Serum biomarkers of tubal ectopic pregnancy: current candidates and future possibilities

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Abstract

Ectopic pregnancy remains a considerable cause of maternal morbidity and mortality worldwide. Currently, it is diagnosed using a combination of transvaginal ultrasound and serial serum β-human chorionic gonadotrophin levels. Diagnosis is often delayed and these tests are time-consuming and costly, both psychologically to the patient and financially to health services. The development of a biomarker that can differentiate a tubal ectopic from an intrauterine implantation is therefore important. In the pre-genomic era, a one-by-one scientific approach has revealed over 20 candidate biomarkers that could be used as a test to diagnose ectopic pregnancy although at present their clinical utility is very limited. These biomarkers cluster into themes: markers of abnormal embryo/trophoblast growth, markers of abnormal corpus luteum function, markers of a growing pregnancy in the Fallopian tube, markers of inflammation and peritoneal irritation, and uterine markers of normal implantation. It is likely that this thematic approach will facilitate the identification of newer biomarkers using microarray technology and inform the development of investigative paradigms using multiple markers at the time of presentation.


Introduction

Tubal ectopic pregnancy is an important cause of maternal morbidity that can be fatal if left undiagnosed due to the risk of potential tubal rupture and haemorrhage. Every year in the UK there are ~11 000 cases of tubal ectopic pregnancy (11.5 per 1000 pregnancies) and four maternal deaths due to ruptured tubal ectopic pregnancies (0.4 per 1000 tubal ectopic pregnancies; Tay et al. 2000, Lewis 2007). The incidence of tubal ectopic pregnancy is increasing not only in the UK but worldwide most likely due to a rising incidence of pelvic inflammatory disease caused by Chlamydia trachomatis infection and the increased use of assisted reproductive techniques (Farquhar 2005, Walker 2007).

Current approaches for diagnosing tubal ectopic pregnancy

In general, the majority of patients presenting with pain or bleeding in early pregnancy have an ultrasound scan to ascertain the viability of the pregnancy and if possible the location of the gestational sac (Gracia & Barnhart 2001). If the ultrasound proves inconclusive, a serum β-human chorionic gonadotrophin (β-hCG) level is ascertained. The diagnosis of an ectopic pregnancy is then based on the combined sonographic findings and serum β-hCG measurements. However, it is difficult to distinguish between an ectopic pregnancy, spontaneous abortion and early ongoing intrauterine pregnancy using a single β-hCG measurement therefore repeated β-hCG measurements are taken (Gracia & Barnhart 2001). Generally, a doubling of serum β-hCG concentrations over 48 h is classed as a normal rise and is therefore suggestive of viable intrauterine pregnancy (Lenton et al. 1982). If the rise is abnormal or β-hCG concentrations are static, then an ectopic pregnancy event more likely. A tubal ectopic pregnancy can then be inferred by repeated β-hCG measurements or confirmed by endometrial curettage or a more invasive laparoscopy.

Difficulties diagnosing tubal ectopic pregnancy

A tubal ectopic pregnancy can be difficult to diagnose at an early stage as nearly a third of all cases do not exhibit clinical signs and 9% have no symptoms prior to rupture (Tay et al. 2000). In addition, when a woman presents with a suspected pregnancy, in up to half the cases the diagnosis is not made on the basis of the initial consultation, scan and β-hCG estimation despite the improvement in the resolution of ultrasonography (Duncan et al. 1995, Robson & O’Shea 1996, Munro et al. 2008). The requirement for serial β-hCG
The diagnosis of tubal ectopic pregnancy is therefore often time-consuming and costly, both psychologically to the patient and financially to health services (CJ Wedderburn, P Warner, WC Duncan, B Graham & AW Horne, unpublished observations). Consequently, a serum biomarker (Atkinson et al. 2001) of tubal implantation, which could accurately identify an ectopic pregnancy at first presentation, would be a major clinical advance (Cabar et al. 2008). A serum biomarker has been defined by the Biomarkers Definitions Working Group as ‘a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention’ (Atkinson et al. 2001). As there are no suitable animal models of tubal ectopic pregnancy, research into putative biomarkers is carried out in women. In most studies, serum concentrations of a putative biomarker are retrospectively measured in cohorts of women with a diagnosis of tubal ectopic pregnancy, ongoing intrauterine pregnancy and spontaneous abortion. This paradigm introduces major problems with interpretation of biomarker utility as the numbers are small, there are gestational differences, the samples are often collected from more than just the cohort of women in whom the diagnosis is difficult and large prospective studies are rare. However, in the pre-genomic era, a logical approach to biomarker identification has resulted in several candidate biomarkers of varying potential. These markers follow several themes (Fig. 1 and Tables 1–5) and it is likely that these themes can inform novel biomarker identification and serum diagnostic strategies in the post-genomic era.

**Markers of abnormal embryo/trophoblast growth**

The first theme and indeed the primary current investigative strategy for the diagnosis of tubal ectopic pregnancy are focused on molecules secreted from the conceptus (Table 1). It is likely that pregnancy implanted in the tubal environment will have abnormal growth kinetics that can be reflected in disordered measurable trophoblast function. This is characterised by the abnormal dynamics of serum β-hCG concentrations in ectopic pregnancy.

