Molecular diagnostics and therapeutics for ectopic pregnancy

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Abstract: Ectopic pregnancies are a serious gynaecological emergency that can be fatal. As such, prompt diagnosis and safe timely treatment is essential. Here, we review the literature on the development of molecularly targeted diagnostics and therapeutics for ectopic pregnancy. A blood-based biomarker that accurately identifies an ectopic pregnancy could be used to offer early diagnostic certainty in cases where ultrasound cannot determine the location of the embryo (“a pregnancy of unknown location”). Molecules examined so far can be broadly grouped into biological themes of relevance to reproduction: (i) Fallopian tube (dys)function, (ii) embryo/trophoblast growth, (iii) corpus luteum function, (iv) inflammation, (v) uterine function and (vi) angiogenesis. While a sensitive and specific biomarker for ectopic pregnancy has yet to be identified, it is possible that improvements in platform technologies or a multi-modal biomarker approach may yield an accurate diagnostic biomarker test. Furthermore, with the advent of better imaging technology, the need for a blood-based biomarker test may be superseded by improvements in ultrasound or magnetic resonance imaging technology. There have been some recent preclinical studies describing molecularly targeted therapeutic approaches for ectopic pregnancy. Notably, bench-to-bedside studies have examined the use of combination gefitinib (orally available epidermal growth factor receptor inhibitor) and methotrexate. Preclinical studies suggest that combination gefitinib and methotrexate is highly effective in inducing placental cell death, and is significantly more effective than methotrexate alone. In early human trials, encouraging preliminary efficacy data have shown that combination gefitinib and methotrexate can rapidly resolve tubal ectopic pregnancies, and large extra-tubal ectopic pregnancies. If a large clinical randomized controlled trial confirms these findings, combination gefitinib and methotrexate could become a new medical treatment option for ectopic pregnancy.

Key words: biomarker / treatment / ectopic pregnancy / gefitinib / methotrexate

Introduction

An ectopic pregnancy occurs when a fertilized ovum implants outside the endometrial cavity. The name ‘ectopic’ is derived from the Greek word ‘ektopos’, meaning ‘out of place’. Complicating 1–2% of all pregnancies, ectopic pregnancies are a life-threatening emergency since they can Invade maternal blood vessels, causing catastrophic haemorrhage. Over 98% of all ectopic pregnancies implant in the Fallopian tube.

While ectopic pregnancies can often be diagnosed by ultrasound, sometimes imaging cannot identify the location of the conceptus. In such clinical situations, termed a ‘pregnancy of unknown location’ (PUL) (Barth et al., 2011), the differential diagnosis is an ectopic pregnancy, miscarriage or even a viable, intrauterine pregnancy. Here, we will review research efforts to develop an accurate molecular biomarker to diagnose ectopic pregnancy in PUL, where the ultrasound findings are inconclusive. Such a biomarker would allow early definitive treatment and improve management (Part 1).

Most ectopic pregnancies are treated surgically (Jurkovic and Wilkinson, 2011). A medical alternative has been in use since the 1990s, namely the administration of the chemotherapeutic methotrexate (Skubisz and Tong, 2012). While effective for smaller ectopic pregnancies, methotrexate is less successful in resolving large ectopic pregnancies. Furthermore, methotrexate is cost-effective only if administered to small ectopic pregnancies where the pretreatment serum hCG concentrations are < 1 500 IU/l (Mol et al., 2008). Thus, there is significant scope for a medical alternative that is considerably more efficacious than methotrexate in rapidly resolving ectopic pregnancies. In this review, we will also describe preclinical and early phase human studies to develop new molecularly targeted treatments for ectopic pregnancy (Part 2).

Part 1: Diagnostics

Current methods for diagnosing ectopic pregnancy

Diagnosis of ectopic pregnancy continues to present a major clinical challenge in obstetrics and gynaecology. Patients with an ectopic
Biomarker development for clinical use

Biomarker development for ectopic pregnancy diagnosis has been steered by hypothesis-driven approaches supported by an understanding of the biology of early pregnancy failure. More recently, recognizing that many of the pathways involved in early pregnancy failure remain poorly understood, unbiased approaches using microarray analysis (Horne et al., 2008) and proteomics (Beer et al., 2011; Brown et al., 2013) have also been explored as a method for biomarker discovery. Biomarker development for clinical use can be divided into five phases (Table I). Disappointingly, although over 20 biomarkers of ectopic pregnancy have been identified (Fig. 1), most have not progressed beyond Phase II investigation.

### Biomarker themes

The biomarkers that have been identified can be categorized according to biological themes.

