for 2 years, which is particularly significant for older women, who are at increased risk of GTN (Seckl et al., 2013).

As part of my PhD I recruited 6 women with pGTD to receive combination gefitinib and methotrexate. My research again demonstrated no undue toxicity from combination therapy,

however, I did not achieve the maximum intended treatment dose of 7 days of oral 250mg gefitinib in addition to the 8-day methotrexate treatment protocol. GTN is a very rare condition, and with the limitations of time, funding and a relatively small catchment population, I was not able to achieve my recruitment of 12 patients over 2.5 years, despite a recruitment rate of 55%. Consequently, I was not able to show any improved efficacy of combination gefitinib and methotrexate treatment compared to multi-dose methotrexate for the treatment of pGTD.

Whilst I believe combination gefitinib and methotrexate treatment holds much promise for the improvement of treatment of GTN, I was not able to demonstrate this as part of my PhD. Nevertheless, our contribution to the literature regarding combination gefitinib and methotrexate treatment for low-risk GTN introduces the first novel treatment approach since methotrexate and actinomycin D were first trialled in the 1950s and 1970s, respectively (Hertz et al., 1956, Goldstein et al., 1972). There is a sound scientific basis for why this novel treatment approach could improve treatment efficacy whilst reducing treatment toxicity (Nilsson et al., 2013), and combination gefitinib and methotrexate treatment for low-risk GTN merits further clinical investigation. Given the experience of my study, where I attempted to recruit only 12 participants in a single centre (albeit the major referral centre in Victoria, Australia), I would recommend this be a collaborative effort between large specialised GTD referral centres with significantly larger catchment populations.

9.5 Concluding Remarks

The research undertaken as part of this thesis has contributed new approaches to the diagnosis and treatment of ectopic pregnancy and low-risk GTN. In the process, it has significantly expanded my research skills, ranging from laboratory techniques, to data analysis, presentation of scientific findings and the publication process. The results of this PhD have addressed the main aim of translating the theory of combined EGFR inhibition and methotrexate treatment to the clinical treatment of disorders of trophoblastic tissues. It is my hope that this proof-in-principle work helps to

secure further funding for larger, randomised trials to progress combination gefitinib and methotrexate treatment for ectopic pregnancy into common clinical practice; that it generates research interest in a novel molecule, MIC-1, to assist in the diagnosis of ectopic pregnancy, as well as the development of a novel treatment approach for low-risk GTN. The culmination of this would be to prevent further loss of life and reproductive potential in women afflicted with these conditions.

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Appendix A: Single-dose Methotrexate Treatment and Monitoring Protocol for Ectopic Pregnancy

Day	Treatment
0	hCG ± D&C
1	hCG, FBE, U&Es, LFTs, Blood group, MTX
4	hCG
7	hCG

<15% decline in hCG between days 4 and 7, repeat methotrexate;

>15% decline in hCG between days 4 and 7, observe weekly until hCG <15 IU/L.

hCG, human chorionic gonadotropin;

D&C, dilation and curettage;

FBE, full blood examination;

U&Es, urea and electrolytes;

LFTs, liver function tests;

MTX, methotrexate.

Adapted from Stovall *et al.* 1991.

Appendix B: Local Guideline for Use of Methotrexate to Treat Ectopic Pregnancy

Methotrexate for Ectopic Pregnancy for Women presenting to all Southern Health Sites - Guidelines

Who

Obstetric and Gynaecology registrars or above after authorisation for use by the Reproductive Medicine Fellow or Gynaecology consultant on-call.

Nursing Staff administrating methotrexate and caring for the patient.

Expected Outcomes

To ensure the safe management of ectopic pregnancy at Southern Health.

Precautions

Contraindications

- Haemodynamic instability
- Severe immunodeficiency
- Leucopenia, thrombocytopenia, high concentrations of liver enzymes or serum creatinine.

Potential Side Effects

- mild stomatitis, gastritis, enteritis, photosensitivity, diarrhoea, transient elevation in liver enzymes in 3%.
- Rare side effects- alopecia, bone marrow suppression, hepatotoxic effects - 2case reports only.

