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Investigation of novel endocrine markers of early pregnancy and later pregnancy health

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Table of contents

1C: Increasing risk of clinical miscarriage with decreasing human chorionic gonadotrophin levels at days 15-17 after conception in an IVF cohort

Stephen Tong, Euan M Wallace and Luk Rombauts.

(Manuscript under preparation for submission)

1D: Increased risk of low birthweight with three embryo transfer and low human chorionic gonadotrophin levels in early pregnancy

Stephen Tong, Luk Rombauts. Joseph L Onwude and Euan M Wallace.

(Manuscript under preparation for submission)

Section 2: Biochemical markers of miscarriage at 6-13 weeks gestation *Orange Section*

2A: Serum concentrations of macrophage inhibitory cytokine 1 (MIC 1) as a predictor of miscarriage

Stephen Tong, Budi Marjono, David A Brown, Sheila Mulvey, Samuel N Breit, Ursula Manuelpillai and Euan M Wallace.

Lancet 2004:363:129-130

2B: Very low Pregnancy associated Plasma Protein-A in asymptomatic women destined for miscarriage

Stephen Tong, Budi Marjono, Sheila Mulvey and Euan M Wallace.

(Submitted to: Fertility and Sterility)

2C: First trimester levels of inhibins and activin A in normal and failing pregnancies

Euan M Wallace, Budi Marjono, Katrina Tyzack and Stephen Tong.

Clinical Endocrinology 2004 (in press)

[redacted]

Red section

3A: Survival of dizygotic twins in early pregnancy

Stephen Tong, Simon Meagher and Beverley Vollenhoven.

Nature 2002;416:142.

3B: Determining zygosity in early pregnancy by ultrasound

Stephen Tong, Beverley Vollenhoven and Simon Meagher.

Ultrasound in Obstetrics and Gynecology 2004;23:36-37.

Section 4: Corpora luteal diameters and miscarriage risk

Pink section

4A: Corpora luteal diameters across 5-13 weeks gestation, and its association with miscarriage

Stephen Tong, Boon Chua, Beverley Vollenhoven, Roshan Shamon, Ruby Biezen, Carol Tong and Simon Meagher.

(Submitted to: Ultrasound in Obstetrics and Gynecology)

Declaration

In accordance with Monash University Doctorate Regulation 17 / Doctor of Philosophy 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 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 7 papers which are either published, or are 'in press' in peer reviewed journals, and 6 unpublished papers, some of which have been submitted for publication. I confirm that I have only included work where I have been involved as a major collaborator and contributed to all aspects of the studies including design, data collection, analysis and interpretation. My relative contribution to each study has been declared in statements inserted at the front of each manuscript.

[redacted]

Stephen Tong, *February 2004*

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Summary

This doctorate sets out to identify and investigate novel endocrine markers of early pregnancy and late pregnancy health. Such studies may lead to new screening or diagnostic markers to improve clinical care and generate new insights into the biology of human reproduction. Five themes were explored: markers measured in very early pregnancy to predict pregnancy success, first trimester biochemical markers of miscarriage, studies relating to early twin pregnancy, description of corpora luteal diameters across early pregnancy and its association with miscarriage, and activin A in the prediction of later pregnancy complications.

Several markers of potential clinical utility were identified. Maternal serum inhibin pro αC levels measured at days 15-17 after fertilization *in vitro* are highly sensitive and specific in predicting whether a clinical pregnancy will result. Maternal serum levels of Macrophage inhibitory cytokine 1 (MIC-1) and Pregnancy Associated Plasma Protein-A (PAPP-A) are extremely depressed in association with miscarriage, with low levels possibly preceding the pregnancy loss. Women with a viable fetus and a very small corpus luteum (CL) at 5-9 week's gestation have a 3.7 times increased risk of miscarriage compared to those with very large CL's. The zygosity of spontaneous twins may be determined at early ultrasound by using the number of CL's as a surrogate marker of ovulation. One CL would suggest that twins are monozygotic whereas two would imply dizygosity.

Studies described in this thesis have also refuted the clinical utility of several markers previously proposed. Neither inhibin A nor pro-αC can accurately predict miscarriage, umbilical arterial Activin A levels cannot predict the presence of neonatal hypoxic ischaemic encephalopathy, and maternal serum activin A levels cannot be used to reliably screen for the presence of a growth restricted fetus.

Several biological insights have been generated. A link has been made between human chorionic gonadotrophin levels at the end of the first week of implantation and both low birthweight, and clinical miscarriage diagnosed ≥8 weeks' gestation. The pathogenic mechanisms causing both these obstetric complications may therefore be occurring very early. Three embryo transfer, when compared with having less embryos transferred, was also strongly associated with low birthweight. Limiting the number of embryos transferred may decrease the incidence of low birthweight and its consequences.

By examining the relative number of twins and singletons amongst women who had double ovulated and fallen pregnant, it was concluded that from conception until 5-9 weeks' gestation the presence of one dizygotic twin embryo does not impede the development of its co-twin. The CL of pregnancy was found to attain its maximal diameter at 6-8 weeks gestation and steadily shrinks from 9 until 13 weeks.

In summary, several promising markers relating to early pregnancy success and twin zygosity have been identified, all of which will require verification. Novel observations relating to the biology of early pregnancy have also been described, including the

changes in CL diameters across early pregnancy, observations relating to very early dizygotic twin losses, and data suggesting possible very early pregnancy origins of clinical miscarriage and low birthweight.

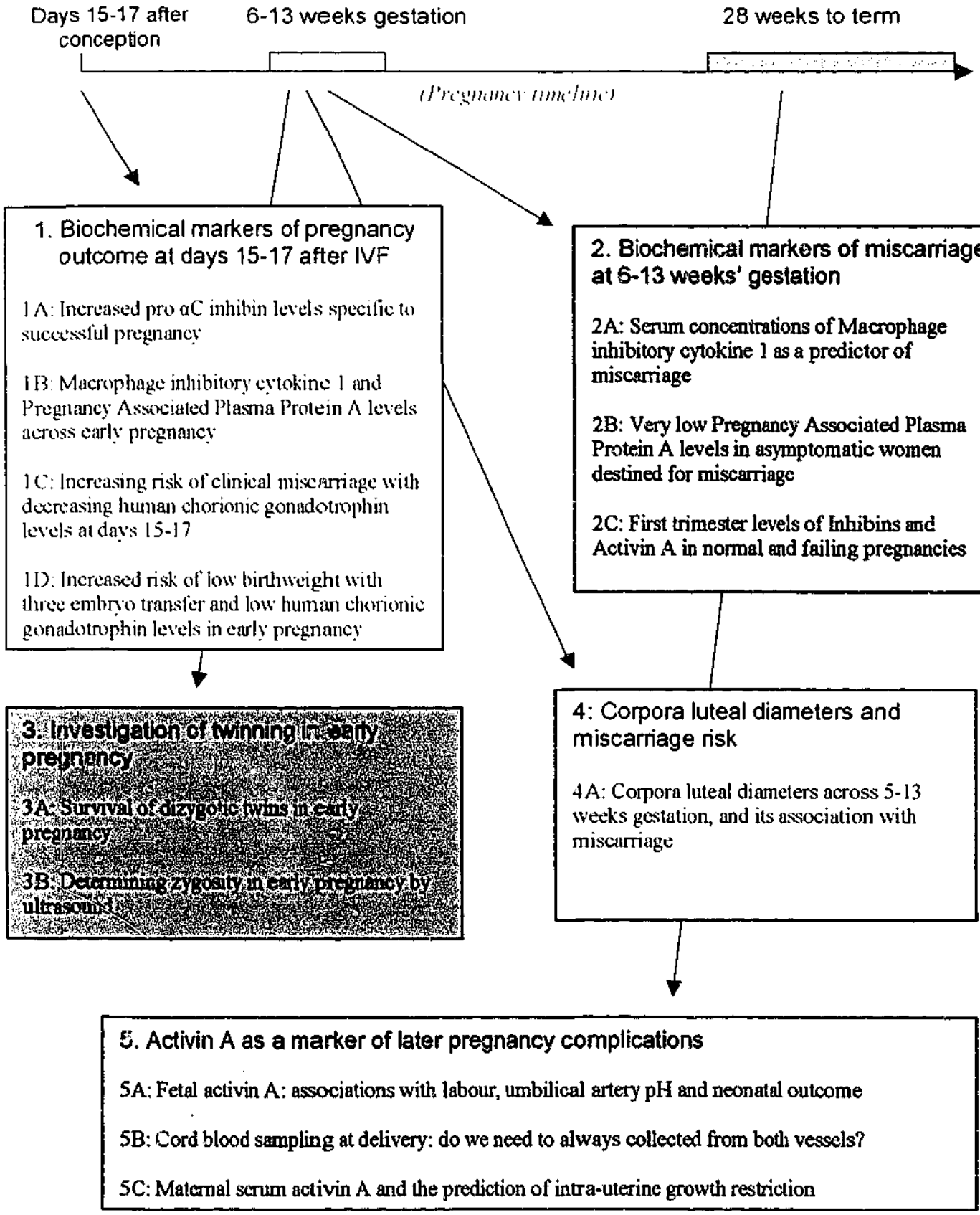
Introduction

Human reproduction is remarkably inefficient. Studies of 18th century parish records on the time taken for newly married Christian virgin couples to conceive (Short, 1978) and more recent large prospective studies (Wang et al., 2003; Wilcox et al., 1988; Zinaman et al., 1996) all suggest that human fecundability, defined as the probability of having a clinically recognised pregnancy after one menstrual cycle in a normal fertile woman, is only 19-30%. Furthermore, if a clinically recognised pregnancy occurs there remains a 9-15% chance that it will miscarry (Gindler et al., 2001; Wilcox et al., 1988; Zinaman et al., 1996).

The theme of the studies undertaken in this doctoral thesis is the investigation of novel endocrine markers of early pregnancy and late pregnancy health. Such studies could lead to the discovery of new screening or diagnostic markers which may be directly useful to improve clinical care. In addition, the investigation of endocrine parameters which vary in association with differing reproductive states can provide important biological insights into the endocrinology and pathophysiology of normal and abnormal reproduction.

In line with the regulations of Monash University, this doctoral thesis comprises of a series of published and unpublished papers. Here, thirteen manuscripts describing original research are presented. They are divided into four sections relating to early singleton and multiple pregnancy, and a fifth investigating later pregnancy. Preceding these sections is a published review article summarising the clinical utility of inhibins and

Investigation of novel endocrine markers of early pregnancy and later pregnancy health



Section 1: Biochemical markers of pregnancy outcome at days 15-17 after IVF

This section encompasses studies exploring the association between pregnancy outcome and analytes measured at days 15-35 after conception (or 4-7 weeks of gestation) in an *in vitro* fertilization (IVF) cohort.

1A. Increased pro-αC inhibin levels specific to successful pregnancy

In light of the fact that maternal serum pro-αC inhibin peaks at around day 16 after conception (Fowler et al., 1998; Illingworth et al., 1996), a study was undertaken to investigate whether levels measured around this time can predict IVF pregnancy success.

1B. Macrophage inhibitory Cytokine 1 and Pregnancy Associated Plasma Protein A levels across early pregnancy

Elsewhere, we report that depressed maternal serum levels of Macrophage inhibitory cytokine 1 (MIC-1;2A), a recently discovered glycoprotein (Lawton et al., 1997), and Pregnancy Associated Plasma Protein A (PAPP-A;2B) are substantially depressed in association with miscarriage. Since both may play important roles in the establishment of pregnancy, this study was undertaken to investigate their ontogeny.

1C. Increased risk of clinical miscarriage with decreasing human chorionic gonadotrophin levels at days 15-17.

Low early human chorionic gonadotrophin (hCG) levels have an established association with preclinical early pregnancy loss (Macklon et al., 2002; Winter et al., 2002), but any

link with later clinical miscarriage has not been properly evaluated. This study tested the hypothesis that low levels may be associated with later miscarriage occurring after clinical pregnancy recognition ($\geq$ 8 weeks' gestation).

1D. Increased risk of low birthweight with three embryo transfer and low human chorionic gonadotrophin levels in early pregnancy

This study explored possible very early origins of low birthweight. It was hypothesised that low early hCG levels may reflect initial difficulties in implantation and are therefore associated with an increased risk of low birthweight. The association between the number of embryos transferred and low birthweight was also evaluated.

Section 2: Biochemical markers of miscarriage in early pregnancy

Utilising a large serum bank prospectively collected as part of a previous Down Syndrome screening study, a number of studies were undertaken to investigate the possible associations between different biochemical markers measured in asymptomatic women and the risk of being subsequently diagnosed with a miscarriage.