#### Marks of Fallopian tube dysfunction

Creatine kinase (CK) is an enzyme that is released when muscle becomes damaged and is currently used in clinical practice as a diagnostic biomarker for myocardial infarction (Costa et al., 2008). The first study of CK as a marker of Fallopian tube damage was encouraging (Lavie et al., 1993). Serum CK concentrations were significantly higher in those with ectopic pregnancy as opposed to those with a missed miscarriage or a viable intrauterine pregnancy. In this study, there was no overlap between the groups and all women with an ectopic pregnancy had a CK level >45 IU/l. However, when used as a marker in an unselected population, although serum CK was significantly higher in ectopic compared with intrauterine pregnancy, there was considerable overlap in the values and, with a cut-off of 45 IU/l, CK only had a sensitivity of 57% and specificity of 67% (Duncan et al., 1995). Subsequent studies are conflicting (Darai et al., 1996; Garcia-Velasco et al., 1996; Korhonen et al., 1996; Lincoln et al., 1996; Qasim et al., 1996; Vandermolen and Borzelleca, 1996; Plewa et al., 1998; Birkhahn et al., 2000; Kurzel et al., 2001). Some have proposed that the differences found in these various studies are explained by the fact that serum CK concentrations are actually a marker of tubal rupture rather than ectopic pregnancy per se (Develioglu et al., 2002; Soundravally et al., 2007).

Other markers of muscle damage have also been investigated, such as myoglobin (Birkhahn et al., 2001) and smooth muscle heavy chain myosin (Birkhahn et al., 2000, 2001), but neither marker is discriminatory enough for clinical use.

Adrenomedullin is a peptide hormone belonging to the calcitonin/calcitonin gene-related peptide/amylin family thought to regulate ciliary beat activity in the Fallopian tube (Liao et al., 2012). Plasma adrenomedullin levels have been confirmed in two small studies to be lower in women with ectopic pregnancies than in viable intrauterine studies (Liao et al., 2012; Brown et al., 2013). This marker warrants further investigation on a larger scale.

#### Marks of abnormal embryo/trophoblast growth

Pregnancy-associated plasma protein-A (PAPP-A) is a large glycoprotein expressed by the trophoblast (and decidua) (Horne et al., 1976). Two early studies showed that the concentration of PAPP-A was much lower in patients with an ectopic pregnancy when compared with a normal intrauterine pregnancy (Bischof et al., 1983; Sinosich et al., 1985). A subsequent larger study found that PAPP-A was undetectable in >80% of samples from women with an ectopic pregnancy, but PAPP-A was also undetectable in >55% of intrauterine spontaneous miscarriages (Sjoberg, 1987). PAPP-A concentrations appear to be very low before 7 weeks gestation and, in isolation, are therefore poorly discriminative between spontaneous miscarriage and ectopic pregnancy (Mueller et al., 2004; Daponte et al., 2005).

Pregnancy-specific β-glycoprotein I (PSG-1 or SP-1) is another product of the placental syncytiotrophoblast (Horne et al., 1976). Serum SP-1 concentrations tend to be lower in ectopic pregnancy than intrauterine pregnancy (Ho and Jones, 1980; Tomehave et al., 1987), but there is poor discrimination between ectopic pregnancy and spontaneous miscarriage. In ectopic pregnancies, serial SP-1 measurements reach a plateau while in viable intrauterine pregnancies, they progressively increase (Mantzavinos et al., 1991; Mueller et al., 2004). The potential role of SP-1 has been further supported more recently by unbiased proteomic discovery (Beer et al., 2011) and requires further investigation.

### Table I Phases of biomarker development.

<table>
<thead>
<tr>
<th>Phase</th>
<th>Description</th>
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<tbody>
<tr>
<td>I</td>
<td>Preclinical/exploratory studies</td>
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<tr>
<td>II</td>
<td>Clinical assay development and validation</td>
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<tr>
<td>III</td>
<td>Retrospective/longitudinal studies</td>
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<tr>
<td>IV</td>
<td>Prospective studies</td>
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<tr>
<td>V</td>
<td>Assessment of biomarker in clinical practice</td>
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</table>
Human placental lactogen (HPL) is also a major placental product that can be detected during the first trimester (Handwerger and Freemark, 2000). An initial study found that there were no differences in serum concentrations between ectopic and intrauterine pregnancies (Kuscu et al., 1993). However, a subsequent study investigating a number of placental proteins showed that HPL levels were lower in those with an ectopic pregnancy compared with a viable intrauterine pregnancy, particularly after 7 weeks gestation (Mueller et al., 2004). Although this finding has been confirmed in a different set of patients (Daponte et al., 2005), the marked overlap in values between groups limits HPL’s utility as a single biomarker.

Activin A is a member of the transforming growth factor-β family, and in pregnancy, the main source of secretion is the trophoblast (Petraglia, 1997). Florio et al. (2007) found that activin A levels in women with an ectopic pregnancy were markedly lower than those with a viable intrauterine pregnancy or spontaneous miscarriage. They reported that activin A performed better than a single measurement of hCG in discriminating between the pregnancy outcomes, and remarkably, an ectopic pregnancy could be identified with a sensitivity of 100% and specificity of 99.6%. Disappointingly, others have not been able to independently validate these results, reporting that activin A had a much lower specificity and sensitivity as a biomarker for ectopic pregnancy (Kirk et al., 2009; Warrick et al., 2012).