Eligibility Criteria

- Clinically stable
- Compliant, able and willing to attend for follow-up scans and blood tests
- Early unruptured ectopic pregnancy
  - Under 8 weeks gestation, hCG ≤3000
  - Minimal free fluid on ultrasound scan
  - Ectopic sac ≤3cm
- No fetal heart pulsation in ectopic sac
- Surgery contraindicated or likely to be difficult
- medically unfit
- cornual / cervical ectopic
- Failed surgical treatment
- Persistent ectopic after conservative tubal surgery
- False negative laparoscopy

It is mandatory that once the decision to give methotrexate is made, the following is appropriately documented in the patient notes:

That you have discussed the following with the patient:

1. Risks/side effects of MTX
2. Possible failure and therefore requirement for surgical management
3. Patient information leaflet has been issued

Single Dose Regime

Methotrexate (50mg/m2 IM or 1mg/kg)

Effective in 80-90% of patients

Arrange presentation to Ward One at Moorabbin following day, suggested time 9am. If at site other than Monash Medical Centre Clayton or Moorabbin please contact the Reproductive Medicine Fellow.

Administration of Methotrexate by nursing staff can then proceed.

Follow-up blood test

Day 1	FBE, LFT, U&E, Creatinine, QHCG
Day 4	HCG
Day 7	QHCG, FBE, U&E, Creatinine, LFT

Notes

1. BHCG's are followed up by the Gynaecology Registrar at Moorabbin (pg 145). When writing out the referral for follow up QHCG, on the pathology form clearly state in clinical info "MTX follow-up" and mark urgent and to call Gynaecology Registrar with result.
2. Expect a rising QHCG from Day 1 to 4
3. Day 4-7 QHCG should decrease by at least 15%, if not
   - d/w Reproductive Medicine Fellow
   - consider laparoscopy or repeat MTX
   - USS
4. Day 1-7 QHCG should fall, if not
   - Consider rpt MTX or surgical treatment
5. Weekly QHCG follow up till negative: average 4 weeks. 3-4% rupture despite falling QHCG
6. Avoid pregnancy for 3 months after MTX

Multidose Methotrexate Regimen

Only indicated for cervical ectopic. Must be discussed with Reproductive Medicine Fellow

- Admit patient to hospital, IV access, X-match
- Day 1
  - FBE, U&E, Creatinine, LFT
  - MTX 1mg/kg IM
- Day 2
  - Leucovorin 6mg IM
- Day 3
  - MTX 1mg/kg IM
- Day 4
  - Leucovorin 6mg IM
  - QHCG
- Day 5
  - MTX 1mg/kg IM
  - QHCG
- Day 6
  - Leucovorin 6mg IM
  - QHCG
- Day 7
  - MTX 1mg/skg IM
  - QHCG
- Day 8
  - Leucovorin 6mg IM
  - FBE, U&E, Creatinine, LFT

- Treatment is discontinued when a decline in 2 consecutive daily QHCG as observed *after* 4 doses of Methotrexate. This is followed up by the Gynaecology Registrar at Moorabbin.

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If an adverse event (actual or 'near miss') occurs document details in the health record and complete an incident report.

Strategic policy	Patient care	***ACHS***	Continuum of care
Reviewer	Dr Jason Tan (Gynaecology Fellow) Obstetrics and Gynaecology, Women's & Children's Program	***Last review date***	April 2008
Authoriser	Assoc Prof B. Vollenhoven Director Gynaecology Southern Health	***Next review date***	April 2011

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Appendix C: Phase II Protocol Paper

Open Access Protocol

BMJ open

Phase II single arm open label multicentre clinical trial to evaluate the efficacy and side effects of a combination of gefitinib and methotrexate to treat tubal ectopic pregnancies (GEM II): study protocol

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ABSTRACT

Introduction: Tubal ectopic pregnancy (tEP) is the most common life-threatening condition in gynaecology. tEPs with pretreatment serum human chorionic gonadotrophin (hCG) levels <1000 IU/L respond well to outpatient medical treatment with intramuscular methotrexate (MTX). TEPs with hCG >1000 IU/L take a significant time to resolve with MTX and require multiple outpatient monitoring visits. Gefitinib is an orally active epidermal growth factor receptor (EGFR) antagonist. In preclinical studies, we found that EP implantation sites express high levels of EGFR and that gefitinib augments MTX-induced regression of pregnancy-like tissue. We performed a phase I toxicity study administering oral gefitinib and intramuscular MTX to 12 women with tEPs. The combination therapy did not cause significant toxicities and was well tolerated. We noted that combination therapy resolved the tEPs faster than MTX alone. We now describe the protocol of a larger single arm trial to estimate the efficacy and side effects of combination gefitinib and MTX to treat stable tEPs with hCG 1000–10 000 IU/L

Methods and analysis: We propose to undertake a single-arm multicentre open label trial (in Edinburgh and Melbourne) and recruit 28 women with tEPs (pretreatment serum hCG 1000–10 000 IU/L). We intend to give a single dose of intramuscular MTX (50 mg/m^2) and oral gefitinib (250 mg) daily for 7 days. Our primary outcome is the resolution of EP to non-pregnant hCG levels <15 IU/L without requirement of surgery. Our secondary outcomes are comparison of time to resolution against historical controls given MTX only, and safety and tolerability as determined by clinical/biochemical assessment.