**Human chorionic gonadotrophin**

Serum measurement of the β-subunit of hCG is currently the only biomarker that is routinely used clinically to aid the diagnosis of ectopic pregnancy. It has been studied...
extensively as a marker of ectopic pregnancy over the last 30 years (Rasor & Braunstein 1977). Much of the early research suggested that serum β-hCG levels were reduced in ectopic pregnancy (Lundstrom et al. 1979, Milwidsky et al. 1980). However, the true value of β-hCG measurement in ectopic pregnancy is based on two crucial concepts. The first of these relates serum β-hCG concentrations to ultrasound findings using the concept of a ‘discriminatory zone’ (Kadar et al. 1981a). The ‘discriminatory zone’ was classified as the minimum serum β-hCG concentration at which an intrauterine gestational sac could be reliably identified on ultrasound assessment. Therefore, if the serum β-hCG concentration was above the proposed discriminatory zone and there was no sign of an intrauterine gestational sac on ultrasonography, an ectopic pregnancy could be diagnosed. The initial suggestion was that the discriminatory hCG concentration lay somewhere between 6000 and 6500 IU/l (Kadar et al. 1981a). However, as imaging technology improved this discriminatory value became lower, initially to 2000 IU/l (Mol et al. 1998a) and lower if there was ultrasound evidence of an adnexal mass or fluid in the pouch of Douglas. At present, most clinics have a ‘discriminatory zone’ of between 1000 and 1500 IU/l, which equates to just over 5 weeks gestation. However, care needs to be taken in interpreting single β-hCG values in the presence of twin gestation or spontaneous abortion. The second concept is the use of serial serum β-hCG measurements 2 days apart (Kadar et al. 1981b). Initial studies suggested that in 48 h the normal percentage increase in serum β-hCG should be at least 66% and that this would not be the case in ectopic pregnancy. However, 15% of normal intrauterine pregnancies showed a suboptimal increase and a strict cut-off for the diagnosis of ectopic pregnancy would lead to unnecessary laparoscopies. Subsequent studies have suggested that the minimum rise, over 48 h, to predict normal viable intrauterine pregnancy was 53% (Barnhart et al. 2004) or even 35% (Seeber et al. 2006) to avoid unnecessary laparoscopies (Condous et al. 2004). Most units currently use a much less conservative cut-off and frequently use multiple serial β-hCG assessments in cases that are difficult to diagnose. Despite its deficiencies, serum β-hCG concentrations combined with transvaginal ultrasonography is widely and routinely used as a biomarker of tubal ectopic pregnancy. However, the key concept of this biomarker is the role for dynamic assessment by measuring serum concentrations several days apart.

**Pregnancy-associated plasma protein-A**

Pregnancy-associated plasma protein-A (PAPP-A) is another large glycoprotein that is produced by the trophoblast although it is also produced by the decidua (Lin et al. 1976). Early studies showed that the concentration of PAPP-A was lower in patients with a tubal ectopic pregnancy when compared with normal intrauterine pregnancy (Bischof et al. 1983, Sinosich et al. 1985). Indeed, the serum concentration of PAPP-A of ectopic pregnancy was sometimes within the

### Table 1 Markers of abnormal embryo/trophoblast growth.

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Description</th>
<th>Utility as biomarker</th>
<th>References</th>
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<tbody>
<tr>
<td>β-Human chorionic gonadotrophin (β-hCG)</td>
<td>Hormone produced by trophoblast that maintains the corpus luteum</td>
<td>Serial measurements used currently in clinical practice</td>
<td>Seppala &amp; Purhonen (1987), Riss et al. (1989), Garcia et al. (1990), Guillaume et al. (1990), Witt et al. (1990), Mantzavinos et al. (1991), Grosskinsky et al. (1993), Kuscu et al. (1993), Cacciapuoti et al. (1994), Dally et al. (1994), Korhonen et al. (1996), O’Leary et al. (1996), Mol et al. (1997), Marill et al. (1999), Condous et al. (2004), Mueller et al. (2004) and Seeber et al. (2006)</td>
</tr>
<tr>
<td>Human placental lactogen (HPL)</td>
<td>Placental hormone that increases insulin production</td>
<td>Not useful as single predictive marker of ectopic pregnancy</td>
<td>Kuscu et al. (1993), Mueller et al. (2004) and Daponte et al. (2005)</td>
</tr>
<tr>
<td>Activin A</td>
<td>Glycoprotein of TGFβ1 superfamily that is produced by the pregnant decidua and ovary</td>
<td>Lower in ectopic. 100% sensitivity and 100% specificity. Discounted in most recent study due to much lower sensitivity and specificity. Need more studies</td>
<td>Florio et al. (2007) and Kirk et al. (2008)</td>
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non-pregnant range (Bischof et al. 1983). A subsequent larger study found that PAPPA was undetectable in 84.8% of samples from women \((n=102)\) with tubal ectopic pregnancy, but it was also undetectable in 55% of intrauterine spontaneous abortions (Sjoberg 1987). It seems that serum PAPPA concentrations are very low before 7 weeks of gestation and in isolation are poorly discriminative between spontaneous abortion and tubal ectopic pregnancy (Mueller et al. 2004, Daponte et al. 2005).