A disintegrin and metalloprotease-12 (ADAM-12) is a glycoprotein produced by the syncytiotrophoblast (Huppertz et al., 2006). A case-control study of patients with ectopic pregnancies and viable intrauterine pregnancies demonstrated that serum ADAM-12 ≤ 48.49 ng/ml was 97% sensitive and 37% specific for ectopic pregnancy (Rausch et al., 2011). We have attempted to validate ADAM-12 in our patient cohort but found that it did not discriminate ectopic pregnancy from other outcomes when including treated persisting PULs, spontaneously resolving PULs and probable ectopic pregnancies (Horne et al., 2012). However, a subsequent study demonstrated that the level of ADAM12 in maternal serum facilitated the detection of ectopic pregnancies and spontaneous miscarriage (Yang et al., 2014). If further studies can confirm the reduction of ADAM12 in the first trimester, a strategy including ADAM12 may be adopted to identify women at high risk of ectopic pregnancy.

Markers of abnormal corpus luteum function

Progesterone has been proposed as a useful test to distinguish a viable pregnancy (higher levels) from a miscarriage or ectopic pregnancy (both lower levels) in multiple studies (Verhaegen et al., 2012). Indeed, this recent meta-analysis found that a single progesterone measurement for women with early pregnancy bleeding and an inconclusive ultrasound can rule out a viable pregnancy (Verhaegen et al., 2012). However, any
possible role for diagnosing ectopic pregnancy, if any, will likely be in combination with other biomarkers.

 Estradiol is secreted by the corpus luteum in response to hCG. Certainly, there are clear differences in serial serum estradiol concentrations in women with ectopic pregnancies when compared with viable intrauterine pregnancies up to 11 weeks gestation (Guillaume et al., 1990). While estradiol concentrations rise continuously in viable pregnancies, they are reduced in ectopic pregnancy, where values plateau at 6 weeks and decline after the 8th week of gestation. However, there is considerable overlap in estradiol concentrations between the different diagnostic groups (Mantzavinos et al., 1991; Grosskinsky et al., 1993; Kuscu et al., 1993), meaning it is not likely to be a useful biomarker to diagnose ectopic pregnancy.

 Inhibin A is a major peptide product of the corpus luteum and its secretion is regulated by hCG (Illingworth et al., 1996). Inhibin A has been demonstrated to be lower in women with ectopic pregnancies compared with viable intrauterine pregnancies (Illingworth et al., 1996; Seifer et al., 1996), and subsequently, it has been shown that inhibin A is lower in ectopic pregnancies than both viable and non-viable intrauterine pregnancies (Segal et al., 2008). However, recent conflicting data (Chetty et al., 2011) do not support its value as a marker of extra-uterine implantation.

**Markers of inflammation**

Cancer Antigen-125 (CA-125) is typically considered as a biomarker of ovarian carcinoma (Canney et al., 1984). However, it is also raised in benign conditions where there is peritoneal irritation or inflammation, such as endometriosis. Brumsted et al. (1990) reported that serum CA-125 levels increase in women with viable intrauterine pregnancies, and women with ectopic pregnancies had a wide range of concentrations, but overall they were lower in women with ectopic pregnancies (Brumsted et al., 1990; Witt et al., 1990). However, a subsequent study suggested that serum CA-125 concentrations were increased, rather than reduced, in women with ectopic pregnancies (Sadovsky et al., 1991) and another study suggested no differences between the clinical groups (Kuscu et al., 1993). The differences in these studies may reflect the proportion of women with spontaneous miscarriages included because it seems this group do have significantly elevated concentrations of serum CA-125 per se (Katsikis et al., 2006).

The possible predictive potential of cytokines, such as interleukin (IL) 6, IL-8, IL receptor 2 and tumour necrosis factor-α (TNF-α) which are primarily associated with inflammation, in the diagnosis of tubal ectopic pregnancy has been assessed (Soriano et al., 2003). The concentrations of IL6, IL8 and TNF-α (but not IL2 receptor) were significantly higher in women with ectopic pregnancy when compared with those with a viable intrauterine pregnancy and miscarriage. Interestingly, serum levels of IL6, IL8 and TNF-α did not differ between those women with a viable intrauterine pregnancy and those who ultimately miscarried.

Using a shotgun proteomics approach that incorporated combinatorial ligand library pre-fractionation (Brown et al., 2013), we identified the glycoprotein fibronectin (FN1), which has a significant role in wound healing (Pankov and Yamada, 2002), as a potential marker of ectopic pregnancy. Receiver operating characteristic curve analysis of the ability of FN1 to discriminate ectopic pregnancy from other pregnancy outcomes suggested that FN1 has diagnostic potential, becoming significant when ‘ambiguous’ medically managed PUL were excluded from our analysis.

**Uterine markers of abnormal implantation**

Leukaemia inhibitory factor (LIF) is a marker of embryo implantation expressed in the endometrium (Aghajanova, 2004). Wegner and Mershon (2001) demonstrated that women with an ectopic pregnancy had lower serum LIF concentrations than those with viable and non-viable intrauterine pregnancies. Nevertheless, there was considerable overlap between the values, and serum LIF concentrations had 73% sensitivity and 72% specificity for diagnosing ectopic pregnancy. Disappointingly, a subsequent larger study failed to find any differences in serum LIF concentrations (Daponte et al., 2005).