Ethics and dissemination: Ethical approval has been obtained from Scotland A Research Ethics Committee (MREC 11/AL/0350), Southern Health Human Research Ethics Committee B (HREC 11180B) and the Mercy Health Human Research Ethics Committee (R12/25).

Data will be presented at international conferences and published in peer-reviewed journals.

Trial registration number: ACTRN12611001056987.

ARTICLE SUMMARY

Article focus

- Protocol of a study to determine:
- Is combination therapy with MTX and gefitinib effective at resolving tEP?
- Is combination therapy with MTX and gefitinib safe and well tolerated?

Key messages

- Tubal ectopic pregnancy (tEPs) with hCG levels <1000 IU/L respond well to treatment with intramuscular MTX.
- tEPs with human chorionic gonadotrophin (hCG) levels >1000 IU/L require multiple hospital visits to resolve with MTX and often require surgery.
- Novel combination therapy of MTX and the oral EGFR antagonist, gefitinib, could reduce the number of hospital visits required to resolve tEPs with hCG levels >1000 IU/L.

Strengths and limitations of this study

- This is a phase II exploratory efficacy trial, and will be the 'first in man' to examine the efficacy of gefitinib and MTX to treat tEPs with hCG levels >1000 IU/L
- This is a 'single arm' trial. The data will be used to inform a future large multicentre randomised controlled trial comparing combination therapy to conventional management of tEPs.
- The combination therapy described also has potential use in other pregnancy disorders where medical regression of placental tissue could be useful, for example, molar disease and regression of placenta accrete postpartum.

Combination gefitinib and methotrexate for ectopic pregnancy

INTRODUCTION

Tubal ectopic pregnancy (tEP) is the most common life-threatening condition in modern gynaecology in both the developed and developing world.[1 2] tEPs with pretreatment serum human chorionic gonadotrophin (hCG) levels <1000 IU/L respond well to outpatient medical treatment with an intramuscular injection of methotrexate (MTX). Indeed, it has been suggested that these tEPs could be managed safely, and equally efficiently by expectant management without medical intervention.[3–5] In contrast, single-dose MTX is only cost-effective in women with serum hCG concentrations <1500 IU/L.[6] In tEPs with higher hCG levels (>60% of total tEPs), emergency laparoscopic surgical excision (with its inherent risks of damage to visceral organs) remains the most effective treatment. tEPs with higher hCG levels take a significant time to resolve with MTX and require multiple outpatient monitoring visits. There, therefore, exists a need for more effective medical treatments for tEPs with higher hCG levels to reduce the need for emergency surgery and reduce the time to resolution associated with MTX management.

Gefitinib is an orally active epidermal growth factor receptor (EGFR) antagonist licensed to treat non-small-cell lung cancer.[7] In preclinical studies, we found that EP implantation sites express high levels of EGFR and that gefitinib augments MTX-induced regression of pregnancy-like tissue.[8] To translate this into clinical care, we performed a phase I single-arm open-label dose-escalation study administering a combination of 250 mg oral gefitinib (one dose (n=3), three daily doses (n=3), seven daily doses (n=6)) and intramuscular MTX (50 mg/m^2) to 12 women with tEPs.[9] The combination therapy did not cause any significant toxicities, and was well tolerated. We noted that resolution (fall in serum hCG to <15 IU/l) with combination therapy was faster than the median time for tEPs to resolve with MTX alone when compared with contemporaneous controls (21 vs 32 days).

OBJECTIVES

The objective of this trial is to evaluate the efficacy and side effects of combination gefitinib and MTX to treat tEPs (hCG 1000–10 000 IU/L).

METHODS AND ANALYSIS

Study design

Phase II single-arm multicentre open label trial (Edinburgh and two sites in Melbourne).

Subjects

Twenty-eight women with tEPs with hCG levels 1000–10 000 IU/L.

Study settings

We intend to recruit patients from gynaecology departments within NHS Lothian (UK), and Southern Health and Mercy Health networks in Melbourne, Australia.

Sample size

We have calculated the sample size using A'Hern's formula for phase II one-stage designs.[10] For treatment of tEPs with hCG levels 1000–10 000 IU/L by MTX /gefitinib to be considered effective, we expect a success rate of at least 90%.[11] However, a success rate of 70% or less would be considered unacceptable. With 80% power and a 5% level of significance, 28 patients are required to enable us to assess whether the proportion of patients with a successful outcome to treatment is ≤70% or ≥90%. If 24, or more, patients have a successful outcome, we can reject the hypothesis that the true efficacy of MTX /gefitinib is ≤70% and progress to a phase III trial.