2A. Serum concentrations of Macrophage Inhibitory Cytokine 1 as a predictor of miscarriage.

MIC-1 is a glycoprotein (Lawton et al., 1997) with possible immunomodulatory actions favouring viability, and is abundantly expressed at the maternofetal interface (Marjono et al., 2003). A possible association between maternal serum MIC-1 levels and miscarriage was investigated.

2B. Very low Pregnancy Associated Plasma Protein A level in asymptomatic women destined for miscarriage.

There has been a body of literature showing that PAPP-A levels are low in serum sampled from women who present with symptomatic miscarriage (Bersinger et al., 1987; Westergaard et al., 1983; Westergaard et al., 1985). This study explored a possible association between PAPP-A and miscarriage in our asymptomatic cohort.

2C. First trimester levels of inhibins and activin A in normal and failing pregnancies.

In view of recent studies suggesting that pro-αC (Illingworth et al., 1996) and inhibin A (Muttukrishna et al., 2002; Phipps et al., 2000) may be useful markers of miscarriage, these analytes were assayed in our serum pool in order to verify or disprove these findings.

Using the corpus luteum as a surrogate marker of ovulation, two ultrasound studies were undertaken relating to twins.

3A. Survival of dizygotic twins in early pregnancy.

It has been previously estimated by mathematical modeling that that only 1 in 50 twins conceived survive (Boklage, 1995). In this study, a novel method was used to directly investigate the survival of dizygotic twins from conception until clinical pregnancy recognition (5-9 weeks gestation) by determining the relative incidences of twin and

singletons present among a group of pregnant women who had spontaneously double ovulated (two corpora lutea).

3B. Determining zygosity in early pregnancy by ultrasound

A retrospective pilot study was undertaken to examine the possibility of determining the zygosity of spontaneous twins by noting the number of corpora lutea (CL). In the presence of same-sexed dichorionic twin pregnancy, the presence of one CL would suggest that twins are monozygotic whereas two CL's would imply dizygosity.

Section 4: Corpora luteal diameters and miscarriage risk

4A: Corpora luteal diameters across 5-13 weeks of gestation, and its association with miscarriage

The diameter of the corpus luteum (CL) is directly related to the level of progesterone secretion in a number of animal models (Griffin & Ginther, 1992) and may therefore reflect endocrine activity. This study was done to describe the changes in CL diameters across 5-13 weeks of gestation and to investigate whether a small CL has any association with an increased risk of miscarriage.

Section 5: Activin A as a marker of later pregnancy complications

At the time that the PhD had just commenced, our group had generated preliminary data showing that activin A increases with acute ovine fetal hypoxia (Jenkin et al., 2001) and

intrauterine growth restriction in humans (subsequently published: (Wallace et al., 2003)). The following studies were undertaken to determine whether these early observations had any possible clinical utility.

5A. Fetal activin A: associations with labour, umbilical artery pH and neonatal outcome.

We investigated whether serum umbilical arterial activin A levels can identify newborns are at risk of neonatal hypoxic ischaemic encephalopathy and explored its associations with labour outcome.

5B Cord blood sampling at delivery: do we need to always collect from both vessels?

Universal cord blood sampling immediately after delivery to measure pH has been advocated for audit and medico-legal reasons (Westgate et al., 1994). In this study we examined the accuracy of our labour ward staff in collecting valid umbilical arterial samples, and go on to question the need to routinely document both arterial and venous pH in all deliveries.

5C Maternal serum activin A and the prediction of intra-uterine growth restriction.

This study looked at whether maternal serum activin A can be used to screen for fetal growth restriction antenatally.

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Review article:

Inhibins and activins: Clinical advances in reproductive medicine

Stephen Tong, Euan M Wallace and Henry Burger.

Declaration regarding the paper entitled:

Inhibins and activins: clinical advances in reproductive medicine

Contributions to the work involved the following:

Name	%contribution	Nature of contribution
Stephen Tong	40%	Major contributor to the writing of the report.
Euan M Wallace	40%	Major contributor to the writing of the report.
Henry G Burger	20%	Major contributor to the writing of the report.

Declaration by co-authors

The undersigned hereby certify that:

(1) they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;
(2) they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
(3) there are no other authors of the publication according to these criteria;
(4) potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journals or other publications, and (c) the head of the responsible academic unit; and
(5) the original data are stored at the following location(s) and will be held for at least five years from the date indicated below:

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Review

Inhibins and activins: clinical advances in reproductive medicine

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Introduction

The discovery of a new hormone is understandably followed by a competitive urgency to seek useful clinical applications. For the glycoprotein hormones inhibin, isolated in 1985 (Ling *et al.*, 1985; Robertson *et al.*, 1985), and activin, identified the following year (Vale *et al.*, 1986), this is no exception. Some 15 years on, we aim to summarize the advances made in understanding the roles of inhibins and activins in human reproduction, and review their current and likely future applications in clinical practice.

Inhibins are heterodimeric glycoprotein hormones comprising a common α-subunit and one of two β-subunits (α-β_A forming inhibin A, α-β_B forming inhibin B). Other β-subunits have been identified, but there is currently no evidence for the existence of additional biologically active inhibin species (Burger *et al.*, 2000). The existence of inhibin was predicted by McCullagh almost 70 years ago, who noted that hypertrophy of the anterior pituitary gland caused by castration could be prevented by injecting a water soluble extract of bovine testis (McCullagh, 1932). However, McCullagh's 'inhibin' hypothesis remained stalled for half a century, until the 1970s, when it was found that FSH from the pituitary gland could be selectively suppressed both *in vitro* and *in vivo* by testicular (Franchimont *et al.*, 1972; Setchell & Jacks, 1974; Keogh *et al.*, 1976) and follicular fluids (De Jong & Sharpe, 1976). It would take another decade until inhibin was finally isolated and characterized from bovine (Robertson *et al.*, 1985) and porcine (Ling *et al.*, 1985) follicular fluid.

During the purification of inhibin, a structurally related substance which stimulated FSH secretion was identified. This substance was termed activin (Vale *et al.*, 1986) and is a dimer of the inhibin β subunit. Three activins exist: activin A (β_A–β_A), activin B (β_B–β_B) and activin AB (β_A–β_B).

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Inhibins and activins are related to the transforming growth factor beta (TGF-β) superfamily, sharing structural homology through the β subunit as well as common receptor pathways (Derynck *et al.*, 1998). Not surprisingly, it has become clear that, in addition to the regulation of FSH secretion, activins and inhibins may have diverse actions in varied tissues, including autocrine regulation of cell proliferation in the male reproductive tract (Risbridger & Cancilla, 2000), erythropoiesis (Yu *et al.*, 1987) and neural cell survival (Schubert *et al.*, 1990). Some recent advances in the understanding of activin and inhibin signalling have occurred recently with the identification of two possible inhibin receptors (Chong *et al.*, 2000; Lewis *et al.*, 2000). However, full consideration of these findings and the activin receptors and signalling mechanisms is outwith the scope of this review and is available elsewhere (Mathews, 1994; Heldin *et al.*, 1997; Derynck *et al.*, 1998; Attisano & Wrana, 2002).

While initially problematic, the eventual purification of inhibin led rapidly to the development of a number of immunoassays (Burger, 1993), the most widely used of which was a radioimmunoassay employing a polyclonal antibody raised against purified bovine inhibin, with specificity directed against an epitope on the α-subunit (McLachlan *et al.*, 1986a). This assay became known as the 'Monash' assay and was the basis of almost all of the initial studies of inhibin in human reproductive pathophysiology. However, it became apparent that this assay also detected circulating α-subunit free from the β-subunit and of uncertain bioactivity (Schneyer *et al.*, 1990). Therefore, while the application of the Monash assay led to important advances in our understanding of inhibin in reproductive physiology, some observations, as will be discussed below, were difficult to explain (Bhasin *et al.*, 1996). The subsequent development of specific two site assays, particularly those performed by Groome and colleagues (Groome *et al.*, 1994, 1996), for inhibin A and B, inhibin α-subunit precursors and the activins, clarified those apparent inconsistencies and revealed the specific changes in inhibins and activins in health and disease, as discussed below.

Although there are a host of different areas of investigation into inhibins and activins in obstetrics and gynaecology, they centre around three major tissues of origin: the ovary, placenta and testis. Therefore, this review will be structured around these sites of origin, affording a logical discussion linking clinical studies to the physiology of these hormones.

Ovary

It was clear from the application of the first inhibin immunoassays that circulating inhibin levels changed during the menstrual cycle

(McLachlan *et al.*, 1987b; Reddi *et al.*, 1990). However, what was not apparent then was that the pattern of secretion of inhibin A and inhibin B differed across the cycle. It is now known that circulating levels of inhibin B rise in the follicular phase and fall in the luteal phase (Groome *et al.*, 1996). In contrast, circulating levels of inhibin A remain low until the late follicular phase, rising to a midcycle peak, followed by a temporary fall and a further rise to a peak in the mid-luteal phase (Groome *et al.*, 1994). These data reflect that inhibin B is principally a product of the developing follicles, most likely under the stimulation of FSH, and that inhibin A is derived from the dominant follicle and corpus luteum. Indeed, when hCG is administered in the luteal phase to sustain the corpus luteum, mimicking the pattern seen in early pregnancy, inhibin A levels continue to increase whereas inhibin B remains low (Illingworth *et al.*, 1996b). During the postpartum period, inhibin B levels rise, presumably reflecting follicular activity in the ovary, while inhibin A levels remain low until ovulation resumes (Burger *et al.*, 2000; Perheentupa *et al.*, 2000). In addition, while there is no direct evidence that inhibin B exerts negative feedback on FSH secretion, the lower levels observed in older premenopausal women at a time of increasing FSH suggests that this is likely. What remains unclear is why there are two almost identical inhibins with presumed similar biological effects.

Consistent with the early hopes that inhibin may prove to be a useful diagnostic tool in reproductive medicine (Findlay, 1986), the insights afforded by the specific assays suggested that inhibin B may be a useful marker of follicular development and that inhibin A may indicate likely corpus luteal function. The possible clinical applications that these insights offer would include the prediction of outcome of and tracking of assisted reproduction, assessment of perimenopausal changes and the diagnosis and surveillance of ovarian cancer.

Assessment of the ovary during Assisted Reproductive Technology (ART)

It has been appreciated for some time that basal early follicular phase FSH levels are predictive of subsequent assisted reproductive outcome with higher levels indicative of likely failure (Scott *et al.*, 1989; Pearlstone *et al.*, 1992). More recently, studies *in vivo* have suggested that the high FSH levels may relate to low ovarian inhibin secretion, suggesting that inhibin may be a useful marker of 'ovarian reserve' and therefore likely ART outcome (Seifer *et al.*, 1996a; Dumesic *et al.*, 2001). Indeed, a subsequent report from Seifer and colleagues confirmed that a low inhibin B level on day 3 was associated with a poorer response to ovulation induction, requiring significantly more gonadotrophin and resulting in fewer oocytes and a lower pregnancy rate (Seifer *et al.*, 1997). Others have confirmed the finding that a low basal inhibin B level and a high FSH level are associated with poorer ART outcome (Hall *et al.*, 1999; Dzik *et al.*, 2000), but have been unable to define useful diagnostic thresholds to predict those outcomes (Hall *et al.*, 1999). Indeed, this is consistent with the work of Seifer *et al.* (1997) who reported some pregnancies in women with apparently poor 'ovarian reserve'. Accordingly, at present, the measurement of basal inhibin B is not a useful component of routine ART, although it may prove to be helpful in defining 'poor responders' for a more critical evaluation of new induction regimens.

Similarly, while it is clear that the administration of exogenous FSH increases total immunoreactive inhibin, circulating inhibin A, pro-αC inhibin and inhibin B levels (McLachlan *et al.*, 1986b; Buckler *et al.*, 1989; Lockwood *et al.*, 1996; Anderson *et al.*, 1998a; Burger *et al.*, 1998a; Casper *et al.*, 2001) and that the nature of this inhibin response, particularly inhibin A and pro-αC, is associated with ART outcome (Hall *et al.*, 1999; Dzik *et al.*, 2000; Eldar-Geva *et al.*, 2000; Casper *et al.*, 2001), the measurement of these proteins adds little, if anything, to the more established use of serum oestradiol and ultrasound tracking. The measurement of the inhibins to predict or track outcome in ART cycles therefore remains of research interest only.