Glycodelin, also known as placental protein-14 (PP14), is a major secretory product of the endometrium and decidua (Ruge et al., 1992). Serum PP14 concentrations increase during early pregnancy until Weeks 8–10 of gestation and then decline (Ruge et al., 1992). Pedersen et al. (1995) found that women with ectopic pregnancy had significantly lower serum concentrations of PP14 than those with intrauterine pregnancies. This was confirmed in a larger cohort of women with ectopic pregnancies (Ruge et al., 1992). A comparison of serum PP14 concentrations in women with spontaneous miscarriage and ectopic pregnancy found that 81% of women with spontaneous miscarriage had PP14 serum concentration levels within the normal range, while 81% of women diagnosed with an ectopic pregnancy had PP14 levels below the 5th percentile of the normal range (Stabile et al., 1994). In addition, the lower concentrations of serum glycodelin in ectopic pregnancy were not dependent on tubal status as this difference was maintained in patients with an unruptured tube, a ruptured tube or a ‘tubal miscarriage’ (Foth and Romer, 2003).

We sought to identify novel decidual markers of intrauterine and ectopic implantation using microarray technology. We used gene profiling of endometrium from gestation-matched women with viable or non-viable intrauterine pregnancies and compared the profiles with those of women with ectopic pregnancies (Horne et al., 2008). This approach revealed that the expression of uterine activin B is increased during decidualization. In addition, we were able to show that women with ectopic pregnancies had a less decidualized endometrium and lower concentrations of serum activin B (Horne et al., 2008).

**Markers of an abnormal angiogenic response**

Vascular endothelial growth factor (VEGF) is a potent angiogenic factor up-regulated by tissue hypoxia that has a vital role in implantation and placentation (Smith, 2000). Daniel et al. (1999) showed that serum VEGF concentrations were significantly higher in women with ectopic pregnancies compared with viable intrauterine pregnancies (although there was only borderline significance between ectopic and failing intrauterine pregnancy). A serum VEGF level >200 pg/ml discriminated between an ectopic and intrauterine pregnancy with a specificity of 90% and a positive predictive value of 86%. Using the same cut-off, Felemban et al. (2002) reported that all of the women in their cohort with an intrauterine pregnancy had a serum VEGF <200 pg/ml, and this cut-off distinguished an ectopic pregnancy with a sensitivity of 88%, specificity of 100% and positive predictive value of 100%. However, another study published around the same time failed to find statistically significant differences (Kucer-Sliutz et al., 2002). Nevertheless, when women with viable intrauterine pregnancies are compared with those with ectopic pregnancies,
serum VEGF correlations do appear to be consistently elevated (Mueller et al., 2004).

Placenta-like growth factor (PIGF) is an angiogenic factor predominantly produced by trophoblasts and present at ectopic pregnancy implantation sites (Plaisier et al., 2007). We reported PIGF mRNA expression was lower in trophoblast cells and sera from women with ectopic pregnancies compared with intrauterine pregnancies (Horne et al., 2011). In a subsequent study, a diagnostic algorithm based on PIGF and soluble fms-like tyrosine kinase-1 (sflt-1) levels suggested that PIGF levels > 15.73 pg/ml can differentiate viable intrauterine from nonviable pregnancies with 86% sensitivity and 67% specificity, but could not differentiate between ectopic pregnancy and a non-viable pregnancy (Daponte et al., 2011).

Challenges and future directions for the development of diagnostics

It is clear that most of the putative biomarkers of ectopic pregnancy investigated to date are not useful in isolation. Thus, an increasing number of recent reports have studied a select few as part of a multiple marker test (Rausch et al., 2011; Butler et al., 2013). Disappointingly, although these multiple marker tests can accurately distinguish an ectopic pregnancy from an intrauterine pregnancy with superior accuracy, they are unable to distinguish between an ectopic pregnancy and a spontaneous miscarriage. However, it is possible that other biomarker combinations yet to be discovered may yield a diagnostic with better test performance characteristics.

In general, future studies examining ectopic pregnancy biomarkers should ideally be performed in prospective cohorts representative of all potential early pregnancy outcomes: ectopic pregnancy, miscarriage and ongoing intrauterine pregnancies (Barnhart and Speicher, 2011). Fundamentally, the question of whether a serum biomarker (or panel of markers) exists that can accurately and specifically detect an ectopic pregnancy is still unanswered. Furthermore, with the advent of better imaging technology, the need for a blood-based biomarker test may be superseded by improvements in ultrasound or magnetic resonance imaging technology.

Table II Methotrexate single-dose protocol.