Inclusion criteria

Women aged between 18 and 45 years; pretreatment serum hCG of 1000–10 000 IU/L (rising or static); ultrasound diagnosis of definite tEP (extrauterine gestational sac with yolk sac and/or embryo, with or without cardiac activity) or probable tEP (inhomogeneous adnexal mass or extrauterine sac-like structure)[12] performed by a clinical team of trained, qualified and experienced ultrasonographers; no clinical evidence of intra-abdominal bleeding; no pallor; no guarding/rigidity on abdominal examination; stable blood pressure and heart rate; haemoglobin on full blood examination at day 1 between 100 and 165 g/L).

Exclusion criteria

Women with a pregnancy of unknown location; evidence of a significant intra-abdominal bleed on ultrasound defined by free fluid above the uterine fundus or the surrounding ovary[13]; women with a history of any significant pulmonary disease; abnormal liver/renal/haematological indices; significant pre-existing dermatological conditions; significant pre-existing gastrointestinal medical illnesses; Japanese ethnicity.

Participant enrolment

All gynaecology consultants within NHS Lothian (UK), Southern Health and Mercy Health (both Australia) will be sent a letter informing them of the study and requesting permission to approach their patients. The clinical research team in NHS Lothian, Southern Health and Mercy Health will approach eligible women, provide them with patient information sheets and offer them the opportunity to discuss the trial, and obtain informed consent. Consent will only be taken once the patient has had ample time to read the patient information sheet and had her questions answered.

Intervention

Eligible women will be given a single-dose intramuscular MTX (50 mg/m^2) injection with seven daily doses oral gefitinib (250 mg). The gefitinib will be started on the same date when the MTX injection is given.

Data collection

Data storage

A log with the patients' name and date of birth will be kept along with their unique study number in a separate file. All the data generated from the study will be stored in an anonymised form in a bespoke database, which will also be password protected. Only anonymised information will be stored on this, and participants will only be identifiable by their study number. All paperwork will be kept in a locked filing cabinet in a locked office. All data will be stored on university server (University of Edinburgh) on a password-protected computer with limited access to the research team, in accordance with the Data Protection Act (UK).

Screening

A member of the research team will carry out a screening visit to assess eligibility. All data will be recorded on a case record form and transferred to a secure database.

Participant log

The clinical research team will keep an electronic log of women who fulfil the eligibility criteria, women who are invited to participate in the study, women recruited and women who leave the trial early. Reasons for non-recruitment (eg, non-eligibility, refusal to participate, administrative error) will also be recorded. We will attempt to collect reasons for non-participation from women who decline to take part after previously providing contact details. During the course of the study, we will document reasons for withdrawal from the study and loss to follow-up (figure 1).

Assessments

To monitor treatment response, we will follow protocols used clinically for medical management with MTX. Serum hCG levels will be measured on days 4, 7 and 11, then weekly until hCG levels drop to non-pregnant levels (<15 IU/L). Medical management will be discontinued and patients will undergo surgery based on their response to MTX and clinical picture (eg, clinical evidence of intra-abdominal bleeding) following standard clinical paradigms documented by the assessing clinician. Participants will be contacted at 3 and 6 months post-treatment to document return of menstrual cycles and any subsequent pregnancies. To monitor safety and tolerability, women will be assessed clinically (history) and biochemically (haematological, renal and liver function tests) on days 4 and 7 (or if elevated, until return to normal physiological levels).

Primary outcome

Our primary outcome is resolution of tEP without requirement for surgery. Resolution is defined by serum hCG levels (the current clinical marker to monitor treatment response) falling to non-pregnant levels (hCG <15 IU/L). We have selected our primary outcome based on the data from our phase I trial where two

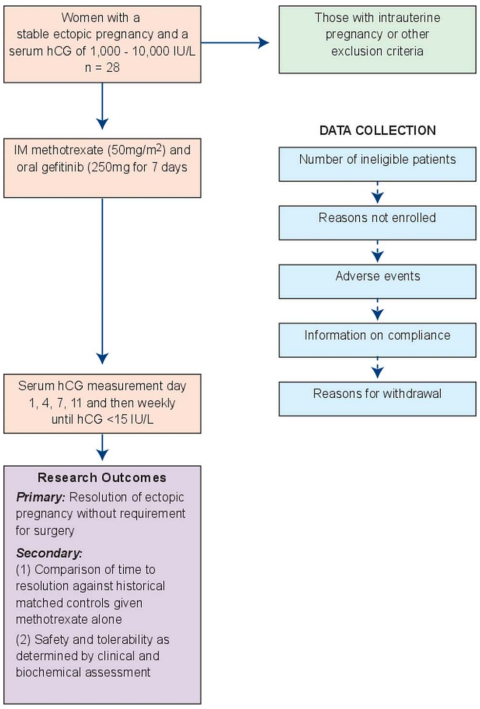

Figure 1 Flow chart of participants involved in the study.

patients recruited with pretreatment hCG levels >1000 IU/L required surgery and previously published data.[14] We are using a cut-off of <15 IU/L, which corresponds to a negative urinary pregnancy test using the most sensitive assays.