**Human placental lactogen**

Human placental lactogen (HPL) is also a major placental product that can be detected during the first trimester (Handwerger & Freemak 2000). It has a somatotrophic effect on the foetal tissues aided by enabling adequate foetal nutrient supply by altering the maternal carbohydrate and lipid metabolism (Walker et al. 1991). 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 involving a number of placental proteins showed that HPL levels were lower in those with tubal ectopic pregnancy compared with normal intrauterine pregnancy, particularly after 7 weeks of 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 its utility as a clinically useful biomarker.

**Activin A**

Activin A is a member of the transforming growth factor-β family that is secreted from the ovary and placenta (Petraglia et al. 1987, Wen et al. 2008). However, in pregnancy, the main source of secretion is the trophoblast (Petraglia et al. 1987). Florio et al. (2007) examined the blood samples of 486 women (viable intrauterine pregnancy \((n=155)\), first-trimester spontaneous miscarriage \((n=305)\), tubal ectopic pregnancy \((n=76)\)) and found that activin A levels in women with tubal ectopic pregnancy were markedly lower than those with intrauterine pregnancy and spontaneous miscarriage \((P<0.0001)\). More importantly, it performed better
Table 3 Markers of growing pregnancy in the Fallopian tube.

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Description</th>
<th>Utility as biomarker</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myoglobin</td>
<td>One of protein components of muscle fibres</td>
<td>Not useful as single predictive marker of ectopic pregnancy</td>
<td>Birkhahn et al. (2001)</td>
</tr>
<tr>
<td>Smooth muscle heavy-chain myosin</td>
<td>One of protein components of muscle fibres</td>
<td>Not useful as single predictive marker of ectopic pregnancy</td>
<td>Birkhahn et al. (2000b, 2001)</td>
</tr>
<tr>
<td>Vascular endothelial growth factor (VEGF)</td>
<td>Potent angiogenic factor that stimulates profound angiogenesis at implantation site</td>
<td>Discounted in largest study to date. Possibly useful as single predictive marker of ectopic pregnancy in selected patient groups</td>
<td>Daniel et al. (1999), Felemban et al. (2002), Kucera-Sliutz et al. (2002), Fasouliotis et al. (2004), Mueller et al. (2004) and Daponte et al. (2005)</td>
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than a single measurement of hCG in discriminating between the different groups (Florio et al. 2007). When using activin A as a single marker, the authors calculated that for their study population (tubal ectopic pregnancy prevalence 14.18%), a tubal ectopic pregnancy could be identified with a sensitivity of 100% and specificity of 99.6% if the cut-off was <0.37 ng/ml. Disappointingly, a preliminary report of the use of activin A as a single biomarker for the diagnosis of tubal ectopic pregnancy in a different population of women has been less encouraging, with a much lower specificity and sensitivity (Kirk et al. 2008). Although it is not being used in clinical practice, activin A remains a potentially important biomarker that is worthy of further study.

**α-Foetoprotein and cell-free foetal DNA**

Unlike the above markers of trophoblast function, which are lower in ectopic pregnancy, it was hypothesised that some embryonic markers, which are normally maintained within the conceptus, would be released during abnormal tubal implantation. α-Foetoprotein (AFP) is a product of both the yolk sac and the foetal liver (Jones et al. 2001) and an initial study suggested that serum AFP was elevated in tubal ectopic pregnancy (Grosskinsky et al. 1993). However, a subsequent study has not confirmed this finding (Kucu et al. 1993) and it is known from serum screening programmes that threatened abortion can increase serum AFP in ongoing pregnancies.

Foetal DNA escaping into the maternal circulation has also been analysed with more positive results. Lazar et al. (2006) examined cell-free foetal DNA, looking at the Sry gene, in blood samples from 58 women. They identified the Sry gene in 15 women who had a tubal ectopic pregnancy and 14 women with an intrauterine pregnancy, and its concentration was significantly higher in those with a tubal ectopic pregnancy ($P<0.001$).

Using a cut-off of >80 GE/ml, this test was able to differentiate a tubal ectopic from an intrauterine pregnancy with sensitivity of 84%, a specificity of 76%, a positive predictive value (PPV) of 83% and a negative predictive value (NPV) of 83% (Lazar et al. 2006). This technology at present is lengthy and complex and cannot be used when the conceptus is female, so its utility as a clinically useful serum biomarker is very limited.

**Markers of abnormal corpus luteum function**

The second theme is the assessment of luteal function by measuring secreted ovarian products (Table 2). Ongoing function of the corpus luteum is fundamental to maternal recognition of pregnancy and the maintenance of early pregnancy. In women, the corpus luteum is rescued from luteolysis by hCG from the implanting blastocyst. Luteal function in early pregnancy is absolutely dependent on logarithmically increasing serum hCG concentrations, and in the absence of normal hCG kinetics, it is suboptimal. It was therefore evident that a single measurement of a luteal product may give a clearer picture of recent hCG dynamics, which are suboptimal in ectopic pregnancy, than a single measurement of hCG.

**Steroids from the corpus luteum**

**Progesterone**

The most obvious luteal cell product is progesterone and there has been much research into its utility as a biomarker of ectopic pregnancy or a pregnancy of unknown location. It was initially reported, in a small cohort, that all women with tubal ectopic pregnancies ($n=29$) had a serum progesterone measurement of <15 ng/ml and this was lower than women ($n=20$) with a viable intrauterine pregnancy (Matthews et al. 1986).
Tumour necrosis factor (TNF) is a cytokine produced by macrophages and T-lymphocytes and secreted by T-lymphocytes and pregnant deciduas. It plays a role in the regulation of the immune response.