Assessment of the perimenopausal ovary

Perhaps the strongest evidence that inhibin B has a negative feedback effect on FSH secretion is the observation that inhibin B levels in older, reproductive age women who have elevated circulating FSH are lower than in younger women and that there is an inverse relationship between these two hormones (Klein *et al.*, 1996; Danforth *et al.*, 1998; Welt *et al.*, 1999; Burger *et al.*, 2000). These data are consistent with our understanding that inhibin B is derived from small antral follicles under FSH stimulation and that the number of recruited follicles declines with advancing age, particularly at the time of menopausal transition (Richardson *et al.*, 1987). The observations are also consistent with those discussed previously in the context of an impaired response to ovulation induction in women with low inhibin B levels. However, do these data indicate that inhibin B is a useful marker of impending menopause and indeed a better marker than FSH?

It is well established that there are significant changes in the pituitary-ovarian axis during and after the transition to menopause encompassing rising FSH and subsequently LH levels and declining oestradiol. More recently, it has become apparent that these changes are most likely secondary to the decline in inhibin B secretion resulting from decreasing functioning antral follicles (Burger *et al.*, 1998b; Burger *et al.*, 1999). The fall in inhibin B reduces negative feedback on FSH secretion permitting increased FSH which, in turn, continues to recruit a dominant follicle from the diminishing ovarian pool. In this way, inhibin A and oestradiol secretion are maintained until late in the transition when the

cycles become more frequently anovulatory and then cease (Burger *et al.*, 1999). Thus, the first endocrine change heralding the perimenopause, when menstrual irregularity is first reported, may be a decline in early follicular inhibin B (Burger *et al.*, 1998b; Burger *et al.*, 1999). However, while data collected from cross-sectional studies would suggest that inhibin B may therefore be a useful marker of the perimenopause (Burger *et al.*, 1995; Klein *et al.*, 1996; Danforth *et al.*, 1998; Welt *et al.*, 1999), an extensive longitudinal study of 150 women over 6 years of a natural menopause indicated that there is no good single endocrine marker of the menopause for any given individual woman (Burger *et al.*, 1999). Furthermore, there is considerable variation in early follicular phase inhibin B concentrations in young regularly cycling women, again making it improbable that it will be a valuable marker for predicting menopause onset (Burger *et al.*, 2000).

Diagnosis and surveillance of ovarian cancer

Considering the origins and physiological roles of inhibins and activins, it is not unreasonable to expect that altered circulating levels may be a feature of ovarian malignancy. This expectation was first fulfilled with the report that serum immunoreactive inhibin was significantly increased in women with a granulosa cell tumour (Lappohn *et al.*, 1989; Kauppila *et al.*, 1992; Healy *et al.*, 1993; Jobling *et al.*, 1994). This has been confirmed and extended in a number of subsequent studies showing that all forms of inhibin are increased in women with a granulosa cell tumour but that total inhibin or inhibin B are most consistently raised (Kauppila *et al.*, 1992; Healy *et al.*, 1993; Jobling *et al.*, 1994; Boggess *et al.*, 1997; Petraglia *et al.*, 1998; Robertson *et al.*, 1999b). As summarized by Lambert-Messerlian (2000), immunoreactive inhibin is elevated in 100% of women with granulosa cell tumours, whilst inhibin B is elevated in 89–100% of cases, pro α-C in 90% and inhibin A in 1–77%. It is clear that, for ovarian cancer monitoring, the nonspecific assay recognizing all forms of circulating inhibin is likely to be of greatest utility (Burger *et al.*, 2001). Importantly, serum immunoreactive inhibin in association with granulosa cell tumours is so markedly elevated beyond the levels observed during the normal menstrual cycle that it is potentially valuable for granulosa cell tumour surveillance in women of all ages (Jobling *et al.*, 1994). However, these ovarian tumours are very rare and a primary screening programme specifically targeted for them is not feasible. Instead, the value of inhibin measurement in women with granulosa cell tumour is as a marker of disease recurrence after treatment when levels fall. In those women who remain disease free, inhibin levels stay within the normal premenopausal range (Jobling *et al.*, 1994) whereas, in those that relapse, levels increase again prior to clinical evidence of tumour recurrence (Kauppila *et al.*, 1992; Jobling *et al.*, 1994). It has yet to be determined whether surveillance of granulosa cell tumour recurrence with immunoreactive inhibin affects prognosis but given its ability in identifying recurrence well before it is clinically apparent, such secondary surveillance has been adopted at our centre.

However, granulosa cell tumours constitute only 5–10% of all ovarian neoplasms (Lappohn *et al.*, 1989). Most (85–90%) ovarian cancers arise from the epithelial surface, the more common types being serous or mucinous cystadenocarcinomas (McGuire *et al.*, 2002). These ovarian epithelial cancers still kill more women than all other gynaecological cancers combined (Healy *et al.*, 1993), principally because clinical presentation is often late and distant spread has already occurred. Unfortunately, compared to the rarer granulosa cell tumours, inhibin has been found to be less sensitive in the detection of epithelial cancers. Nonetheless, inhibin has a useful role in the management of these tumours, particularly the mucinous cystadenocarcinomas. In approximately 80% of women with a mucinous tumour, immunoreactive inhibin was found to be elevated, whether the tumour was overtly malignant or of borderline malignancy (Healy *et al.*, 1993). As with granulosa cell tumours, subsequent studies have confirmed that total immunoreactive inhibin, rather than specific inhibin dimers, is the best marker (Robertson *et al.*, 1999b; Ala-Fossi *et al.*, 2000) with 70–80% of women having elevated levels. This compares most favourably with the traditional ovarian cancer marker CA125 which is increased in only a minority of women with a mucinous cystadenocarcinoma (Jacobs & Bast, 1989). In contrast to granulosa cell tumours, the more modest elevation of inhibin levels in association with mucinous tumours argues against its use in premenopausal women who normally have circulating inhibin (Wallace, 1996). Any practical application would therefore be limited to postmenopausal or oophorectomized women.

In addition, inhibin is not a sensitive marker of nonmucinous (serous) ovarian epithelial tumours. Elevated total immunoreactive inhibin has been observed in only 5% to 35% of women with a serous adenocarcinoma (Healy *et al.*, 1993; Lambert-Messerlian *et al.*, 1997; Menon *et al.*, 2000) with even poorer sensitivity reported for the more specific inhibin assays (Burger *et al.*, 1996; Lambert-Messerlian *et al.*, 1997; Petraglia *et al.*, 1998; Menon *et al.*, 2000). However, CA125 is a good marker for these tumours (Jacobs & Bast, 1989) and a two marker screening strategy utilizing immunoreactive inhibin and CA125 has been suggested to detect both serous and mucinous ovarian carcinomas (Lambert-Messerlian *et al.*, 1997; Robertson *et al.*, 1999a). Unfortunately, serum inhibin is elevated in a number of benign ovarian conditions (Healy *et al.*, 1993; Burger *et al.*, 1996; Ala-Fossi *et al.*, 2000), and may be idiopathically increased in postmenopausal women. This would argue against inhibin as a screening marker where it has been estimated that the specificity required of a marker for general application should be 99·6% or more (Jacobs, 1994). Therefore, at present, population screening for ovarian cancer using inhibins remains a research exercise.

Surveillance for recurrence after treatment using total inhibin as a marker would appear worthwhile. A new two-site monoclonal antibody based assay for immunoreactive inhibin, with both sites on the α-subunit, has been developed recently and may be useful for screening evaluation (Robertson *et al.*, 2002). Activin A has also been assessed as a possible ovarian tumour marker. Unfortunately, while it is elevated in some women with late stage epithelial cancers, it is not consistently so (Lambert-Messerlian *et al.*, 1997; Menon *et al.*, 2000) and is therefore unlikely to find utility as a marker.

The potential use of inhibin in the context of ovarian cancer may not be restricted to post-treatment surveillance. Often, particularly when these tumours are advanced and undifferentiated, it is difficult to assign the tissue of origin thus compromising planning of appropriate therapy. However, Gurusinghe *et al.* (1995) showed that granulosa cell tumours and mucinous cystadenocarcinomas, but not serous tumours, stain positively for both the inhibin α- and β_A-subunits, as would be expected from the serum studies discussed above. Others have confirmed that ovarian tumours stain positively and may be useful in the differential diagnosis of some malignancies (Flemming *et al.*, 1996; Pelkey *et al.*, 1998). There is debate about the site of immunohistochemical staining which may depend on whether the tumour tissue is examined fresh frozen or after fixation (Burger *et al.*, 2001). However, more recently, it was shown that adenocarcinomas of various primary origins may display immunostaining for inhibin (McCluggage & Maxwell, 1999) and that advanced ovarian malignancy may not stain, or have reduced staining, for inhibin (Ala-Fossi *et al.*, 2000; Gebhart *et al.*, 2000). These observations suggest that, while inhibin immunohistochemistry will certainly have a role in the assessment of abdominal malignancy, definitive diagnoses on those findings alone cannot be expected.

Placenta

Early pregnancy assessment

As discussed previously, despite the lack of an immediate application for inhibins prior to conception in ART, there are several potential applications for inhibin and activin in pregnancy. Inhibins and activins are produced in abundant amounts throughout pregnancy by the feto-placental unit (McLachlan *et al.*, 1987a; Healy *et al.*, 1988; Petraglia *et al.*, 1993). Both activin A and inhibin A have been found in the decidua (Petraglia *et al.*, 1990), fetal membranes (Petraglia *et al.*, 1993), the developing fetus (Jaffe *et al.*, 1993; Roberts & Barth, 1994) and the placenta (Muttukrishna *et al.*, 1997a; Wallace *et al.*, 1997b). Circulating inhibin levels are at detectable levels by 4 weeks of gestation (Lockwood *et al.*, 1997), peak at 9–10 weeks (Tovanabutra *et al.*, 1993) before falling to a plateau between 15 and 20 weeks (Abe *et al.*, 1990; Qu *et al.*, 1991; Tabei *et al.*, 1991; Tovanabutra *et al.*, 1993) after which levels rise such that peak levels are achieved at term. Of the two dimers, the placenta only secretes inhibin A (Wallace *et al.*, 1997b), which is detectable in maternal serum, whereas the chorion trophoblast secretes both inhibin A and B into the amniotic fluid (Wallace *et al.*, 1997b). Inhibin B levels remain essentially undetectable in maternal serum throughout pregnancy. The low levels detectable at term most probably represent cross-reactivity of the very large amount of circulating inhibin A in the inhibin B assay (Petraglia *et al.*, 1997). In early pregnancy, the corpus luteum secretes significant amounts of pro-αC inhibin (Illingworth *et al.*, 1996b). Activin A is also present in maternal serum throughout pregnancy (Lockwood *et al.*, 1997), increasing progressively such that peak levels are attained at 37 weeks (Schneider-Kolsky *et al.*, 2000a).

Following the development of the specific inhibin assays and the recognition that the placenta secretes principally inhibin A, a number of investigators explored this protein as a marker of early pregnancy viability. In a small number of women with a spontaneous pregnancy presenting with early pregnancy bleeding, Illingworth *et al.* (1996b) reported that inhibin A levels were unaltered in those with early pregnancy failure compared to those with an on-going pregnancy. However, they did observe lower pro-αC inhibin levels in the former group. In women undergoing *in vitro* fertilization–embryo transfer, Lockwood *et al.* (1996) reported that inhibin A levels at 4 weeks were significantly lower in eight women who were to eventually have a miscarriage compared to those who were to have ongoing pregnancies. Importantly, five of those eight had preclinical 'biochemical pregnancies'. In a larger study, Treetampinich *et al.* (2000) also found that inhibin A was able to identify nonviable 'biochemical pregnancies', and to do so earlier than β-hCG, but observed that once a clinical pregnancy was evident (at approximately 6 weeks gestation), inhibin A was unable to discriminate the pregnancies that were eventually successful from those that miscarried (Treetampinich *et al.*, 2000). We have also shown that activin A is not a useful marker of early pregnancy failure (unpublished observations). More recently, a multiple marker approach, including the use of inhibin A, to discriminate nonviable from viable pregnancies demonstrated that such a biochemical approach does not offer adequate sensitivity or specificity to find clinical acceptance. In any event, inhibin A was a poorer marker than progesterone (Phipps *et al.*, 2000). Together, these studies suggest that inhibins and activins are not useful as markers of pregnancy viability. Indeed, it is difficult to foresee that any biochemical marker could replace ultrasound assessment in this setting.