Secondary outcome

I. Time to resolution (categorical variable) compared with historical controls of similar pretreatment serum hCG levels (identified by an individual blinded to the study).
II. Safety and tolerability as determined by clinical and biochemical assessment. Both MTX and gefitinib have the potential to affect haematological, renal and liver function.

Proposed analyses

Given this is a single arm efficacy trial, the majority of the data will be expressed as descriptive statistics.

Ethics and dissemination

Ethical approval has been obtained from the Scotland A Research Ethics Committee (LREC 12/SS/0005) (UK), the Southern Health Human Research Ethics Committee B (SH HREC 11180B) and the Mercy Health Human Research Ethics Committee (R12/25) (both

Australia). Data will be presented at international conferences and published in peer-reviewed journals. We will make the information obtained from the study available to the public through national bodies and charities (eg, Ectopic Pregnancy Trust).

Adverse events

Participants will collect information about adverse events in their treatment diaries. However, they will be instructed to contact the clinical research team at any time after consenting to join the trial if they have an event that requires hospitalisation or an event that results in persistent or significant disability or incapacity. Any serious adverse events that occur after joining the trial will be reported in detail in the participant's medical notes, followed up until resolution of the event and reported to the ACCORD Research Governance (http://www.accord.ed.ac.uk) and QA Office based at the University of Edinburgh, or the Southern Health/Mercy Health Human Research Ethics Committees and Therapeutic Goods Administration of Australia's Office of Scientific Evaluation immediately or within 24–72 h.

DISCUSSION

If effective, we believe that this combination (gefitinib and MTX) could become standard of care for stable tEPs. The combination also has potential use in other pregnancy disorders. There may be other important conditions where medical regression of pregnancy tissue could be useful, for example, women with complete molar pregnancies and persistent molar disease and women with placenta accrete postpartum (to avoid hysterectomy).

Regarding the safety of gefitinib, data from post marketing surveillance representing over 92 000 patients exist and have shown that EGFR inhibitors are well tolerated and largely free of serious side effects (Food and Drug Administration (FDA) report).[15] Of note, the data on tolerability are based on patients taking gefitinib daily on an ongoing, indefinite basis, after primary treatment of cancer. Diarrhoea and skin rash are the most common side effects (20–30%). The skin rash, described as acneiform, can be severe, but is generally self-limited. Skin rashes occur within a month of initiation of treatment, but rarely in the first week. Interstitial lung disease (ILD) is a very rare but a serious side effect of gefitinib. It is a thickening of the lung parenchyma that can be fatal in a third of cases. Of the 31 045 patients in the USA who took gefitinib (reported to the FDA), 84 developed ILD (0.3%). We plan to administer seven 250 mg gefitinib tablets, one daily for only 7 days, in addition to MTX. This is an extremely short duration of treatment compared with gefitinib's current marketing indications and existing data usage. We would not expect this short course to have an adverse long-term effect on fertility but we will be assessing participants 3 and 6 months posttreatment to document return of menstrual cycles and any subsequent pregnancies.

We do not anticipate that this will be the final trial to determine whether further exploration of combination therapy with gefitinib and MTX is worthwhile. We hope that the study will generate sufficient 'signal' that gefitinib and MTX may be effective and safe, to support a funding application for a larger trial with a comparative group. Such a trial could be designed as an 'equivalence' trial in terms of treatment efficacy between conventional management and the gefitinib/MTX comparison. It would aim to test the hypothesis that gefitinib/MTX was superior in a range of outcomes prioritised by consumer groups and clinicians. We anticipate that these outcomes could include: time to resumption of normal activities, SF-36 at intervals after treatment and patient satisfaction scores. Outcomes of a subsequent pregnancy are also important but would require long-term follow-up studies. We anticipate that focus groups and surveys of patients and clinicians would be required to define the outcomes (other than efficacy) of these studies.