The Interleukin-2 receptors are protein expressed on the surface of T-lymphocytes, and Interleukin-6 is a pro-inflammatory cytokine secreted by T-lymphocytes. These cytokines are involved in the regulation of the immune response and are produced by macrophages, T-lymphocytes, and pregnant deciduas.

Proteins such as Cancer antigen-125 (MUC16), Cancer antigen-1, and Cancer antigen-125 (MUC16) are cell-surface antigens that are primarily tumour markers. They are found in the female genital tract and pregnant deciduas.

Interleukin-2 is a cytokine produced by activated T-lymphocytes, and Interleukin-8 is a chemokine produced by macrophages. Both are involved in the regulation of the immune response.

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Table 4: Markers of inflammation and peritoneal irritation.

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Description</th>
<th>Utility as biomarker</th>
<th>References</th>
</tr>
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<tbody>
<tr>
<td>Interleukin-2 receptors</td>
<td>Protein expressed on surface of T-lymphocytes</td>
<td>Not useful as single predictive marker of ectopic pregnancy</td>
<td></td>
</tr>
<tr>
<td>Interleukin-6</td>
<td>Pro-inflammatory cytokine secreted by T-lymphocytes</td>
<td>Potentially useful. Higher in ectopic compared with viable and non-viable pregnancies. Need more studies</td>
<td></td>
</tr>
<tr>
<td>Interleukin-8</td>
<td>Chemokine produced by macrophages</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tumour necrosis factor (TNF)</td>
<td>Cytokine involved in regulation of the immune response</td>
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A follow-up study, using this cut-off, reported that all ectopic pregnancies were identified at the patient’s first visit to the emergency department and that none were mistaken for viable intrauterine pregnancies (Yeko et al. 1987). A larger study around the same time also found serum progesterone measurement to be a valuable diagnostic test for tubal ectopic pregnancy (Buck et al. 1988). Using a cut-off of 20 ng/ml, women with an ectopic pregnancy (n=89), when compared with spontaneous abortion (n=27) or ongoing intrauterine pregnancy (n=77), could be predicted with a sensitivity of 92%, a specificity of 84%, a PPV of 90% and NPV of 87% (Buck et al. 1988). However, not all studies had the same success. Riss et al. (1989) were only able to diagnose a tubal ectopic pregnancy, using the cut-off of 15 ng/ml, with a sensitivity of 93%, specificity of 47% and perhaps most crucially a PPV of only 30%. They concluded that tubal ectopic pregnancy could not be identified on the basis of serum progesterone measurement alone. Subsequent studies have reported mixed results. Guillaume et al. (1990) investigated a larger population of 205 women with tubal ectopic pregnancy (n=100), viable intrauterine pregnancy (n=69) and threatened abortion (n=36) and found that all but one of the women with a tubal ectopic pregnancy had serum P levels <23 ng/ml and this had a sensitivity of 99% and a specificity of 100%. However, serum progesterone levels are significantly lower in both tubal ectopic pregnancies (n=32; P<0.001) and spontaneous abortions (P<0.001) than viable intrauterine pregnancies (Witt et al. 1990). Crucially, there was no significant difference in serum progesterone concentrations in women with an ectopic pregnancy when compared with those who had a spontaneous abortion and this reduced the predictive value of progesterone. Subsequent studies have highlighted the considerable overlap in progesterone concentrations between diagnostic groups. In one study, 31% of viable intrauterine pregnancies, 23% of abnormal intrauterine pregnancies and 52% of tubal ectopic pregnancies had a serum progesterone concentration between 10 and 20 ng/ml (Gelder et al. 1991). Many subsequent studies into the utility of progesterone as a biomarker for tubal ectopic pregnancy had similar results. Serum progesterone levels are significantly lower in tubal ectopic pregnancy but that they are not discriminative enough to be diagnostic in isolation (Mantzavinos et al. 1991, Grosskinsky et al. 1993, Kuscu et al. 1993, Daily et al. 1994, Ledger et al. 1994, McCord et al. 1996, O’Leary et al. 1996, Mol et al. 1998b, Buckley et al. 2000, Condous et al. 2004, Mueller et al. 2004). However, the concept that progesterone can be a marker of serial hCG dynamics

Table 5: Uterine markers of normal implantation.

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<thead>
<tr>
<th>Category</th>
<th>Biomarker</th>
<th>Description</th>
<th>Utility as biomarker</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Glycoselhin (placental protein-14)</td>
<td>Major secretory product of endometrium</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Activin B</td>
<td>Glycoprotein of TGFβ1 superfamily that is produced by pregnant decidua and enhances FSH secretion</td>
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still has some merit. When the value of a single serum progesterone measurement (<25 ng/ml) is compared with serial hCG measurements (n=402), it was significantly more sensitive (P<0.05) in the diagnosis of ectopic pregnancy than two hCG measurements of 48 h apart (Stovall et al. 1992). Some centres have added progesterone assessment to the standard hCG/ultrasound diagnostic protocol; however, its value as a useful discriminatory tool in practice has been questioned (Daponte et al. 2005, Katsikis et al. 2006). It is clear that progesterone concentrations are lower in tubal ectopic pregnancy and this probably does reflect hCG dynamics. However, the poor discriminatory capabilities of a single serum progesterone measurement mean that its value as a serum biomarker does not support its routine use in clinical practice.