<table>
<thead>
<tr>
<th>Day</th>
<th>Protocol</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>• Assess patient for suitability</td>
</tr>
<tr>
<td></td>
<td>Exclusion criteria</td>
</tr>
<tr>
<td></td>
<td>- Clinical suspicion of haemodynamic instability, or suspicion the ectopic pregnancy may have ruptured</td>
</tr>
<tr>
<td></td>
<td>- Ultrasound evidence of gestational sac size &gt; 3.5 cm, presence of fetal cardiac activity, significant blood in the abdomen</td>
</tr>
<tr>
<td></td>
<td>- Deranged tests of liver or renal function, low haemoglobin count</td>
</tr>
<tr>
<td></td>
<td>- Pretreatment serum hCG &gt; 3000 IU/l (RCOG) or &gt; 5000 IU/l (ACOG)</td>
</tr>
<tr>
<td></td>
<td>• Administer a single dose of i.m. methotrexate, 50 mg/m²</td>
</tr>
<tr>
<td></td>
<td>• Administer Anti-D if rhesus-negative blood group</td>
</tr>
<tr>
<td>Follow-up monitoring</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Regular clinical/outpatient review of patient</td>
</tr>
<tr>
<td></td>
<td>• Measure serum hCG levels on post-treatment Days 4 and 7</td>
</tr>
<tr>
<td></td>
<td>• Check for 15% serum hCG decrease between Days 4 and 7</td>
</tr>
<tr>
<td></td>
<td>• Then measure serum hCG weekly until reaching non-pregnant level</td>
</tr>
<tr>
<td></td>
<td>If the results are less than the expected 15% decrease, re-administer 50 mg/m² and repeat serum hCG measurements on Days 4 and 7 after the second dose. This can be repeated as necessary</td>
</tr>
<tr>
<td></td>
<td>If, during follow-up, hCG levels plateau or increase, consider repeating a further methotrexate dose</td>
</tr>
<tr>
<td></td>
<td>Offer surgery if there is suspicion during follow-up that the ectopic pregnancy has ruptured or is actively bleeding</td>
</tr>
</tbody>
</table>

Adapted from the Guidelines written by the Royal College of Obstetricians and Gynaecologists (RCOG, 2004) and the American College of Obstetricians and Gynecologists (ACOG, 2008).

Part 2: Therapeutics

Current treatment for ectopic pregnancy

Ectopic pregnancies are treated either by surgical excision or by medical treatment using methotrexate. A small number of women may be offered expectant management where no treatment is given, and the ectopic is closely monitored in the expectation that it will spontaneously resolve (Jurkovic and Wilkinson, 2011).

While surgical excision of ectopic pregnancies is safe, risks remain. They include complications arising from having a general anaesthetic administered and injury to major abdominal structures (bowel, major abdominal vessels). Furthermore, removal or incision of the Fallopian tube during the operation may negatively affect future fertility.

A medical alternative to surgery has been increasingly used to treat ectopic pregnancy: the i.m. administration of the anti-folate antagonist methotrexate (reviewed elsewhere; Skubisz and Tong, 2012). The outpatient clinical methotrexate protocol, pioneered by Stovall et al. (1991) and now in widespread use, is summarized in Table II. Methotrexate acts on the enzyme dihydrofolate reductase and is a competitive inhibitor of folic acid. By preventing folic acid from being incorporated into purines and thymidine, it blocks RNA and DNA synthesis. Rapidly dividing trophoblast tissue is particularly sensitive to methotrexate. This is highlighted by the fact that methotrexate has revolutionized treatment of metastatic choriocarcinoma, making a previously fatal disease one with a high rate of cure (Skubisz and Tong, 2012).

The potential benefits of more efficacious therapeutics to manage ectopic pregnancies medically

A medical therapeutic that is more effective than methotrexate could substantially improve the treatment of women diagnosed with ectopic pregnancies for a number of reasons. First, methotrexate has an unacceptably high rate of failure when used to treat large ectopic pregnancies (e.g. serum hCG > 5000 IU/l; Menon et al., 2007) and such cases are usually offered surgery. It is possible a more efficacious medical therapeutic could replace surgery for these ectopic pregnancies of large size.
Secondly, two studies that performed cost analyses on clinical trial data concluded that methotrexate management was only cost-effective if pretreatment serum hCG was < 1500 IU/l (Mol et al., 1999; Sower et al., 2001). Once the pretreatment serum hCG was > 1500 IU/l, medical treatment was more costly than surgery, due to the expense arising from monitoring patients (tracking serum hCG levels to non-pregnant levels) and costs incurred from the need to operate on those failing medical management (Mol et al., 2008). Thus, there may be a role for a new medical therapeutic for ectopic pregnancies with a pretreatment hCG < 5000 IU/l, if it resolves ectopic pregnancies more rapidly than methotrexate.

Thirdly, an efficacious medical therapeutic could be particularly useful in the setting of extra-tubal ectopic pregnancies, where the conceptus has implanted at sites such as the cervix, at the scar of a previous Caesarean section or the uterine interstitium/cornua. Extra-tubal ectopic pregnancies are notoriously difficult to treat, even with surgery, and are associated with higher maternal mortality. An efficacious option to avoid an operative approach would be extremely useful in the management of these rare subtypes of ectopic pregnancy.