In contrast, the expectant management of ectopic pregnancy requires serial biochemical surveillance, traditionally with hCG. Inhibin A levels are lower in women with an ectopic pregnancy than in those with a normal pregnancy (Seifer *et al.*, 1996b) with apparently better discrimination than that afforded by hCG. However, whether this translates into more rapid diagnosis has yet to

be demonstrated. It has also been suggested that, because the half-life of inhibin A is much shorter than that of hCG, inhibin A might prove to be a better marker of persistent trophoblastic disease following conservative management of ectopic pregnancy (D'Antona *et al.*, 1998a). Unfortunately, the return of ovarian function leading to a rise in circulating inhibin A complicated the interpretation of surveillance inhibin A level, leaving hCG as the preferred marker (D'Antona *et al.*, 1998a). Similarly, while an early report suggested that immunoreactive inhibin levels fell more rapidly than hCG following treatment of gestational trophoblastic disease (Yohkaichiya *et al.*, 1989), offering the potential for inhibin to replace hCG in this setting, it became apparent subsequently that some cases of persistent or recurrent tumour detected by rising hCG may be missed by inhibin (Badonnel *et al.*, 1994). Activin A has also been assessed as a marker of trophoblastic disease and similarly found wanting (Lambert-Messerlian *et al.*, 1998).

Human chorionic gonadotrophin, particularly the free β-subunit of hCG (fβhCG), is also the single most important serum marker for Down's syndrome. On average, maternal serum levels of fβhCG are increased two-fold or more in pregnancies with a Down's syndrome fetus in both the first and second trimester of pregnancy. Not surprisingly, inhibins and activins have also been assessed as possible markers. The first report that inhibin may be a useful marker of Down's syndrome was by Van Lith *et al.* (1992) who observed increased maternal inhibin levels in association with fetal Down's syndrome using an assay that measured 'total' immunoreactive inhibin. While subsequent studies were less promising (Spencer, 1993; Cuckle, 1994; Wallace *et al.*, 1994), it was suggested that specific inhibin isoforms may be altered in pregnancies with a Down's syndrome fetus while other inhibins may not (Wallace *et al.*, 1994). Indeed, the development of the specific inhibin assays has confirmed this, showing that while inhibin A is significantly elevated almost two-fold in association with Down's syndrome, pro-αC is less discriminatory (Cuckle *et al.*, 1995; Wallace *et al.*, 1996; D'Antona *et al.*, 1998b; Lambert-Messerlian *et al.*, 1998). Accordingly, the addition of inhibin A to existing second trimester maternal serum marker combinations increases the detection rate by 10–15% (Aitken *et al.*, 1996; Cuckle *et al.*, 1996) such that inhibin A is now widely used in preferred marker combinations (Wenstrom *et al.*, 1997). Unfortunately, while initial data suggested that inhibin A may also be useful as a first trimester marker (Wallace *et al.*, 1995), more recent and extensive evaluation has been much less promising (Noble *et al.*, 1997; Spencer *et al.*, 2001). Most recently, the possibility that specific molecular weight forms of inhibin A, rather than 'total' inhibin A, might afford improved discrimination between Down's syndrome and normal pregnancies has been addressed (Thirunavukarasu *et al.*, 2002). Inhibin A exists as a number of molecular weight forms in maternal serum (Thirunavukarasu *et al.*, 2001), as it does in other biological fluids (Robertson *et al.*, 1996; Robertson *et al.*, 1997). The relative abundancy of these various forms alters across pregnancy with increasing amounts of small molecular weight inhibins as gestation advances (Thirunavukarasu *et al.*, 2001). Unfortunately, while Down's syndrome placentae contained more large molecular weight inhibins than chromosomally normal placentae, this difference was not reflected in maternal serum where there were no differences between cases and controls. This suggests that further improvements in serum screening for Down's syndrome will not be afforded by measuring specific inhibin A isoforms (Thirunavukarasu *et al.*, 2002).

Assessment of mid to late pregnancy

Complications of late pregnancy have also attracted the attention of research endocrinologists interested in inhibins and activins. In particular, potential roles and applications have been suggested in the regulation of parturition and in the pathogenesis of pre-eclampsia. As might be predicted from the ontogeny of inhibins and activins in pregnancy (Fowler *et al.*, 1998), and in contrast to early pregnancy and ovarian studies, most interest has focussed on activin.

There has been considerable speculation that activin A may have a role in the onset and or control of parturition, particularly preterm labour. In cross-sectional studies, maternal serum activin A was observed to increase markedly during the final weeks of normal pregnancy (Petraglia *et al.*, 1994; Muttukrishna *et al.*, 1996), and maternal serum and amniotic fluid concentrations of activin A are higher in women in labour, particularly preterm labour, than in gestation matched nonlabouring women (Petraglia *et al.*, 1993; Petraglia *et al.*, 1997; Florio *et al.*, 1999). Keelan *et al.* (1999) have also found a significantly higher level of activin A in placental tissues in women who had spontaneously laboured compared to those who had a Caesarean section. *In vitro*, activin A stimulates release of prostaglandin, a hormone tightly associated with the onset of labour, from an amnion-derived cell line (Petraglia *et al.*, 1993). Finally, activin binding sites have been identified in rat myometrium (Draper *et al.*, 1997), consistent with activin exerting a direct effect on this tissue.

For activin to have a role in labour is certainly attractive. It offers the prospect of new predictive tests for preterm delivery and of new therapeutic interventions to suppress and stimulate labour. However, more recently, longitudinal studies of term and preterm labour have suggested that it is most unlikely that activin has a direct role in labour or delivery (Coleman *et al.*, 2000; Schneider-Kolsky *et al.*, 2000a). Activin A levels in the peripheral maternal circulation do not increase in the last 3 weeks of pregnancy, remain unchanged during either spontaneous or induced labour and are similar in women delivering vaginally and in those delivered by elective Caesarean section (Schneider-Kolsky *et al.*, 2000a). The same investigators also demonstrated, using

immunohistochemistry, that activin receptors are not localized to pregnant myometrial smooth muscle, either before or during labour, suggesting that myometrial smooth muscle does not have target sites for activin A in late pregnancy or during labour (Schneider-Kolsky *et al.*, 2001). It has also been shown that peripheral activin A levels cannot discriminate between women in threatened preterm labour who subsequently deliver preterm and those who did not (Coleman *et al.*, 2000). Most recently, in a large prospective study, the measurement of activin in asymptomatic women in mid-pregnancy proved to be of no value in predicting those women who subsequently delivered preterm (Wang *et al.*, 2002). However, that study utilized a rather insensitive 'free' activin assay (Wong *et al.*, 1993) which has a lower limit of detection above the levels of activin A expected in maternal serum at the gestations studied. It would certainly be worthwhile undertaking this study, employing a more sensitive activin assay before finally closing the door on the potential for activin A as a marker of preterm delivery. Nonetheless, at present, the bulk of available data suggest that activin will not find a clinical application in the assessment of labour and delivery, either at term or preterm.

Activin and inhibin levels are also increased significantly in association with hypertensive disorders of pregnancy (Petraglia *et al.*, 1995; Muttukrishna *et al.*, 1997b; Laivuori *et al.*, 1999; Silver *et al.*, 1999; D'Antona *et al.*, 2000), particularly pre-eclampsia, a systemic pregnancy complication that remains a major cause of maternal morbidity and mortality and a common indication for preterm delivery. While pre-eclampsia is associated with reduced renal function, it would appear that the mechanism underlying the increased activin levels is increased placental production rather than reduced clearance (Manuelpillai *et al.*, 2001). What underlies the increased production remains unknown. Recent studies *in vitro* suggest that impaired placental oxygenation, which is a feature of established pre-eclampsia, is unlikely to be a causative mechanism (Manuelpillai *et al.*, 2002). Whether oxidative stress or cytokine activation (Mohan *et al.*, 2001) underlies the altered inhibin and activin secretion in pre-eclampsia remains to be examined. In addition, the observation of increased activin in established pre-eclampsia may offer new insights into possible mechanisms of disease progression (Manuelpillai *et al.*, 2001). Pre-eclampsia is characterized by widespread maternal vascular endothelial cell dysfunction (Roberts *et al.*, 1989a). It is therefore of interest that activin β_A subunit and the activin receptors have been localized to both maternal (Schneider-Kolsky *et al.*, 2001) and placental endothelial cells (Schneider-Kolsky *et al.*, 2001). In addition, it has been shown that activin and follistatin may regulate some endothelial cell functions including proliferation and angiogenesis (McCarthy & Bicknell, 1993; Kozian *et al.*, 1997; Breit *et al.*, 2000). It is therefore possible that the very high levels of maternal serum activin A observed in association with pre-eclampsia may have, at least in part, a role in the endothelial damage that leads to the pathological vascular adaptations in both the mother and the placenta. However, such a role has yet to be proven and as such does not translate into any immediate clinical application. Nonetheless, it is possible that maternal serum activin A levels may increase with disease progression and that tracking of the levels could be useful in disease monitoring. However, again, there are no published data to confirm this at present. More promising is the observation that maternal activin A and inhibin A are increased much earlier in pregnancy in women who are well but who subsequently develop pre-eclampsia (Cuckle *et al.*, 1998; Aquilina *et al.*, 1999; Muttukrishna *et al.*, 2000). While published data remain limited, it would appear that activin A is a more sensitive marker of subsequent pre-eclampsia than inhibin A, and that activin A performs best as a marker when measured at 20–24 weeks of pregnancy, rather than earlier, and when predicting severe disease with an onset before 34 weeks of pregnancy (Muttukrishna *et al.*, 2000). Further studies are clearly required to define whether activin has a potential role as a routine marker of pre-eclampsia, but it is likely that it would only have adequate sensitivity and specificity in combination with other biochemical markers such as hCG and AFP or in combination with uterine artery Doppler flow studies (Farrell *et al.*, 1999).

Finally, recent sheep and human studies have suggested that placental and/or fetal activin secretion is increased in response to feto-placental hypoxia (Jenkin *et al.*, 2001a; Jenkin *et al.*, 2001b) and in association with intrauterine fetal growth restriction (Schneider-Kolsky *et al.*, 2000b; Bobrow *et al.*, 2002). Further evaluation of these preliminary reports is required but it is an exciting possibility that activin may prove to be a useful marker of fetal compromise. In addition, in the neonatal brain, activin production is increased by hypoxaemia possibly as a neuroprotective response (Wu *et al.*, 1999). Together, these findings offer many new potential applications for the measurement and administration of activin in perinatal medicine.

Testis

It was the original work by McCullagh (1932), who used aqueous testicular extracts to prevent the formation of 'castration cells' in the pituitary, that predicted the existence of inhibins. While it took many decades to confirm this prediction, it is clear that the testis is the main source of inhibins in males (Ishida *et al.*, 1990) and that, unlike the ovary, the testis produces inhibin B and not inhibin A (Illingworth *et al.*, 1996a) in addition to producing large amounts of free α-subunit (Robertson *et al.*, 1996). Furthermore, it is now apparent that, within the adult testis, the Sertoli cell is the principal source of inhibin, as initially suggested by rat Sertoli cell culture (Le Gac & de Kretser, 1982), immunolocalization of inhibin subunit proteins (Cuevas *et al.*, 1987) and by localization of mRNA expression in the rat testis (Roberts *et al.*, 1989b) and, more recently, as confirmed in the human by

the immunolocalization of both the α-subunit and the β_B-subunit to the Sertoli cell (Bergh & Cajander, 1990; Andersson *et al.*, 1998). However, studies *in vitro* using rat Leydig cell cultures have shown that Leydig cells can also secrete inhibin (Risbridger *et al.*, 1989), as may germ cells (Andersson *et al.*, 1998). Indeed, as recently reviewed by Meachem *et al.* (2001), the localization and secretion of the two inhibin subunits in the testis may alter with age, possibly reflecting changing regulation of inhibin B secretion before, during and after puberty. Nonetheless, the observations that inhibin B is undetectable in men with Sertoli cell only syndrome, despite normal testosterone levels (Andersson *et al.*, 1998), and that LH or hCG administration to men does not increase circulating inhibin B levels (Young *et al.*, 2000; Kinniburgh & Anderson, 2001) suggests that the Leydig cell contribution to circulating inhibin B must be very small. More likely than being a primary source of inhibin B, the Leydig cells probably have a role in the regulation of inhibin production by the Sertoli cell (Tena-Sempere *et al.*, 1999). Over recent years, a number of clinical studies have significantly furthered our understanding of inhibin and the regulation of testicular function, highlighting possible applications for inhibin measurement in men.