Oestradiol

The corpus luteum of women also secretes oestradiol (E2) in response to hCG and again could function as a luteal marker of pregnancy dynamics. A preliminary study in 205 women confirmed that all of the ectopic pregnancies (n=100) had an E2 level <650 pg/ml and all but one of the intrauterine pregnancies had a value >650 pg/ml (Guillaume et al. 1990) giving a sensitivity of 100% and specificity of 99%. However, again there was no difference in serum E2 when women with tubal ectopic pregnancy were compared with those with non-viable intrauterine pregnancies (Witt et al. 1990). There are differences in serial serum E2 concentrations in tubal ectopic pregnancy when compared with viable intrauterine pregnancies measured up to 11 weeks of gestation (Mantzavinos et al. 1991). While E2 concentrations rose continuously in viable pregnancies, they were reduced in tubal ectopic pregnancy and the values plateaued after the sixth week and declined after the eighth week of gestation. However, like progesterone assessment, E2 concentrations overlapped between different diagnostic groups (Mantzavinos et al. 1991, Grosskinsky et al. 1993, Kuscu et al. 1993) and the considerable overlap meant that identifying a discriminatory cut-off value was not possible and E2 assessment as a biomarker has not entered clinical practice.

Peptides from the corpus luteum

Relaxin and renin

Relaxin is a well-recognised peptide hormone produced by the corpus luteum during pregnancy (Weiss et al. 1976). It has been shown to be elevated shortly after conception and remain steady until the 15th week of gestation (Szlachter et al. 1982). Preliminary assessment showed that serum relaxin concentrations in patients with a tubal ectopic pregnancy (n=9) were significantly lower than that in those (n=13) with a viable intrauterine pregnancy (Garcia et al. 1990). However, 67 out of 74 (91%) patients with either a spontaneous miscarriage or tubal ectopic pregnancy had low concentrations of relaxin and as such it too was poorly discriminatory as a biomarker of ectopic pregnancy (Witt et al. 1990). The ovaries are now known to be a source of extrarenal renin and its production has been shown to rise when ovulation is followed by pregnancy (Sealey et al. 1987). It has been reported that active renin was significantly decreased in women with an ectopic pregnancy when compared with those with an ongoing intrauterine pregnancy or spontaneous miscarriage (Meunier et al. 1991). However, although a low active renin (<30 pg/ml) in combination with low hCG could predict a tubal ectopic pregnancy, the specificity of 76% and PPV of 75% suggested little clinical utility and further assessment has been limited (Meunier et al. 1991).

Inhibin A

Inhibin A is a major peptide product of the corpus luteum whose secretion is regulated by hCG (IlIlingworth et al. 1996). Although inhibin A is produced by the trophoblast, the corpus luteum is the major source of inhibin A in early pregnancy (Treetamnich et al. 2000). Serum dimeric inhibin A and pro-α-C inhibin were lower in women with tubal ectopic pregnancies than ongoing intrauterine pregnancies (IlIlingworth et al. 1996, Seier et al. 1996) and fell more precipitously than hCG after treatment for ectopic pregnancy due to its shorter half-life (D’Antona et al. 1998). A recent prospective study has suggested that inhibin A was lower in ectopic pregnancies (n=17) than both ongoing (P<0.0002) and failing intrauterine (P<0.0002) pregnancies and it may be a better marker to differentiate between a failing pregnancy and tubal ectopic pregnancy than hCG (Segal et al. 2008). It thus is an attractive potential biomarker and more studies are required to assess its utility in clinical practice.

Markers of growing pregnancy in the Fallopian tube

The circular layer of smooth muscle that surrounds the Fallopian tube can be disrupted by a growing ectopic pregnancy. Indeed, the integrity of the tubal musculature is particularly disrupted during tubal rupture. It is clear that the damaged Fallopian tube will release potentially measurable compounds into the circulation and these could be used as biomarkers of tubal implantation. Markers of muscle damage have been investigated as potential markers of tubal ectopic pregnancy (Table 3).