The rise of molecularly targeted therapeutics in medicine

The last few decades have seen the emergence of exciting molecularly targeted therapeutics: drugs designed to specifically block molecules that have a major role in disease pathogenesis. The major advantage of molecular targeted therapeutics is that, being highly specific in their actions, they can be particularly effective in targeting the disease and often have a low rate of significant side effects. Examples of highly successful molecular targeted therapeutics include denosumab to treat osteoporosis and bone cancers (Yasuda, 2013) (denosumab blocks the receptor activator of nuclear factor κ-B (RANKL) ligand, a key signalling molecule for bone removal), and B-Raf inhibitors (e.g. vemurafenib) which have been remarkably efficacious against malignant melanoma (Jang and Atkin, 2014). While not yet clinically available, trials of nivolumab have been promising against a range of cancers (Robert et al., 2013). Nivolumab is a therapeutic antibody targeting PD1 (Programmed cell death 1). PD1 dampens the immune system and by countering this, nivolumab increases the host immune response against the cancer.

The focus of research for newer-generation molecularly targeted therapeutics has been to treat cancers, autoimmune diseases and other chronic illnesses of old age, but none (of which we are aware) has been licensed for gynaecological disorders. Below, we examine the research to develop molecular targeted approaches to treat ectopic pregnancy.

Mifeprisone treatment of ectopic pregnancy

In early pregnancy, the early conceptus is critically dependent on progesterone from the corpus luteum to survive. Mifeprisone (RU486) is a competitive progesterone receptor antagonist and is commonly used to induce medical termination of pregnancy. It binds to the progesterone receptor and induces a conformational change, blocking its ability to act as a transcription factor.

A systematic meta-analysis found adding mifeprisone to methotrexate resulted in a non-significant improvement of 13% higher rates of success compared with methotrexate alone (Mol et al., 2008). The analysis included data from two trials (Gazvani et al., 1998; Rozenberg et al., 2003) and the P-value was 0.05, raising the possibility that mifeprisone is effective in treating ectopic pregnancy but that these trials are underpowered. Thus, adding mifeprisone to methotrexate to treat ectopic pregnancy merits further consideration. Unfortunately, we have been unable to identify ongoing trials of mifeprisone to treat ectopic pregnancy in the clinical trials registries.

Leukaemia inhibitory factor inhibitor and targeted nanoparticle delivery of chemotherapeutics

LIF is a member of the IL6 family of cytokines and plays a critical role in implantation in women. Krishnan et al. (2013) proposed the use of PEGLA, a novel LIF inhibitor, to treat ectopic pregnancy. They confirmed that ectopic implantation sites express the LIF receptor and found PEGLA treatment blocked downstream signalling in OE-E6/E7 ells (an oviduct cell line). Furthermore, PEGLA inhibited HTR-8/SV neo (a trophoblast cell line) adhesion to OE-E6/E7, and blocked first trimester explant outgrowths.

We previously described a nanoparticle approach to treat ectopic pregnancy (Kaitu’u-Lino et al., 2013). EnGenIC Delivery Vehicles (EDVs) are bacterially derived spheres of 400 nm diameter that can stably encapsulate a range of drugs (while derived from bacteria, there is no genetic material and they are therefore sterile and organic). Millions of EDVs can be injected i.v. for therapy. They stably encapsulate drugs, the payload only released once the EDVs are endocytosed into target cells. Importantly therefore, EDVs can facilitate delivery of drugs to target tissues. Targeting is achieved by coating the surface of EDVs with antibodies specific to antigens highly expressed on the surface of the target tissue.

We loaded EDVs with the chemotherapeutic agent doxorubicin and coated the surface with epidermal growth factor receptor (EGFR) antibodies (Kaitu’u-Lino et al., 2013) (EGFR is highly expressed on the placental membranes, meaning these EDVs will target trophoblast). The EDVs induced greater inhibition of JEG3 (trophoblast cell line) subcutaneous xenografts in non-obese diabetic/severe combined immunodeficiency (NOD/SCID) mice, compared with EDVs coated with antibodies targeting an irrelevant antigen. EGFR-targeted EDVs were readily taken up by human placental explants and induced apoptosis. Furthermore, EGFR-targeted EDVs administered to JEG3 cells induced a dose-dependent inhibition of cell viability, proliferation and apoptosis. Thus, EDVs may represent a promising novel nanoparticle approach to treating ectopic pregnancy.

Combination gefitinib and methotrexate to treat ectopic pregnancy

EGFR signalling switches on a potent cell survival response. A search on BioGPS (www.BioGPS.org) shows that placenta has, by far, the highest expression of the EGFR compared with all other tissue types. Furthermore, the placenta relies heavily on EGFR signalling (Ferretti et al., 2007) which promotes cytotrophoblast motility (LaMarca et al., 2008), blocks apoptosis (Johnstone et al., 2005) and protects placental cells when exposed to stressors (Wolff et al., 2007). Therefore, inhibiting EGFR signalling could negatively affect placental survival and could be a novel approach to treat ectopic pregnancy.