Fetal, neonatal and childhood testicular function

The fetal testis produces inhibin B prior to birth, as indicated by the expression of the mRNAs for the inhibin subunits (Tuuri *et al.*, 1994), the immunolocalization of the subunit proteins (Majdic *et al.*, 1997) and the presence of almost adult levels of inhibin B in cord blood of term male, but not female, infants (Wallace *et al.*, 1997b). Indeed, the lower FSH levels observed in male compared to females fetuses may be secondary to the circulating inhibin B in males (Reyes *et al.*, 1974; Wallace *et al.*, 1997b). Following delivery, there is a transient stimulation of the infant male hypothalamo-pituitary-gonadal axis reflected by increased inhibin B, testosterone and gonadotrophin secretion (Winter, 1982; Burger *et al.*, 1991; Andersson *et al.*, 1998) with peak levels being attained at 3–6 months of age (Andersson *et al.*, 1998; Byrd *et al.*, 1998). While the control of this phenomenon remains uncertain, it has been suggested that this transient function is crucial to the postnatal development of both Sertoli and Leydig cell function and number (Anderson *et al.*, 1998; Main *et al.*, 2000). The secretion of inhibin B by the neonatal testis in this activity would appear to be stimulated by FSH (Main *et al.*, 2000), as it is in prepubertal boys (Raivio *et al.*, 1997), and may reflect Sertoli cell number (Raivio *et al.*, 1997; Ramaswamy *et al.*, 1999). However, unlike testosterone and the gonadotrophins, which become undetectable by 6–9 months postnatal age, inhibin B remains detectable throughout childhood (Andersson *et al.*, 1998). Because inhibin B may be a marker of Sertoli cell number and Sertoli cell number is related to future sperm production, it is attractive to think that childhood inhibin B levels may predict an individual's future spermatogenic potential and fertility. However, unlike in the adult, inhibin B production in childhood is independent of germ cells (Andersson *et al.*, 1998) and so individuals infertile with Sertoli cell only syndrome have been shown to have received normal inhibin B levels prepubertally but undetectable levels postpubertally (Andersson *et al.*, 1998). Childhood inhibin B is therefore not a useful predictor of future fertility. In contrast, inhibin B may be useful to determine whether testes are present in infants with bilateral cryptorchidism, as may the measurement of serum Mullerian inhibiting substance (Lee *et al.*, 1997), and this may be helpful in assessing babies with ambiguous genitalia (Kubini *et al.*, 2000).

Assessment of adult testicular function

As already discussed, the control of inhibin B secretion in the adult differs from that in childhood. Prepubertally, inhibin B production is independent of germ cells whereas, in the adult, circulating inhibin B is correlated with both spermatogenesis and FSH (Anawalt *et al.*, 1996; Illingworth *et al.*, 1996a; Anderson *et al.*, 1997; Jensen *et al.*, 1997; Pierik *et al.*, 1998). Thus, as testicular damage/suppression increases, the sperm count and inhibin B fall and FSH rises. This is in contrast to the earlier studies of total immunoreactive inhibin in men with various testicular disorders (de Kretser *et al.*, 1989) in which inhibin levels were normal despite severe testicular damage, azoospermia and elevated FSH. It is now clear that the majority of the inhibin measured in those men was probably pro-αC inhibin and not biologically active inhibin B. Indeed, in a study of prospective testicular damage in men undergoing chemotherapy, inhibin B levels progressively fell as FSH levels increased and pro-αC inhibin levels increased (Wallace *et al.*, 1997a), which is consistent with FSH stimulation of α-subunit production (Bergh & Cajander, 1990) and explains the 'normal' total immunoreactive inhibin levels observed by de Kretser *et al.* (1989).

The positive correlation between inhibin B levels and sperm count in both normal (Illingworth *et al.*, 1996a; Anderson *et al.*, 1997; Jensen *et al.*, 1997) and infertile (Illingworth *et al.*, 1996a; Pierik *et al.*, 1998) men suggests that inhibin B may be a valuable tool in the assessment of the infertile male. Indeed, several investigators have observed that, as testicular dysfunction increases, inhibin B levels fall (Pierik *et al.*, 1998; Bohring & Krause, 1999; von Eckardstein *et al.*, 1999). However, it remains unclear whether inhibin B is sensitive and specific enough to correctly predict the successful extraction of sperm from men with non-obstructive azoospermia (von Eckardstein *et al.*, 1999; Ballesca *et al.*, 2000).

In contrast to ovarian cancer, there are very few reports of inhibin in testicular cancer. At present, while circulating inhibin B levels may be elevated in men with Sertoli cell tumours and immunohistochemical localization of the inhibin subunits may

assist in differentiating between some tumour types (Gilcrease *et al.*, 1998; Ulbright *et al.*, 2000; Henley *et al.*, 2002), inhibin does not have an established role in the diagnosis and/or management of men with testicular malignancy.

Conclusion

Over the past 5 years, the application of the highly sensitive and specific assays for the various inhibins and activins has led to significant advances in our understanding of male and female reproductive health and disease.

The fact that these hormones are actively secreted by the reproductive tract has led to intensive efforts to find clinical uses of these hormones as early markers of disease. However, it has often been the case that, while an initial observation of altered levels of inhibins or activins in a particular disease is described, subsequent studies find that the observation falls short of a clinically useful test. Nevertheless, two clear clinical applications have emerged. The measurement of inhibin A significantly enhances the sensitivity of existing second trimester screening for Down's syndrome and 'total' immunoreactive inhibin is exquisitely sensitive in detecting granulosa cell tumours of the ovary with levels decreasing in response to treatment, and increasing again in the event of a relapse.

It is expected that improved insights into male and female reproduction afforded by the studies summarized here will lead to further advances and new investigations. Hopefully, administered inhibins and/or activins may also prove to be useful as interventions, either preventative or therapeutic.

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Section 1: Biochemical markers of pregnancy outcome at days 15-17 after IVF

Manuscript 1A:

Increased day 15-17 serum Pro-αC inhibin levels specific to successful pregnancy

Stephen Tong, Luk Rombauts, Annegien Mulder, Budi Marjono, Joseph L Onwude and Euan M Wallace.

Declaration regarding the paper entitled:

Increased day 15-17 serum Pro-αC inhibin levels specific to successful pregnancy (1A)

(submitted)

Contributions to the work involved the following:

Name	% contribution	Nature of contribution
Stephen Tong	40%	Conceived and designed the study, collected samples and performed the pro-αC assay, statistically analysed data, and was a major contributor in the writing of the report.
Luk Rombauts	12%	Supervised and implemented the study, and contributed to the writing of the report.
Annegien Mulder	10%	Collected patient details and contributed to the writing of the report.
Budi Marjono	10%	Helped with the pro-αC assay and contributed to the writing of the report.
Joseph L Onwude	8%	Performed most of the statistical data analysis and contributed to the writing of the report.
Euan M Wallace	20%	Supervised and helped to design the study, and was a major contributor in the writing of the report

Declaration by co-authors

The undersigned hereby certify that:

(1) they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;
(2) they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
(3) there are no other authors of the publication according to these criteria;
(4) potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journals or other publications, and (c) the head of the responsible academic unit; and
(5) the original data are stored at the following location(s) and will be held for at least five years from the date indicated below:

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Increased day 15-17 serum Pro-αC inhibin levels specific to successful pregnancy

Abbreviated title: Pro-αC levels in the prediction of pregnancy

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Key words: Pro-αC, inhibin, IVF, marker, embryo transfer, pregnancy

Abstract

In early pregnancy, serum levels of the luteal derived hormone pro-αC inhibin peak by the second week after conception. Whether this early rise is biologically important and a consistent feature of only successful pregnancy is unknown. We undertook a prospective cross-sectional study to determine whether serum pro-αC inhibin levels at days 15-17 are predictive of a successful clinical IVF pregnancy and compared levels between fresh embryo transfer (ET) and frozen-thawed ET. Median (95%CI) pro-αC inhibin levels were 68 (57-76) pg/mL in 204 women who did not become clinically pregnant after ET, significantly lower than in either 90 women who became clinically pregnant after fresh ET, 3139 (1684-4220) pg/mL, or in 39 women with a successful frozen ET 877 (678-1111) pg/mL. Pro-αC was highly sensitive and specific in predicting clinical pregnancy success, but did not improve on the performance of human chorionic gonadotrophin (hCG). Pro-αC inhibin levels were not correlated with progesterone or hCG. Levels were no higher in singleton compared to multiple pregnancies and did not increase across gestation, confirming a luteal source. The increase in circulating pro-αC inhibin in very early pregnancy is highly specific to clinical pregnancy suggesting a possible biological role in early gestation.

Introduction

The inhibins are glycoprotein hormones belonging to the transforming growth factor-β superfamily and thought to have diverse roles in human reproduction (1, 2). Inhibin comprises an α-subunit linked by a disulphide bond to either a β_A (inhibin A) or β_B (inhibin B) subunit. Of these two dimers, only inhibin A is detectable in the peripheral circulation in pregnancy (3, 4). However, both the α- and β- subunits are produced as pre-pro-proteins giving rise to a large number of different molecular weight forms relating to different stages of processing (5). Indeed, it is now recognised that many of these different molecular weight forms of inhibin A are present in the maternal circulation (4), including inhibin with a partially processed α-subunit, termed pro-αC inhibin.

Most pregnancy-related hormones which are detectable in the maternal circulation rise progressively across the first few weeks of pregnancy. These include the luteal derived steroid progesterone, and fetoplacentally derived hormones such as human chorionic gonadotrophin (hCG), oestradiol, prolactin and other inhibin forms, such as activin A and inhibin A (3, 6). In contrast, maternal serum levels of pro-αC inhibin appear to peak at around day 16 after conception (6, 7), then gradually decline. Although a secondary rise in pro-αC inhibin occurs in early second trimester and continues until the end of pregnancy, absolute levels do not surpass those seen in very early pregnancy (6). Given these apparent differences in the ontogeny of pro-αC inhibin compared to most other pregnancy hormones, it is conceivable that the early rise in pro-αC inhibin reflects an, as yet uncharacterised, important biological role for these inhibin forms in the establishment of early pregnancy. Accordingly, we

hypothesised that if pro-αC inhibin did have such a role then detectable levels should rise consistently with the establishment of pregnancy and that pro-αC inhibin measured by around day 16 after conception in women undergoing IVF may be a useful predictor of whether a clinical pregnancy will result. We therefore undertook a prospective study to evaluate pro-αC inhibin levels at day 13-14 after embryo transfer, in both fresh and frozen embryo transfer (ET) cycles, and to assess the clinical utility of pro-αC inhibin as a predictor of subsequent pregnancy success.

Illingworth *et al* (7) showed that the administration of hCG at around day 7 after ovulation in non-pregnant women increases pro-αC inhibin levels. In addition, a recent study has shown that pro-αC inhibin starts to fall very quickly upon administration of the antiprogestagen mifepristone, in women undergoing medical termination of pregnancy (8). In light of these data, we also explored whether any correlation existed between Pro-αC inhibin and hCG, or progesterone.

Subjects and methods

Subjects and samples

We undertook a prospective, cross-sectional observational study of 334 women undergoing IVF. Blood was collected at days 13-14 post embryo transfer, equivalent to the 15-17th day after conception, or by convention, four weeks of gestation. Bloods were also collected from some women at a time equivalent to 22-24 days (n=73) and 32-35 days (n=39) gestation.

Baseline patient information was collected including age, current IVF treatment cycle number, and whether the ET cycle was fresh or frozen-thawed. Women were grouped according to whether they became clinically pregnant or not, and if so, whether they had a singleton or twin pregnancy. Clinical pregnancy was defined by a positive pregnancy test (hCG) and an early ultrasound scan at around six weeks of gestation confirming the presence of one or two gestational sacs with a fetal pole and a visible fetal heart beat. The non-pregnant group included both women with a negative pregnancy test and those who had had an initially positive pregnancy test but ultimately no demonstrable viable gestation on ultrasound (i.e. biochemical pregnancies). Approval for the study was granted by the Monash IVF Ethics Committee.

Treatment protocols

Women undergoing fresh IVF cycles were administered the GnRH agonist nafarelin (Synarel; Searle, High Wycombe, UK) for pituitary desensitisation followed by subcutaneous recombinant human follicle stimulating hormone (rhFSH; Gonal-F; Ares-Serono, Geneva, Switzerland). When the dominant follicle or follicles were

>17mm in diameter, both nafareline and rhFSH were discontinued, and prior to oocyte retrieval, 5000-10,000 IU of HCG (Profasi; Serono Laboratories, Geneva, Switzerland) was administered. The oocytes were fertilised *in vitro* either with or without sperm microinjection. In fresh cycles, embryos were transferred after 3 days. After embryo transfer, luteal support was provided by administration of 400 mg daily progesterone vaginal suppositories.