Creatine kinase

Creatine kinase (CK) is an enzyme that is released when muscle becomes damaged and is currently used as a diagnostic biomarker for myocardial infarction (Costa et al. 2008). The first study of CK as a marker of Fallopian
tube damage produced some encouraging results (Lavie et al. 1993). Serum CK concentrations were significantly higher in those with tubal pregnancy \((n=17)\) as opposed to those with missed abortion \((n=17)\) or normal pregnancy \((n=17)\). In this study, there was no overlap between the groups and all women with a tubal ectopic pregnancy had a CK level > 45 IU/l. As well as being higher than ongoing pregnancies \((n=20)\) or missed abortions \((n=20)\), CK in tubal ectopic pregnancies \((n=20)\) was also higher \((P<0.0001)\) than that in women with acute appendicitis \((n=10)\) or pelvic inflammatory disease \((n=20;\) Chandra & Jain 1995). However, when used as a marker in an unselected population, although serum CK was significantly higher in tubal ectopic \((P<0.0001)\) compared with intrauterine pregnancy (ongoing pregnancy, complete abortion and incomplete abortion), there was considerable overlap in the values and with a cut-off of 45 IU/l had a sensitivity of 57% and specificity of 67% \((Duncan et al. 1995)\). Some subsequent studies have shown no significant increases in serum CK concentrations in ectopic pregnancy \((Darai et al. 1996, Garcia-Velasco et al. 1996, Korhonen et al. 1996, Lincoln et al. 1996, Qasim et al. 1996, Vandermolen & Borzelleca 1996, Plewa et al. 1998)\). However, others suggest that there are indeed increases in serum CK in tubal ectopic pregnancy \((Birkhahn et al. 2000a)\) although it did not work well as a screening tool in a high-risk population \((Birkhahn et al. 2001, Kurzel et al. 2001)\). The differences in these studies may be because serum CK concentrations may actually be a marker of potential tubal rupture rather than tubal ectopic pregnancy per se \((Develioglu et al. 2002)\). When a study population of women with normal intrauterine pregnancies, unruptured ectopic pregnancies and ruptured ectopic pregnancies was examined, serum CK concentrations were significantly higher in the patients with ruptured tubal ectopic pregnancy compared with unruptured tubal ectopic pregnancy \((P=0.003)\) and normal intrauterine pregnancy \((P<0.0001;\) Develioglu et al. 2002). Indeed, CK concentrations appeared to be affected by tubal location as they were significantly higher in those with ischemic as opposed to ampullary pregnancies. This observation has been confirmed in a more recent study where CK levels were also higher in ischemic tubal ectopic pregnancies and ruptured ectopic pregnancies \((Soundravally et al. 2007)\). It is likely that as a tubal ectopic pregnancy grows and progresses towards rupture, then serum CK concentrations are increased. However, despite the initial excitement, this has not proven to be a clinically useful discriminator.

**Myoglobin and smooth muscle heavy-chain myosin**

Other markers of muscle damage have also been investigated as potential biomarkers of tubal damage in ectopic pregnancy. Myoglobin is a small intracellular protein component of muscle fibres \((Wodzig et al. 1997)\), which is released as a result of striated muscle degradation. There was no correlation between its serum concentration and pregnancy location \((Birkhahn et al. 2001)\). This may be because it is debatable whether it is expressed in smooth muscle fibres \((Enoki & Morimoto 2000)\) and it has a very short circulatory half-life \((Klocke et al. 1982)\). It does not appear to have any validity as a candidate biomarker of ectopic pregnancy. Neither does myoglobin and smooth muscle heavy-chain myosin \((MYH11)\), another marker of muscle destruction \((Katoh et al. 1995)\). Although this marker is significantly elevated in women with tubal ectopic pregnancies \((Birkhahn et al. 2000b)\), the wide spread of values in each diagnostic group made its utility as a marker suspect. A larger study confirmed that it was higher in ectopic pregnancy but it was not discriminatory enough for clinical use with a PPV of 22% and an NPV of 91% \((Birkhahn et al. 2001)\).

**Vascular endothelial growth factor**

Vascular endothelial growth factor (VEGF) is a potent angiogenic factor that plays a key role in vascular growth, permeability and remodelling \((Neufeld et al. 1999)\). It has a major role in the regulation of angiogenesis in the corpus luteum \((Fraser & Duncan 2005)\) and the endometrium \((Fraser et al. 2008)\). It also has a vital role during implantation and placenta and its expression is stimulated by tissue hypoxia \((Ladoux & Frelin 1993)\). It was therefore hypothesised that implantation in the unfavourable Fallopian tube would be associated with increased tissue hypoxia and that, despite a role in endometrial and luteal development, serum VEGF would be increased in tubal ectopic pregnancy \((Daniel et al. 1999)\). Indeed, serum VEGF concentrations were significantly higher in tubal ectopic pregnancy compared with normal intrauterine pregnancy although there was only borderline significance between tubal ectopic pregnancy and failing intrauterine pregnancy \((Daniel et al. 1999)\). In this study, serum VEGF of > 200 pg/ml could discriminate a tubal ectopic pregnancy from an intrauterine pregnancy with a specificity of 90% and a PPV of 86%. Using the same cut-off, Felemban et al. (2002) reported that all 15 women they tested with an intrauterine pregnancy had a serum VEGF of < 200 pg/ml and they were able to distinguish a tubal ectopic pregnancy with a sensitivity of 88%, specificity of 100% and PPV of 100%. Another study at the same time \((n=84)\), however, failed to find statistical differences between serum VEGF concentrations between women with a tubal ectopic pregnancy and those with a failing intrauterine pregnancy \((Kucera-Sliutz et al. 2002)\) and the discrimination was poor with a sensitivity of 56%, specificity of 51% and PPV of 53%. However, when normal intrauterine pregnancy is compared with tubal ectopic pregnancy, serum VEGF correlations do appear to be consistently
Markers of inflammation and peritoneal irritation

It is clear that tubal ectopic pregnancy is associated with peritoneal irritation and indeed this irritation can cause the pelvic pain leading to presentation. As such, markers of peritoneal irritation such as MUC16 and inflammation such as various cytokines have been investigated as possible biomarkers of tubal ectopic pregnancy (Table 4).