Gefitinib is a molecularly targeted drug that selectively blocks EGFR signalling by inhibiting the tyrosine kinase domain of EGFR (Herbst et al., 2005). The last few decades have seen the emergence of exciting molecularly targeted therapeutics: drugs designed to specifically block molecules that have a major role in disease pathogenesis. The major advantage of molecular targeted therapeutics is that, being highly specific in their actions, they can be particularly effective in targeting the disease and often have a low rate of significant side effects. Examples of highly successful molecular targeted therapeutics include denosumab to treat osteoporosis and bone cancers (Yasuda, 2013) (denosumab blocks the receptor activator of nuclear factor κ-B (RANKL) ligand, a key signalling molecule for bone removal), and B-Raf inhibitors (e.g. vemurafenib) which have been remarkably efficacious against malignant melanoma (Jang and Atkin, 2014). While not yet clinically available, trials of nivolumab have been promising against a range of cancers (Robert et al., 2013). Nivolumab is a therapeutic antibody targeting PD1 (Programmed cell death 1). PD1 dampens the immune system and by countering this, nivolumab increases the host immune response against the cancer.

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Leukaemia inhibitory factor inhibitor and targeted nanoparticle delivery of chemotherapeutics

LIF is a member of the IL6 family of cytokines and plays a critical role in implantation in women. Krishnan et al. (2013) proposed the use of PEGLA, a novel LIF inhibitor, to treat ectopic pregnancy. They confirmed that ectopic implantation sites express the LIF receptor and found PEGLA treatment blocked downstream signalling in OE-E6/E7 ells (an oviduct cell line). Furthermore, PEGLA inhibited HTR-8/SV neo (a trophoblast cell line) adhesion to OE-E6/E7, and blocked first trimester explant outgrowths.

We previously described a nanoparticle approach to treat ectopic pregnancy (Kaitu’u-Lino et al., 2013). EnGenIC Delivery Vehicles (EDVs) are bacterially derived spheres of 400 nm diameter that can stably encapsulate a range of drugs (while derived from bacteria, there is no genetic material and they are therefore sterile and organic). Millions of EDVs can be injected i.v. for therapy. They stably encapsulate drugs, the payload only released once the EDVs are endocytosed into target cells. Importantly therefore, EDVs can facilitate delivery of drugs to target tissues. Targeting is achieved by coating the surface of EDVs with antibodies specific to antigens highly expressed on the surface of the target tissue.

We loaded EDVs with the chemotherapeutic agent doxorubicin and coated the surface with epidermal growth factor receptor (EGFR) antibodies (Kaitu’u-Lino et al., 2013) (EGFR is highly expressed on the placental membranes, meaning these EDVs will target trophoblast). The EDVs induced greater inhibition of JEG3 (trophoblast cell line) subcutaneous xenografts in non-obese diabetic/severe combined immunodeficiency (NOD/SCID) mice, compared with EDVs coated with antibodies targeting an irrelevant antigen. EGFR-targeted EDVs were readily taken up by human placental explants and induced apoptosis. Furthermore, EGFR-targeted EDVs administered to JEG3 cells induced a dose-dependent inhibition of cell viability, proliferation and apoptosis. Thus, EDVs may represent a promising novel nanoparticle approach to treating ectopic pregnancy.

Combination gefitinib and methotrexate to treat ectopic pregnancy

EGFR signalling switches on a potent cell survival response. A search on BioGPS (www.BioGPS.org) shows that placenta has, by far, the highest expression of the EGFR compared with all other tissue types. Furthermore, the placenta relies heavily on EGFR signalling (Ferretti et al., 2007) which promotes cytotrophoblast motility (LaMarca et al., 2008), blocks apoptosis (Johnstone et al., 2005) and protects placental cells when exposed to stressors (Wolff et al., 2007). Therefore, inhibiting EGFR signalling could negatively affect placental survival and could be a novel approach to treat ectopic pregnancy.

Gefitinib is a molecularly targeted drug that selectively blocks EGFR signalling by inhibiting the tyrosine kinase domain of EGFR (Herbst et al., 2005).
et al., 2004). We have undertaken a programme of translational research to examine whether gefitinib could be combined with methotrexate to enhance its efficacy.

We first performed preclinical experiments where we found combining methotrexate and gefitinib was significantly more potent in inducing placental cell death in vitro than treating with either drug alone (Nilsson et al., 2013). The combination was more potent in blocking EGFR signalling and inducing apoptosis compared with either alone. Adding gefitinib to methotrexate induced significantly greater decreases in the JEG3 tumour volume xenografted s.c. in NOD/SCID mice, compared with methotrexate alone. Furthermore, we demonstrated that combining these agents doubled the rates of resorption of eutopic fetuses in immunocompetent mice, compared with either drug alone. Our preclinical data supported the exciting premise that combining gefitinib with methotrexate may be a promising treatment for ectopic pregnancy.

Gefitinib is licensed for use to treat non-small cell lung cancer. Post-marketing surveillance of 31 045 people exposed to gefitinib has been reported to the FDA (Cohen et al., 2004). Common side effects include a transient skin rash and diarrhoea. Gefitinib is associated with a rare but significant side effect of interstitial lung disease (ILD), a thickening of the lung parenchyma (0.3% incidence). However, it is possible the development of gefitinib-related ILD occurs mainly when there is co-existing lung cancer. The median time to develop ILD while taking gefitinib is 42 days and risk factors associated with ILD include age >55 years and male sex (Cohen et al., 2004). For these reasons, we felt administering a short course of gefitinib to women will help to avoid all these risk factors for ILD and is likely to be safe.