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Competing interests AH is funded by an MRC Clinician Scientist Fellowship and MRC Centenary Award (G0802808) and holds grants from the Chief Scientist's Office Scotland (CZH/4/688) (HC coinvestigator) and Well-being of Women. HC holds an MRC DCS Grant (G003611), an MRC Centre Grant (G1002033) and research collaboration funding from Bayer Pharma AG. UN, TJ and ST are joint holders of patents that relate to the use of EGFR inhibition in treating ectopic pregnancies.

Ethics approval Ethical approval has been obtained from Scotland A Research Ethics Committee (MREC 11/AL/0350), Southern Health Human Research Ethics Committee B (HREC 11180B) and the Mercy Health Human Research Ethics Committee (R12/25).

Provenance and peer review Not commissioned; externally peer reviewed.

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BMJ Open

Phase II single arm open label multicentre clinical trial to evaluate the efficacy and side effects of a combination of gefitinib and methotrexate to treat tubal ectopic pregnancies (GEM II): study protocol

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Appendix D: Common Terminology Criteria for Adverse Events

These are excerpts of the document pertaining to the most common side effects reported by participants during the conduct of studies comprising this thesis, namely, abdominal pain, rash, nausea, diarrhoea, lethargy, dizziness and deranged liver function tests. A full version of the 196-page document is available from:

http://evs.nci.nih.gov/ftp1/CTCAE/CTCAE_4.03_2010-06-14_QuickReference_8.5x11.pdf.

Accessed 23 May 20, 2014.

Gastrointestinal disorders					
	Grade				
Adverse Event	**1**	**2**	**3**	**4**	**5**
Abdominal distension	Asymptomatic; clinical or diagnostic observations only; intervention not indicated	Symptomatic; limiting instrumental ADL	Severe discomfort; limiting self care ADL	-	-
Definition: A disorder characterized by swelling of the abdomen.					
Abdominal pain	Mild pain	Moderate pain; limiting instrumental ADL	Severe pain; limiting self care ADL	-	-
Definition: A disorder characterized by a sensation of marked discomfort in the abdominal region.					
Anal fistula	Asymptomatic; clinical or diagnostic observations only; intervention not indicated	Symptomatic; altered GI function	Severely altered GI function; tube feeding, TPN or hospitalization indicated; elective operative intervention indicated	Life-threatening consequences; urgent intervention indicated	Death
Definition: A disorder characterized by an abnormal communication between the opening in the anal canal to the perianal skin.					
Anal hemorrhage	Mild; intervention not indicated	Moderate symptoms; medical intervention or minor cauterization indicated	Transfusion, radiologic, endoscopic, or elective operative intervention indicated	Life-threatening consequences; urgent intervention indicated	Death
Definition: A disorder characterized by bleeding from the anal region.					
Anal mucositis	Asymptomatic or mild symptoms; intervention not indicated	Symptomatic; medical intervention indicated; limiting instrumental ADL	Severe symptoms; limiting self care ADL	Life-threatening consequences; urgent intervention indicated	Death
Definition: A disorder characterized by inflammation of the mucous membrane of the anus.					

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Skin and subcutaneous tissue disorders					
	Grade				
Adverse Event	**1**	**2**	**3**	**4**	**5**
Pruritus	Mild or localized; topical intervention indicated	Intense or widespread; intermittent; skin changes from scratching (e.g., edema, papulation, excoriations, lichenification, oozing/crusts); oral intervention indicated; limiting instrumental ADL	Intense or widespread; constant; limiting self care ADL or sleep; oral corticosteroid or immunosuppressive therapy indicated	-	-
Definition: A disorder characterized by an intense itching sensation.					
Purpura	Combined area of lesions covering <10% BSA	Combined area of lesions covering 10 - 30% BSA; bleeding with trauma	Combined area of lesions covering >30% BSA; spontaneous bleeding	-	-
Definition: A disorder characterized by hemorrhagic areas of the skin and mucous membrane. Newer lesions appear reddish in color. Older lesions are usually a darker purple color and eventually become a brownish-yellow color.					
Rash acneiform	Papules and/or pustules covering <10% BSA, which may or may not be associated with symptoms of pruritus or tenderness	Papules and/or pustules covering 10 - 30% BSA, which may or may not be associated with symptoms of pruritus or tenderness; associated with psychosocial impact; limiting instrumental ADL	Papules and/or pustules covering >30% BSA, which may or may not be associated with symptoms of pruritus or tenderness; limiting self care ADL; associated with local superinfection with oral antibiotics indicated	Papules and/or pustules covering any % BSA, which may or may not be associated with symptoms of pruritus or tenderness and are associated with extensive superinfection with IV antibiotics indicated; life-threatening consequences	Death
Definition: A disorder characterized by an eruption of papules and pustules, typically appearing in face, scalp, upper chest and back.					