Cancer antigen 125

Cancer antigen 125 (MUC16) is typically associated as a biomarker of ovarian carcinoma (Canney et al. 1984). However, it is raised in benign conditions with peritoneal involvement such as endometriosis and uterine fibroids. It is also elevated during early pregnancy and therefore may be more of a marker than peritoneal irritation or inflammation. Indeed, some ectopic pregnancies (n = 15) were included in an ovarian carcinoma screening study and all had low concentrations of serum MUC16 (Halila et al. 1986). When serum concentrations in early pregnancy were investigated, MUC16 increases in normal early pregnancy (Brumsted et al. 1990) and subjects with tubal ectopic pregnancy had a wide range of MUC16 concentrations, but overall they were lower than those seen in normal pregnancy (Brumsted et al. 1990, Witt et al. 1990). Further evidence, however, suggested that serum MUC16 concentrations were increased, rather than reduced, in tubal ectopic pregnancy (Sadovsky et al. 1991) and another study suggested no differences between diagnostic groups (Kuscu et al. 1993). At present, the conflicting studies suggest that serum MUC16 is not a biomarker for tubal ectopic pregnancy. The differences in these studies may reflect the proportion of patients with spontaneous abortion included as it seems that this group do have significantly elevated concentrations of serum MUC16 per se (Katsikis et al. 2006).

Interleukin-2 receptors, interleukin-6, interleukin-8 and tumour necrosis factor

The possible predictive potential of cytokines, which are primarily associated with inflammation (interleukin-2 receptors (IL2R), interleukin-6 (IL6), IL8 and tumour necrosis factor (TNF)), in the diagnosis of tubal ectopic pregnancy has been assessed (Soriano et al. 2003). When the sera of 72 women (33 with viable intrauterine pregnancy, 22 with miscarriage and 17 with tubal ectopic pregnancy) were examined, there was no difference in the serum concentration of IL2R between the three groups. However, the concentration of IL6, IL8 and TNF were significantly higher in tubal ectopic pregnancy when compared with those with a viable intrauterine pregnancy (P = 0.03, P = 0.0001, P = 0.0007 respectively) and miscarriage (P = 0.05, P = 0.0001, P = 0.002: Soriano et al. 2003). In addition, serum levels of IL6, IL8 and TNF did not differ between those with viable intrauterine pregnancy and those who miscarried. These positive findings, however, still need to be replicated prospectively in a much larger cohort of women.

Uterine markers of normal implantation

The normal interaction between the pregnancy and the uterine decidua can result in the detection of uterine products in the maternal circulation. It can be anticipated that this normal secretion may be disrupted in the presence of tubal implantation and that uterine products may have a facility as a biomarker for tubal ectopic pregnancy (Table 5).

Leukaemia inhibitory factor

One such uterine marker is leukaemia inhibitory factor (LIF). Although LIF is a cytokine of the IL6 family with a role in inflammation, it has been shown to have a key function in implantation (Senturk & Arici 1998). It was initially hypothesised that its involvement in inflammation be more important than its expression during normal implantation and that serum LIF would be elevated in tubal ectopic pregnancy (Wegner & Mershon 2001). However, that study suggested that women with a tubal ectopic pregnancy (n = 11) had the lowest mean serum LIF concentrations. Nevertheless, there was considerable overlap between the values in ectopic pregnancy and normal and failing intrauterine pregnancies and the test had a 73% sensitivity and a 72%
specificity for the diagnosis of ectopic pregnancy (Wegner & Mershon 2001). However, a larger study failed to find any differences in serum LIF concentrations between the patients with an ectopic pregnancy and those with an abnormal intrauterine pregnancy (Daponte et al. 2005).

**Glycodelin (placental protein-14)**

Glycodelin, also known as placental protein-14 (PAEP), is a major secretory product of the endometrium and decidua (Ruge et al. 1992). In normal ovulatory cycles, its production increases as the secretory changes in the endometrial tissue progress (Julkunen et al. 1986). In addition, serum PAEP concentrations increase gradually during early first-trimester pregnancy until weeks 8–10 of gestation and then decline (Ruge et al. 1992). Pedersen et al. (1991) found, in a small cohort of 20 women, that women with ectopic pregnancy had significantly lower serum concentrations of PAEP. This was confirmed in a larger cohort of women with a tubal ectopic pregnancy (n=59: Ruge et al. 1992). A comparison of serum PAEP concentrations in women with spontaneous abortion (n=32) and ectopic pregnancy (n=26) found that 81% of women with spontaneous abortion had PAEP serum concentration levels within the normal range while 81% of women diagnosed with an ectopic pregnancy had PAEP levels below the fifth percentile of the normal range (Stabile et al. 1994). In addition, the lower concentrations of serum glycodelin in ectopic pregnancy was not dependent on tubal status as this difference was maintained in patients with an unruptured tube, a ruptured tube or a tubal miscarriage (Foth & Romer 2003). Although glycodelin seems to be a potentially useful biomarker worthy of further study, it is notable that it was not discriminatory when used in a large multi-biomarker study (Daponte et al. 2005).