In frozen-thawed cycles, embryos were maintained in culture for 2-3 days before freezing. Unlike fresh IVF cycles, a number of different transfer regimens were used, as briefly described on table 1.

Assays:

Serum hCG (immunometric assay) and progesterone (competitive immunoassay) were assayed at the time of blood collection using an automated system (VitrosECi /OrthoDiagnostics/ Rochester, New York). Excess serum was frozen at –20°C for subsequent measurement of pro-αC inhibin (Oxford Bionnovation/ Oxford, London). All assays were done according to manufacturer's instructions. The intra and inter assay coefficient of variation for all assays was <10%. All assays were undertaken blinded to the clinical outcome.

Statistical analyses:

Levels of all analytes were not normally distributed. Therefore, data were expressed as median (95%CI) and comparisons between groups undertaken using either Kruskal-Wallis or Mann-Whitney U tests. Correlations between hormone levels were performed using Spearman's correlation.

The performance of pro-αC inhibin in predicting viable pregnancy was determined by a logistic regression model which included pregnancy outcome, treatment method (fresh versus frozen embryo transfer) and the categorised pro-αC levels at the cut-off level. Statistical tests were done using state 8.1 (Stata Corporation, New Jersey).

Results:

The mean (SD) age of all women in the study was 34.1 (4.9) years and the median (95% CI) current IVF treatment cycle number was 3 (3-4). Further baseline data for the women, grouped according to gestation at blood sampling, are summarised in table 2.

Pro-αC inhibin

At days 15-17, the median (95%CI) pro-αC inhibin levels in the 204 women who did not become clinically pregnant was 68 (57-76) pg/ml. Median levels were significantly higher in the 107 women with a singleton pregnancy, 1372 (1003-1963) pg/ml ($p<0.0001$) and in the 23 women who had a twin pregnancy, 2000 (897-3735) pg/ml ($P<0.0001$) (Figure 1).

Figure 2 summarises pro-αC inhibin levels, stratified according to whether the embryo transferred was either fresh or frozen-thawed. The median (95% CI) pro-αC inhibin levels at day 15-17 in those 90 women who had a clinical pregnancy after fresh ET was 3139 (664-5720) pg/mL, a 54 fold increase compared to those 96 women who had a fresh ET but did not become clinically pregnant, 58 (35-107) pg/ml, $p<0.0001$. The median (95% CI) pro-αC inhibin levels at 13-14 days post-transfer in those 39 women who had a clinical pregnancy after frozen-thawed ET was 877 (563-128) pg/mL, a 12 fold increase compared to those 108 women who had a frozen-thawed ET but did not become clinically pregnant, 72 (41-136) pg/mL, $p<0.0001$. Low numbers in the non-pregnant cohort in both subgroups prohibited a similar comparison at the latter two gestational periods.

Across gestation, the median (95% CI) pro-αC inhibin levels in the clinical pregnancy group remained significantly higher in those who had a fresh ET compared to frozen-thawed ET at days 15-17, 3139 (664-5720) pg/mL vs 877 (563-128) pg/mL, p<0.0001; 4561 (2542-7358) pg/mL vs 551 (378-766) pg/mL at days 22-24, p<0.0001; and 2560 (1292-3432) pg/mL vs 529 (357-856) pg/mL (p<0.0014) days 32-35 after ET (Figure 2). Levels within either the pregnant fresh or frozen embryo transfer groups did not vary significantly across gestation: p=0.14 for both comparisons (Kruskal-Wallis).

Pro-αC inhibin levels in women with a clinically pregnancy following a fresh ET did not differ, at any gestation, whether a twin or singleton pregnancy resulted (Kruskal-Wallis for viable singletons vs multiples in fresh embryo cohort from all 3 gestations p=0.42; 6 groups). This analysis was not repeated for the frozen ET group because of insufficient numbers of multiple pregnancies.

The Pro-αC inhibin levels at day 15-17 in the frozen embryo transfer group who underwent different regimens are shown on table 3. There were no differences in levels between the non pregnant and clinically pregnant women who had the HRT regimen, but there were clear differences in the pregnant versus non-pregnant subgroups amongst the natural cycle regimen, or the clomiphene citrate regimen.

Correlation of Pro-αC levels with progesterone and-hCG

Table 4 shows the levels of hCG and progesterone levels at day 15-17. Correlations between analytes were limited to those cases where a clinical pregnancy eventuated. There were thirteen cases where progesterone levels were not available. Neither

serum hCG (r=0.14; p=0.11) nor progesterone (r=0.078,p=0.16) were correlated with pro-αC levels.

The clinical pregnancy group was further stratified according to whether the ET was fresh or frozen. There was a significant negative correlation (r=-0.328, p=0.045) between pro-αC and progesterone in the frozen ET group (n=38) but not in the 85 women in the fresh ET group (r=0.07, p=0.5). The correlations between between pro-αC and hCG were not significant in either the frozen (r=0.219,p=0.18) or fresh (r=0.028, p=0.79) ET group

Assessment of Pro-αC inhibin as a predictive marker of clinical pregnancy

At 95% specificity, a pro-αC level of 308 pg/mL at 15-17 days after ET afforded a 83% sensitivity in the prediction of a successful clinical pregnancy. In comparison, for the same specificity hCG afforded a sensitivity of 90%. A combination of pro-αC inhibin and hCG was not better than hCG alone (table 5).

When assessing the sensitivity of pro-αC in predicting pregnancy in the fresh ET group alone (n=186), a cut off of 450 pg/mL provided 80.2% sensitivity at 95% specificity. At the same cut-off and specificity, in the frozen-thawed ET group (n=147), pro-αC provided 79.5% sensitivity in predicting a successful pregnancy. Therefore, the performance of pro-αC inhibin as a marker was not statistically different in fresh compared to frozen-thawed transfer cycles

In light of the possibility that the lack of any increase of pro-αC in the HRT clinical pregnancy subgroup (table 3) might have adversely affected the performance of the entire frozen ET group, we undertook a post-hoc analysis to determine the performance of pro-αC in predicting pregnancy, excluding this group. Since pro-αC

levels among women undergoing the clomiphene citrate regimen were no different to those who underwent the natural cycle (p=0.58), these two groups were combined for this analysis. At 95% specificity, pro-αC inhibin levels was 100% and hCG was 96.7% sensitive respectively in predicting pregnancy success.

Discussion

In this study, we report the largest series to date on maternal serum pro-αC inhibin levels measured during early IVF pregnancies and we have been the first to evaluate its sensitivity as a predictive marker of achieving a successful clinical pregnancy at 6 weeks gestation. We have found that serum levels of pro-αC inhibin are consistently and considerably increased at 13-14 days after IVF ET in those women destined to achieve a clinical pregnancy. However, pro-αC inhibin does not improve upon the predictiveness of outcome already afforded by hCG.

Currently, women undergoing IVF treatment are provided with their first indication of likely success from hCG levels measured at a time corresponding to about day 16 after conception. hCG is often very good at predicting whether a clinical pregnancy will occur, particularly if it is highly elevated (9, 10). However, when levels are around 50-100 mIU/L, it may be difficult to accurately predict whether that IVF cycle will be successful (9). Other markers have been investigated, including inhibin A (11). Whilst inhibin A certainly increases in association with pregnancy success, there is too much overlap between successful and unsuccessful pregnancies for inhibin A to be clinically useful (11).

In contrast, levels of pro-αC inhibin at days 15-17 were highly specific and sensitive in predicting pregnancy success. Nonetheless, pro-αC inhibin did not add to the usefulness of hCG, the currently used clinical marker. However, the 90% sensitivity at 95% specificity that we observed for hCG in our cohort may be better than that reported by others (9, 12). It is therefore possible that other centres might find that pro-αC inhibin is more predictive than we have, particularly centres which administer hCG parenterally as luteal support. In this setting the exogenous hCG administration

may mask endogenous levels and render interpretation difficult if not wholly inaccurate. Pro-aC inhibin may also prove to be a useful diagnostic tool in other disorders of early pregnancy, such as ectopic pregnancy, although this was not explored in this current study. Finally, we did not investigate whether pro-αC at days 15-17 may be able to further discriminate those who have a clinically recognised pregnancy, but later miscarry.

We found that amongst those women undergoing frozen-thawed ET who underwent either a natural cycle regimen (expected to have only one corpus luteum) or clomiphene citrate (expected to have had ≥1 corpus luteum), both pro-αC and hCG appear to offer extremely high sensitivities in predicting a subsequent clinical pregnancy. For this analysis, we excluded the HRT subgroup (expected to have no luteal tissue) after we found that there was no rise in pro-αC in this group. Since this was a post-hoc finding, it requires confirmation in a future study of larger numbers. Nonetheless, if this is verified, it may suggest that in these particular regimens, both these hormones might be extremely sensitive and specific at predicting clinically pregnancy outcome. The fact that the predictive ability may significantly increase in these specific regimens, especially when compared with fresh cycles, may be explained by the fact that with less luteal tissue, the spread of serum levels pro-αC might be tighter in both the pregnant and non-pregnant groups.

Whilst the relative contributions of the corpus luteum and the fetoplacental unit to circulating serum levels of inhibin A in early pregnancy remains uncertain (6, 7, 13), it is generally accepted that the predominant source of pro-αC inhibin is the corpus luteum (13, 14). Our data clearly confirm this. We reported that pro-αC inhibin levels are no higher with advancing gestation, are increased in proportion to the expected

amount of luteal tissue present, and are no higher if the pregnancy is multiple rather than single. The fact that we observed that the levels of pro-αC inhibin in women with a frozen-thawed embryo pregnancies are significantly higher than levels previously reported (13, 14) reflects that the women in this current study were treated with protocols where there is often luteal function present whilst in previous reports (13, 14), women receiving frozen-thawed embryo transfers underwent a protocol of complete ovarian suppression and therefore had a total absence of luteal tissue.

Since there is an obvious discrimination in pro-αC inhibin levels between successful and unsuccessful cycles and since it has been previously shown that administration of exogenous hCG rescues luteal production of pro-αC inhibin (7), it is likely that hCG is the physiological stimulus for the higher luteal pro-αC inhibin production observed in the successful pregnancies. However, we were unable to confirm an association between circulating pro-αC inhibin levels and either progesterone (8) or hCG (7) at day 15-17 in the maternal serum. We did find a weak negative correlation between pro-αC inhibin levels and progesterone in the viable frozen ET pregnancies, but since all other correlations were found to be strongly non-significant, this may be a type I error and interpretation should be cautious.

Whilst there appeared to be no convincing correlations between peripheral levels of pro-αC inhibin progesterone (8) or hCG, our study design does not exclude important paracrine relationships. The more convincing experimental design, in the case of progesterone, would be to undertake interventional studies, such as withholding luteal support and to administer mifepristone concurrently.

Clearly, pro-αC inhibin is not essential for successful pregnancy since protocols employing complete ovarian suppression causing the absence of luteal tissue are

compatible with successful pregnancy (14). However, the fact that an early rise in pro-αC levels appears to be a consistent feature specific to clinical pregnancy suggests that it may have a biological role. In this regard, implantation has been previously shown to be optimal at days 8-10 following fertilisation (15). It has been reported recently that in the week immediately following implantation, an early hCG rise and an abrupt increase in progesterone, is associated with less pregnancy losses (16). It is therefore possible that the progressive rise of pro-αC inhibin across this same period (7) may reflect the fact that these inhibin forms might also play important roles such as the facilitation of implantation and/or decidualisation. In further support of this premise are prior immunohistochemical studies which have reported that the α-subunit is only weakly staining in the non-pregnant endometrium but becomes strongly localised to the decidualised stroma (17). Such speculation should be tempered with some caution since it is presently believed that only the dimeric inhibin forms are biologically active and we have investigated an IVF cohort, not spontaneous pregnancies.

In summary, we have demonstrated a massive and specific early rise of serum po-αC inhibin levels in association with clinical pregnancy. The biological relevance of this, if any, remains uncertain but the current data suggest further study of pro-αC inhibins in early pregnancy would be merited.

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TABLE 1: Descriptions of the different types of regimens used in frozen-thawed ET cycle cohort.

Regimen
HRT cycle (n=19)
• +/- preceded by the oral contraceptive pill and followed by downregulation
• Progynova 4 – 6mg/day
• Progesterone pessaries 200 mg three times a day vaginally from three days before frozen embryo transfer
Natural cycle (n=68)
• +/- HCG to induce ovulation
• No luteal support
Clomiphene citrate (n=51)
• +/- HCG to induce ovulation
• +/- Oestradiol valerate to prime endometrium

There were nine patients who had a frozen ET whom we were unsure of the regimen.