Gastrointestinal disorders					
	Grade				
Adverse Event	**1**	**2**	**3**	**4**	**5**
Nausea	Loss of appetite without alteration in eating habits	Oral intake decreased without significant weight loss, dehydration or malnutrition	Inadequate oral caloric or fluid intake; tube feeding, TPN, or hospitalization indicated	-	-
Definition: A disorder characterized by a queasy sensation and/or the urge to vomit.					
Obstruction gastric	Asymptomatic; clinical or diagnostic observations only; intervention not indicated	Symptomatic; altered GI function; limiting instrumental ADL	Hospitalization indicated; elective operative intervention indicated; limiting self care ADL; disabling	Life-threatening consequences; urgent operative intervention indicated	Death
Definition: A disorder characterized by blockage of the normal flow of the contents in the stomach.					
Oral cavity fistula	Asymptomatic; clinical or diagnostic observations only; intervention not indicated	Symptomatic; altered GI function	Severely altered GI function; TPN or hospitalization indicated; elective operative intervention indicated	Life-threatening consequences; urgent intervention indicated	Death
Definition: A disorder characterized by an abnormal communication between the oral cavity and another organ or anatomic site.					
Oral dysesthesia	Mild discomfort; not interfering with oral intake	Moderate pain; interfering with oral intake	Disabling pain; tube feeding or TPN indicated	-	-
Definition: A disorder characterized by a burning or tingling sensation on the lips, tongue or entire mouth.					
Oral hemorrhage	Mild; intervention not indicated	Moderate symptoms; medical intervention or minor cauterization indicated	Transfusion, radiologic, endoscopic, or elective operative intervention indicated	Life-threatening consequences; urgent intervention indicated	Death
Definition: A disorder characterized by bleeding from the mouth.					

Gastrointestinal disorders					
	Grade				
Adverse Event	**1**	**2**	**3**	**4**	**5**
Diarrhea	Increase of <4 stools per day over baseline; mild increase in ostomy output compared to baseline	Increase of 4 - 6 stools per day over baseline; moderate increase in ostomy output compared to baseline	Increase of >=7 stools per day over baseline; incontinence; hospitalization indicated; severe increase in ostomy output compared to baseline; limiting self care ADL	Life-threatening consequences; urgent intervention indicated	Death
Definition: A disorder characterized by frequent and watery bowel movements.					
Dry mouth	Symptomatic (e.g., dry or thick saliva) without significant dietary alteration; unstimulated saliva flow >0.2 ml/min	Moderate symptoms; oral intake alterations (e.g., copious water, other lubricants, diet limited to purees and/or soft, moist foods); unstimulated saliva 0.1 to 0.2 ml/min	Inability to adequately aliment orally; tube feeding or TPN indicated; unstimulated saliva <0.1 ml/min	-	-
Definition: A disorder characterized by reduced salivary flow in the oral cavity.					
Duodenal fistula	Asymptomatic; clinical or diagnostic observations only; intervention not indicated	Symptomatic; altered GI function	Severely altered GI function; tube feeding, TPN or hospitalization indicated; elective operative intervention indicated	Life-threatening consequences; urgent intervention indicated	Death
Definition: A disorder characterized by an abnormal communication between the duodenum and another organ or anatomic site.					

CTCAE 4.03 - June 14, 2010 : Gastrointestinal disorders
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Nervous system disorders					
	Grade				
Adverse Event	**1**	**2**	**3**	**4**	**5**
Lethargy	Mild symptoms; reduced alertness and awareness	Moderate symptoms; limiting instrumental ADL	-	-	-
Definition: A disorder characterized by a decrease in consciousness characterized by mental and physical inertness.					
Leukoencephalopathy	Asymptomatic; small focal T2/FLAIR hyperintensities; involving periventricular white matter or <1/3 of susceptible areas of cerebrum +/- mild increase in subarachnoid space (SAS) and/or mild ventriculomegaly	Moderate symptoms; focal T2/FLAIR hyperintensities, involving periventricular white matter extending into centrum semiovale or involving 1/3 to 2/3 of susceptible areas of cerebrum +/- moderate increase in SAS and/or moderate ventriculomegaly	Severe symptoms; extensive T2/FLAIR hyperintensities, involving periventricular white matter involving 2/3 or more of susceptible areas of cerebrum +/- moderate to severe increase in SAS and/or moderate to severe ventriculomegaly	Life-threatening consequences; extensive T2/FLAIR hyperintensities, involving periventricular white matter involving most of susceptible areas of cerebrum +/- moderate to severe increase in SAS and/or moderate to severe ventriculomegaly	Death
Definition: A disorder characterized by diffuse reactive astrocytosis with multiple areas of necrotic foci without inflammation.					
Memory impairment	Mild memory impairment	Moderate memory impairment; limiting instrumental ADL	Severe memory impairment; limiting self care ADL	-	-
Definition: A disorder characterized by a deterioration in memory function.					
Meningismus	Mild symptoms	Moderate symptoms; limiting instrumental ADL	Severe symptoms; limiting self care ADL	Life-threatening consequences; urgent intervention indicated	Death
Definition: A disorder characterized by neck stiffness, headache, and photophobia resulting from irritation of the cerebral meninges.					