The identification of uterine markers using a genomic approach

The strategies highlighted above have investigated secreted molecules whose role in normal pregnancy development has been reported and therefore a logical approach has suggested them as possible candidate biomarkers that are altered in tubal ectopic pregnancy. We sought to identify novel decidual markers of intrauterine and ectopic implantation using array 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 tubal ectopic pregnancies (Horne et al. 2008). This approach revealed that activin B is secreted by the decidua and its expression is increased during decidualisation. In addition, women with tubal ectopic pregnancy had a less decidualised endometrium and lower concentrations of serum activin B (P<0.01; Horne et al. 2008).

**Discussion**

Currently, there is sadly no stand-alone diagnostic biomarker for tubal ectopic pregnancy that has been adequately tested and yields accurate results. However, in general, it seems that markers of normal trophoblast and decidual function are lower in tubal ectopic pregnancy as are markers of normal luteal function. By contrast, serum markers of tubal damage, tubal implantation or peritoneal inflammation tend to be increased in women with tubal ectopic pregnancy. Certain serum biomarkers have been shown initially to be of discriminatory value, but subsequent studies have then found them to be of limited use (e.g. PAEP). A number of biomarkers (e.g. E2, PAPPA, MUC16) can distinguish a tubal ectopic from a viable intrauterine pregnancy but are unable to distinguish the former from a non-viable failing intrauterine pregnancy. Other markers (e.g. VEGF, CK, progesterone) have been studied extensively in relation to ectopic pregnancy, but the results have not been consistently convincing enough for them to enter routine clinical practice. The considerable difference in the results of these studies is likely due to limitations in their study design and the nature of the biomarkers themselves. Often the actual cohort that was studied was very small and the prevalence of tubal ectopic pregnancy within the study population is not constant or clinically relevant. The authors also frequently found it difficult to match the subjects for gestational age. This was partly due to the fact that often they were looking retrospectively at patients who attended their emergency department and also due to the general nature of a tubal ectopic pregnancy, which is typically difficult to age. Furthermore, the majority of the studies did not take into account the precise location of the tubal ectopic pregnancy. Develioglu et al. (2002) found that there was a significant difference in the level of CK between ampullary and isthmic ectopic implantations, thus the exclusion of this variable in other studies may also have affected their results. Furthermore, the serum biomarkers can also limit their own use, as often they do not follow a steady pattern (increase or decrease) over a normal gestation. This means that if the subjects are not matched for gestational age large differences can be seen within the same group. An ideal marker would be one that remained constant from an early stage of pregnancy, but in practice this is yet to be found. The accuracy of serum assays has also improved over the last decades and this
may partially account for why many biomarkers have gradually been found to be of limited use. Differing results between the studies may also be the artefact of different methods of biomarker identification and the reagent used to detect them.

As a result of the limited success of single serum biomarker measurement, many researchers have started to investigate the possibility of using multiple serum biomarker analysis in order to diagnose tubal ectopic pregnancy. O’Leary et al. (1996) examined progesterone and β-hCG levels and found, in preliminary studies, that a plasma β-hCG < 3000 IU/l and a plasma progesterone < 40 nmol/l could predict a tubal ectopic pregnancy with a sensitivity of 88% and specificity of 82%. A single serum progesterone measurement may be useful in identifying an abnormal pregnancy (although not specifically a tubal ectopic) in women with a serum β-hCG measurement < 1000 IU/l with a sensitivity of 94% and specificity of 100% (Dart et al. 1998). A number of other multiple serum biomarker studies have focused on the development of an equation that could accurately diagnose a tubal ectopic pregnancy by combining the assay results of several biomarkers. An equation that amalgamated the serum level of both MUC16 and E2 predicted the probability of both miscarriage and 21 out of 29 cases (72%) of tubal ectopic pregnancy (Witt et al. 1990). A triple marker analysis (Mueller et al. 2004) had a high sensitivity and specificity in diagnosing tubal ectopic pregnancy (97.7 and 92.4% respectively), but again showed limitations in its utility with unexpected results in some women. A logical approach to multiple biomarker testing is to combine possible independent markers from the groups described above. Current information would support further assessment of hCG or activin A as a trophoblast marker, progesterone or inhibit A as a luteal marker, VEGF or TNF as a tubal marker and activin B or glycodelin as a uterine marker in various combinations. For the future, an ideal serum biomarker would be one or more that could be accurately and quickly assayed, preferably in an emergency department setting. However, it would also have to be an inexpensive measure in order to have true value clinically. Fundamentally, the question of whether a serum biomarker exists that can accurately and specifically detect a tubal ectopic pregnancy is still unanswered. Furthermore, with the advent of better imaging techniques, a serum biomarker may be superseded by ultrasound-related technology.

**Search strategy and selection criteria**

The following databases were searched: PubMed, MEDLINE and the Cochrane Library using the search term ‘ectopic pregnancy’ alone and in combination with ‘diagnosis’, ‘screening’, ‘biomarkers’, ‘hormones’, ‘hCG’, ‘progesterone’, ‘oestradiol’, ‘activin’, ‘VEGF’ and ‘CK’. Reference lists of articles identified by this search were then used to look for further studies and related articles. Only articles published in English were searched. The articles were found by using the contents of the Edinburgh University library and also Internet resources provided by the library itself. Articles that could not be sourced electronically and were not available in the university library were requested from other libraries in the UK.

**Declaration of interest**

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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