TABLE 2: Baseline data of the study cohort.

Gestational age (days)	Number	Age: Mean (SD)	Treatment cycle number Median (95%CI)	Type of ART
15-17	334	34.4 (5.0)	3 (3-4)	**Fresh:** MI 132 IVF 54 **Frozen:** MI 98 IVF 49
22-24	73	33.5 (4.3)	3 (2.7-4.0)	**Fresh:** MI 34 IVF 17 **Frozen:** MI 14 IVF 8
32-35	39	33.6(4.9)	3 (2-3.1)	**Fresh:** MI 21 IVF 9 **Frozen:**

				MI 7 IVF 2
Differences: **(P value)**	-	0.28 (ANOVA)	0.27 (Kruskal-Wallis)	-

Fresh: Fresh IVF cycle. Frozen: Frozen-thawed embryo transferred. IVF = *in vitro* fertilisation without microinjection; MI = IVF with sperm microinjection.

TABLE 3: Levels of day 15-17 pro-αC in the frozen-thawed ET group according to the type of regimen undertaken.

Regimen and outcome	**Pro-αC levels**
	Median (95%CI) pg/ml
HRT cycle	
Clinical pregnancy (n=7):	30.1 (17.2-263.7)
No clinical pregnancy (n=12):	35.23 (17.07-50.35)
	p=0.58
Natural cycle	
Clinical pregnancy (n=22):	976 (707-1114)
No clinical pregnancy (n=46):	72 (57.3-92.7)
	p=<0.0001
Clomiphene citrate	
Clinical pregnancy (n=8):	1500 (823-1971)
No clinical pregnancy (n=43):	102 (68-126)
	p=<0.0001

TABLE 4: Progesterone and hCG levels at days 15-17, stratified by pregnancy outcome at the early pregnancy ultrasound.

Hormone	No clinical pregnancy	Singleton	Twins
Progesterone			
	30	33	60
	(21.5-36.5)	(25.2-49.6)	(38.1-143.0)
n=	191	101	23
hCG			
	1	253	421
	(1-1)	(224.8-294)	(352-573)
n=	204	107	23

Levels shown as medians (95% CI).

TABLE 5: Sensitivities of hCG and Pro-αC at days 15-17 in predicting the presence of a successful clinical pregnancy

Set Specificity	hCG	Pro-αC	Combined
95%	90.1%	83.0%	86.1%
90%	97.0%	84.5%	93.0%
85%	99.2%	86.0%	98.5%
80%	100%	86.8%	100%

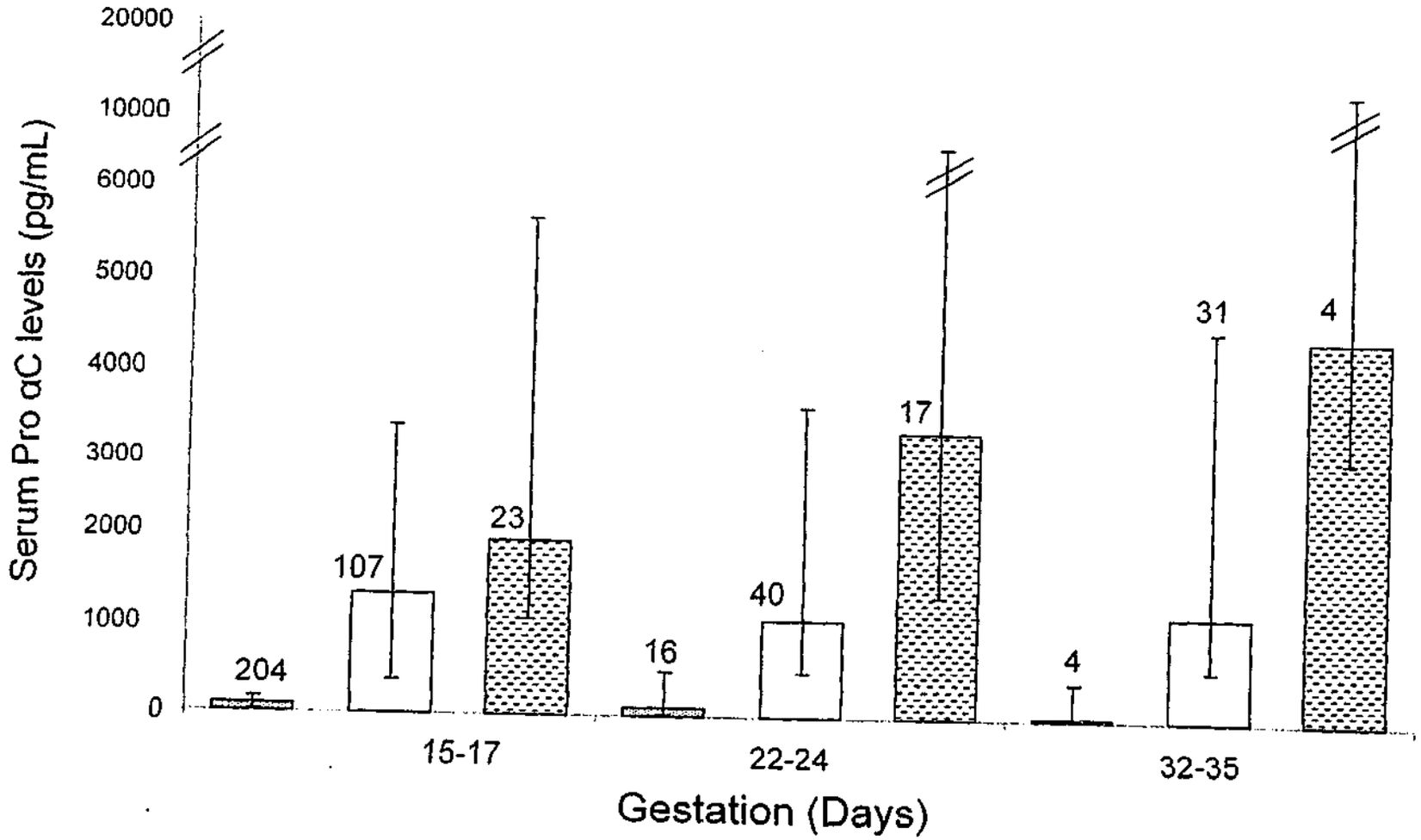

FIG 1: Serum Pro αC levels (Median and 95% CI) in the entire cohort according to gestation at sample collection. No clinical pregnancy (▒), singleton gestation (□), twins (▒). Numbers in each cohort shown above bars. Pro αC levels amongst singleton vs twins pregnancies at all gestations: $p>0.09$ all comparisons, Mann-Whitney U. Pro-αC levels across gestation within single or twin cohorts: $p>0.44$; Kruskal-Wallis.

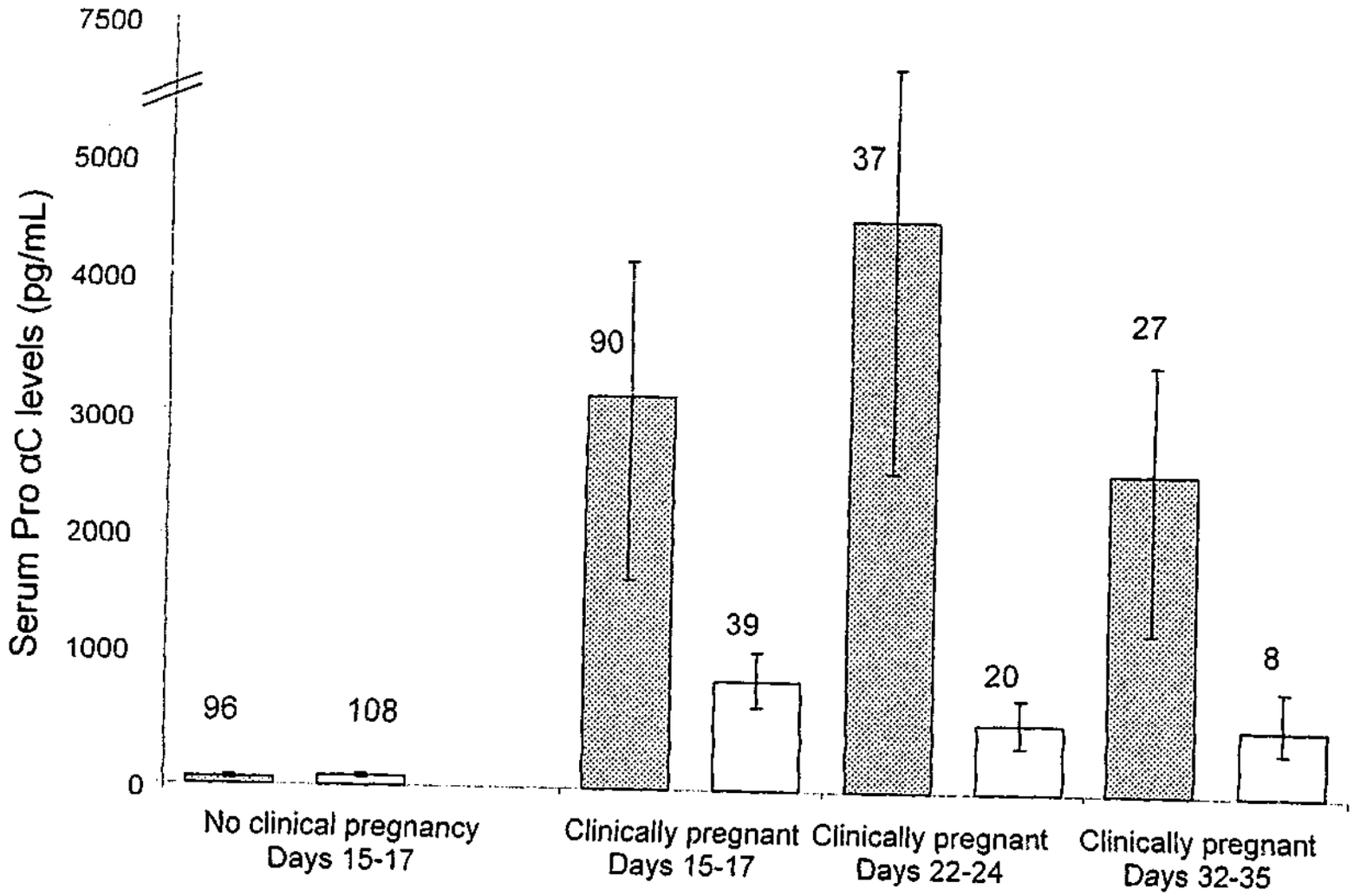

FIG 2: Serum Pro αC levels (Median and 95% CI) grouped to whether there was a clinical pregnancy and whether the embryo transfer was either fresh (▒) or frozen-thawed (□) . Numbers in each cohort shown above bars. Of the no clinical pregnancy subgroup, levels at days 20-21 and days 30-32 were not included due to low numbers within each subgroup.

Manuscript 1B:

Maternal serum levels of Macrophage inhibitory cytokine-1 and Pregnancy Associated Plasma Protein-A across very early pregnancy after IVF

Stephen Tong, Luk Rombauts, David Brown, Annegien Mulder, Budi Marjono, Samuel N Breit and Euan M Wallace.

Declaration regarding the paper entitled:

Maternal serum levels of Macrophage inhibitory cytokine-1 and Pregnancy-Associated Plasma Protein-A across very early pregnancy after IVF (1B)

(submitted)

Contributions to the work involved the following:

Name	% contribution	Nature of contribution
Stephen Tong	30%	Conceived and designed study, collected samples and performed PAPP-A assay, analysed data and was a major contributor in the writing of the report.
Luk Rombauts	15%	Supervised and implemented study, and contributed to the writing of the report.
David Brown	15%	Performed the MIC-1 assay and contributed to the writing of the report.
Annegien Mulder	10%	Collected clinical details and contributed to the writing of the report.
Budi Marjono	5%	Helped perform the PAPP-A assay and contributed to the writing of the report.
Sam Breit	5%	Developed the MIC-1 assay, major contributor in the writing of the report.
Euan M Wallace	20%	Major supervisor, and was a major contributor in the writing of the report.