We therefore progressed to a Phase I single arm, open-label study of 12 participants diagnosed with ectopic pregnancy with a pretreatment serum hCG <3000 IU/l (Skubisz et al., 2013). Participants were administered methotrexate following the current protocols for medical management (Table II). In addition, they were administered 250 mg of oral daily gefitinib in a dose escalation protocol: one dose n = 3, three doses n = 3 and seven doses n = 6. We found the treatment was safe with no clinical or biochemical evidence of serious pulmonary, renal, hepatic or haematological toxicity.

This trial yielded highly encouraging, though preliminary, efficacy data. The 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. Notably, the median time for the ectopic pregnancies to resolve with combination therapy was 34% shorter compared with methotrexate alone (21 compared with 32 days). One participant was treated successfully for an ectopic pregnancy in her only remaining Fallopian tube (the other tube having been removed previously as treatment for a prior ectopic pregnancy). She subsequently conceived spontaneously and delivered a healthy child at term, suggesting that Fallopian tubes exposed to combination gefitinib and methotrexate can remain fertile.

We have also published a case series of eight women with extra-tubal ectopic pregnancies treated with 7 days of oral gefitinib and i.m. methotrexate (Horne et al., 2014). Five were interstitial (cornual) ectopic pregnancies and three were Caesarean section scar ectopic pregnancies. Pretreatment serum hCG levels ranged between 2458 and 48 550 IU/l, and six women had pretreatment hCG levels >5000 IU/l. All women met with treatment success and promptly resumed menstruation. Three women subsequently conceived spontaneously, and all were successful intrauterine pregnancies (two were born at term, and, at the time of writing, the third woman is in the second trimester).

We have progressed to a Phase II single-arm trial, administering combination gefitinib and methotrexate to 28 women with stable ectopic pregnancies of larger size, defined as a pretreatment serum hCG between 1000 and 10 000 IU/l (see our published protocol, Horne et al., 2013). We included ectopic pregnancies where there was fetal cardiac activity. Our power calculation concluded we required at least 24 of the 28 participants to be cured medically without surgery in order to negate the null hypothesis that the true efficacy of gefitinib and methotrexate to treat ectopic pregnancies is ≤70% (and to demonstrate an efficacy of 90%). We have just completed recruitment.

Collectively, our work has suggested that combination gefitinib and methotrexate shows promise as a new medical treatment to treat ectopic pregnancy. To translate this into standard of care, a large clinical randomized controlled trial is required. We are currently designing such a trial and hope to perform the study in the coming years.

**Conclusion**

An accurate serum biomarker that can accurately diagnose an ectopic pregnancy in cases where there is a PUL would significantly improve clinical care. While many candidate biomarkers have been screened, an optimal clinical test has yet to be developed. Despite this, there are reasons for optimism that such a test is possible. First, it still remains biologically plausible to us that when a conceptus implants ectopically, there are likely to be measureable proteins released at differential levels in the maternal blood compared with women with intrauterine pregnancies, and these await discovery. Secondly, as the understanding of the molecular biology of both ectopic and intrauterine early pregnancies continues to improve, it will allow for further hypothesized-based investigations of other candidate biomarkers. Lastly, significant advances in platform technologies will greatly aid the hunt for biomarkers. These include the ready commercial availability of ultrasensitive quantitative enzyme-linked immunosorbent assay systems to detect almost any desired protein, bioplex technologies that can simultaneously measure a number of proteins in the same sample, and mass spectroscopy.

Even if combination gefitinib and methotrexate is ultimately found not to be useful in the treatment of ectopic pregnancy, we believe that there is significant scope to develop molecularly targeted approaches that are far more effective than methotrexate alone. Given that a successful therapy is the one that only needs to efficiently kill placental tissue within the ectopic pregnancy, it seems very possible that new treatment options that are considerably more efficacious than methotrexate can be devised. If achieved, such a therapeutic could reduce the number of women exposed to surgery and its risks, allow for a more rapid resolution of ectopic pregnancies and even decrease the high rates of maternal losses caused by ectopic pregnancy in the developing world.

**Authors’ roles**

All three authors made substantial contributions to the writing of this review. A.W.H. and S.T. conceived the overall structure. A.W.H. wrote Part 1 (diagnostics), M.M.S. and S.T. wrote Part 2 (therapeutics). All authors provided further intellectual input to generate the final version.
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**Conflict of interest**
S.T. is a named inventor of a patent that relates to epidermal growth factor receptor inhibition in ectopic pregnancy treatment.

**References**


Soriano D, Hugol D, Quang NT, Darai E. Serum concentrations of interleukin-2R (IL-2R), IL-6, IL-8, and tumor necrosis factor alpha in patients with ectopic pregnancy. *Fertil Steril* 2003; 79:975–980.