CTCAE 4.03 - June 14, 2010 : Nervous system disorders
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Nervous system disorders					
	Grade				
Adverse Event	**1**	**2**	**3**	**4**	**5**
Cognitive disturbance	Mild cognitive disability; not interfering with work/school/life performance; specialized educational services/devices not indicated	Moderate cognitive disability; interfering with work/school/life performance but capable of independent living; specialized resources on part time basis indicated	Severe cognitive disability; significant impairment of work/school/life performance	-	-
Definition: A disorder characterized by a conspicuous change in cognitive function.					
Concentration impairment	Mild inattention or decreased level of concentration	Moderate impairment in attention or decreased level of concentration; limiting instrumental ADL	Severe impairment in attention or decreased level of concentration; limiting self care ADL	-	-
Definition: A disorder characterized by a deterioration in the ability to concentrate.					
Depressed level of consciousness	Decreased level of alertness	Sedation; slow response to stimuli; limiting instrumental ADL	Difficult to arouse	Life-threatening consequences	Death
Definition: A disorder characterized by a decrease in ability to perceive and respond.					
Dizziness	Mild unsteadiness or sensation of movement	Moderate unsteadiness or sensation of movement; limiting instrumental ADL	Severe unsteadiness or sensation of movement; limiting self care ADL	-	-
Definition: A disorder characterized by a disturbing sensation of lightheadedness, unsteadiness, giddiness, spinning or rocking.					
Dysarthria	Mild slurred speech	Moderate impairment of articulation or slurred speech	Severe impairment of articulation or slurred speech	-	-
Definition: A disorder characterized by slow and slurred speech resulting from an inability to coordinate the muscles used in speech.					

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	Grade				
Adverse Event	**1**	**2**	**3**	**4**	**5**
Activated partial thromboplastin time prolonged	>ULN - 1.5 x ULN	>1.5 - 2.5 x ULN	>2.5 x ULN; hemorrhage	-	-
Definition: An abnormal laboratory test result in which the partial thromboplastin time is found to be greater than the control value. As a possible indicator of coagulopathy, a prolonged partial thromboplastin time (PTT) may occur in a variety of diseases and disorders, both primary and related to treatment.					
Alanine aminotransferase increased	>ULN - 3.0 x ULN	>3.0 - 5.0 x ULN	>5.0 - 20.0 x ULN	>20.0 x ULN	-
Definition: A finding based on laboratory test results that indicate an increase in the level of alanine aminotransferase (ALT or SGPT) in the blood specimen.					
Alkaline phosphatase increased	>ULN - 2.5 x ULN	>2.5 - 5.0 x ULN	>5.0 - 20.0 x ULN	>20.0 x ULN	-
Definition: A finding based on laboratory test results that indicate an increase in the level of alkaline phosphatase in a blood specimen.					
Aspartate aminotransferase increased	>ULN - 3.0 x ULN	>3.0 - 5.0 x ULN	>5.0 - 20.0 x ULN	>20.0 x ULN	-
Definition: A finding based on laboratory test results that indicate an increase in the level of aspartate aminotransferase (AST or SGOT) in a blood specimen.					
Blood antidiuretic hormone abnormal	Asymptomatic; clinical or diagnostic observations only; intervention not indicated	Symptomatic; medical intervention indicated	Hospitalization indicated	-	-
Definition: A finding based on laboratory test results that indicate abnormal levels of antidiuretic hormone in the blood specimen.					
Blood bilirubin increased	>ULN - 1.5 x ULN	>1.5 - 3.0 x ULN	>3.0 - 10.0 x ULN	>10.0 x ULN	-
Definition: A finding based on laboratory test results that indicate an abnormally high level of bilirubin in the blood. Excess bilirubin is associated with jaundice.					
Blood corticotrophin decreased	Asymptomatic; clinical or diagnostic observations only; intervention not indicated	Symptomatic; medical intervention indicated	Hospitalization indicated	-	-

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