Declaration by co-authors

The undersigned hereby certify that:

(6) they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;
(7) they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
(8) there are no other authors of the publication according to these criteria;
(9) potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journals or other publications, and (c) the head of the responsible academic unit; and
(10) the original data are stored at the following location(s) and will be held for at least five years from the date indicated below:

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Maternal serum levels of Macrophage inhibitory cytokine-1 and Pregnancy-Associated Plasma Protein-A across very early pregnancy after IVF

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Summary

Recently we demonstrated that maternal serum levels of macrophage inhibitory cytokine-1 (MIC-1), a Th-2 type cytokine, and pregnancy associated plasma protein-A (PAPP-A) are significantly depressed in asymptomatic women destined to miscarry, suggesting important roles for these proteins in successful pregnancy. We did a prospective cross sectional study to investigate the ontogeny of these hormones across very early pregnancy in women undergoing *in vitro* fertilisation. Serum levels were assayed at 15-17 (4^{th} week of gestation; n=308), 22-25 (5^{th} week; n=79) and 32-35 (6-7^{th} week; n=45) days after embryo transfer. By days 15-17, MIC-1 was significantly increased ($p<0.0001$) in women with both singleton (n=96) and twin pregnancies (n=22), compared to those who did not become pregnant (n=190), and levels continued to rise as gestation advanced. In contrast, levels of PAPP-A were not raised at days 15-17 in either pregnant cohort but were significantly higher in twin pregnancies, though not singletons, at days 22-24 and were increased in both pregnant groups by days 32-35. That MIC-1 levels are increased by the end of the first week of implantation is consistent with a possible role in immunomodulation and/or implantation.

Keywords: pregnancy associated protein-A, macrophage inhibitory cytokine 1, implantation, IVF, immunomodulation

Introduction

The successful establishment and maintenance of pregnancy involves alteration of the maternal immune response such that the fetal semi-allograft is tolerated (Norwitz et al., 2001). An important aspect facilitating this tolerance is thought to include a deliberate shift of the immune response favouring the activity of type II helper T cells (Th2) which facilitate cell-mediated immunity over type I helper T cells (Th1) that are involved in signalling B cells to produce antibodies (Makhseed et al., 2000; Delves and Roitt, 2000). Data in support of this shift include *in vivo* mouse studies showing that the administration of certain Th1 cytokines to pregnant mice induces abortion (Chaouat et al., 1990), whilst administration of interleukin 10, a Th2 cytokine, can prevent fetal wastage (Chaouat et al., 1995). There is also evidence that women with a history of recurrent miscarriage show a Th1 response bias rather than Th2 when they are immunologically challenged with placental antigens (Marzi et al., 1996; Raghupathy et al., 1999).

Macrophage Inhibitory Cytokine-1 (MIC-1), first described in 1997 (Bootcov et al., 1997), is a member of the transforming growth factor-β (TGF-β) superfamily. It has been identified in a diverse range of human tissues (Lawton et al., 1997; Moore et al., 2000) but is most abundantly expressed in placenta. In particular, it has been immunolocalised to syncytiotrophoblast (Marjono et al., 2003). Experimentally, MIC-1 has Th-2 cytokine activity, inhibiting tumour necrosis factor-α (TNF-α) secretion, itself a major Th1 cytokine, from activated human macrophages. Recently, we have shown that maternal serum levels of MIC-1 are consistently and profoundly depressed at 6-13 weeks gestation in women destined to be diagnosed with a miscarriage (Tong et al., 2004) and that low

levels may significantly precede miscarriage. Taken together, these data suggest that MIC-1 may have an important immunomodulatory role in early pregnancy and that low levels may be associated with an increased risk of pregnancy failure.

In light of this possibility, it would be important to understand the ontogeny of MIC-1 at the beginnings of pregnancy. Maternal serum MIC-1 levels across early pregnancy, prior to the sixth week of gestation, have never been reported. Here we describe levels from the end of the first week of implantation (days 15-17) until the seventh week of pregnancy in a cohort of women who conceived following embryo transfer after *in vitro* fertilisation.

Although pregnancy associated plasma protein-A is widely recognised as a marker for Down Syndrome (Crossley et al., 2002), its biological function remained entirely unknown until recently. Lawrence *et al* (1999) reported that PAPPA is a protease of insulin-growth factor binding protein-4 (IGFBP-4). By cleaving IGFBP-4, PAPP-A can release greater amounts of bioactive IGF I and II, growth factors with important roles in a variety of placental functions, including steroidogenesis (Nestler, 1990), glucose and amino acid transport (Kniss et al., 1994) and trophoblast invasion (McKinnon et al., 2001). As with MIC-1 (Tong et al., 2004) we and others (Westergaard et al., 1983, 1985; Bersinger et al., 1987) have also shown that serum levels of PAPP-A are depressed in association with miscarriage. It has been previously shown that maternal serum PAPP-A levels in IVF pregnancies do not start increasing until the sixth week of gestation (day 28 after fertilisation *in vitro*) (Bersinger et al., 1986; Johnson et al., 1993; Bersinger et al., 1995; Bischof et al., 1989). However, with the exception of one (Bersinger et al., 1995), all of these studies did not compare levels in those women who were successfully

pregnant with women who did not conceive. The one study which did include a non-pregnant comparison group found a very small but significant rise in PAPP-A by the fifth gestational week (Johnson et al., 1993). In view of these discrepancies, we sought to clarify at which gestation PAPP-A in pregnancy begins to increase by measuring levels in larger numbers of women than previously reported. We have also used a sensitive ELISA to detect PAPP-A rather than the less sensitive radioimmunoassays used in all previous studies.

Materials and methods

Subjects and samples

Blood collected at days 15-17 (or the 4th week of gestation equivalent), 22-24 (5th week of gestation) and 32-35 (6-7th week of gestation) days after egg pick up and *in vitro* fertilisation were assayed for MIC-1 and PAPP-A. Baseline patient information, including age, current IVF treatment cycle number, whether the embryo transfer (ET) cycle was fresh or frozen was collected.

The women were grouped according to whether they became clinically pregnant, and if so, whether they had a singleton or twin pregnancy. A clinical pregnancy was defined by an early pregnancy ultrasound scan confirming the presence of one or two gestational sacs with a fetal pole and a visible heart beat. This ultrasound is usually offered at around 6 weeks of gestation, or equivalent to days 28-35 after egg pick-up. The non-pregnant group included both women with a negative pregnancy test and those who had had an initially positive pregnancy test but ultimately no demonstrable viable gestation on ultrasound (i.e. biochemical pregnancy). Approval for this study was granted by the Monash IVF Ethics committee.

In light of previous studies suggesting that it was unlikely that PAPP-A would be elevated by days 15-17 (Bersinger et al., 1986; Johnson et al., 1993; Bersinger et al., 1995; Bischof et al., 1989), we restricted sample analysis for PAPP-A to 139 cases. On the basis of those results, we opted to analyse further samples at this gestation only for

MIC-1 (n=308), but not PAPP-A. At later gestational ages (days 22-24; n=79 and 32-35; n=45) we analysed all samples for both analytes.

Analysis of samples.

All samples were centrifuged at 3500rpm for 10 minutes, the sera collected and stored at -20 °C until further assay. PAPP-A was measured using a commercial ELISA (Diagnostic Systems Laboratory, Texas, USA) according to the manufacturer's instructions. MIC-1 was measured using an in-house ELISA as previously described (Brown et al., 2002). The limits of sensitivity were 0.013 mIU/L for PAPP-A and 7.8 pg/mL for MIC-1. The inter and intra-assay coefficient of variations was <10% for both assays.

Statistical analysis

Hormone levels were expressed as the median and 95% confidence interval. Baseline details were compared using the student's T-test or chi-squared test as appropriate. Since levels were not normally distributed, comparison between analytes were made using the Mann-Witney-U test.

Results:

The baseline details of the entire cohort are shown on table 1. Figure 1 shows MIC-1 levels across gestation according to whether or not women became clinically pregnant with a singleton or twin, or did not become pregnant. Across the three gestational time periods, MIC-1 levels in the singleton and twin cohorts increased. At days 15-17 median (95%CI) MIC-1 levels in 96 singleton, 519 (475-566), and 22 twin pregnancies, 513 (437-576), were significantly increased compared to levels amongst the 190 who remained non-pregnant, 398 (372-438), $p<0.0001$ both comparisons against both pregnant groups. Levels between singleton and twins were no different ($p=0.93$). By days 22-24, median (95%CI) MIC-1 levels of 44 women who had a singleton were 947 (886-1111), significantly higher ($p<0.0001$) than 17 women who did not become clinically pregnant, 479 (357-620) but not higher ($p=0.16$) than 18 women who had twins, 1085 (922-1343). By days 32-35, MIC-1 levels amongst 31 women who became clinically pregnant with a singleton, 4021 (3768-5198), were significantly higher than levels amongst 4 who were not clinically pregnant, 413 (226-463); $p=0.0048$. At this gestation the MIC-1 levels in women with twins, 9361 (5876-13203), was significantly higher than in singletons ($p=0.0019$).

Figure 2 shows PAPP-A levels across the same gestational periods. At days 15-17, there was no difference in PAPP-A levels between the three groups ($p=0.98$). At days 22-24, median (95%CI) PAPP-A levels of 18 women who had twins were 0.040 (0.035-0.043), significantly higher compared to 17 women who did not become clinically pregnant, 0.027 (0.025-0.039), $p=0.015$ and higher than 44 women who had a singleton pregnancy

0.032 (0.030-0.036), p=0.0016. Levels amongst the singleton and non-pregnant groups were no different (p=0.23). By days 32-35, PAPP-A levels amongst 31 women who became clinically pregnant with a singleton, 0.056 (0.050-0.069) or twins 0.117 (0.076-0.160) were significantly higher then levels amongst 4 who were not clinically pregnant, 0.035 (0.023-0.036); 0.0066 and <0.0001 respectively. The difference between singleton and twins at this gestation remained significant (p=0.0019).

Discussion:

In this study, we have described maternal serum MIC-1 levels in the beginnings of pregnancy for the first time, and clarified the timing of the PAPP-A rise in a cohort of women undergoing *in vitro* fertilisation.

Our observation that MIC-1 levels are already significantly increased by the end of the first week of implantation (days 15-17 after egg pick-up and *in vitro* fertilisation) and increases across gestation is consistent with the previous observations that the first trimester placenta expresses high levels of MIC-1 mRNA (Moore et al., 2000) and that MIC-1 is immunolocalised in both first trimester trophoblast and maternal decidua (Marjono et al., 2003). It is at this time that the placental villous development including growth of anchoring cytotrophoblast cell columns into the decidua is first occurring. However, what roles, if any, MIC-1 has at this time of implantation and early placentation remain largely unexplored.

MIC-1, also called PL74 (Scott et al., 2000) has been shown to inhibit proliferation and induce apoptosis in an extravillous cytotrophoblast cell line (Li et al., 1999) and to inhibit the *in vitro* activation of the matrix metalloproteases –2 and –9 in first trimester trophoblast explants (Marjono et al., 2002). These observations suggest that MIC-1 may act to limit trophoblast invasion *in vivo*. In support of this MIC-1 has been reported to inhibit tumour cell proliferation and growth (Tan *et al.*, 2000; Graichen *et al.*, 2002; Albertoni *et al.*, 2002) and to induce apoptosis *in vitro* in a prostate tumour cell line (Liu *et al.*, 2003). In contrast however, MIC-1 has been shown to induce gastric cancer cell line invasion via increased uPA activity (Lee et al., 2003). These studies demonstrate that

there may be marked differences in the effects of MIC-1 between cell types and between *in vivo* and *in vivo*. A comprehensive understanding of the role(s) of MIC-1 in early pregnancy will only be afforded by studies in primary human tissues. That MIC-1 levels are profoundly depressed in association with miscarriage (Tong et al., 2004) and that other TGFβ family members, such as TGFβ3, inhibin and activin, have been shown to have a number of roles in early placental development (Caniggia et al., 1997; Caniggia et al 1999; Petraglia et al 1989) suggest that MIC-1 is likely to be of physiological importance.

In contrast to MIC-1, we have observed that peripheral levels of PAPP-A are not noticeably increased by days 15-17 after IVF. Instead, levels start to increase at around 22-24 days, or the fifth week of gestation. This confirms the findings of one prior study (Johnson et al., 1993) and is earlier than the sixth week rise reported by most other studies (Bersinger et al., 1986; Johnson et al., 1993; Bersinger et al., 1995; Bischof et al., 1989). Significantly, we have been able to study greater numbers of women, and rather than a radioimmunoassay (Bersinger et al., 1986; Johnson et al., 1993; Bersinger et al., 1995; Bischof et al., 1989), we have used an ultrasensitive ELISA for PAPPA. The increase in PAPP-A at this early stage of pregnancy, while present, is slight and only apparent in those women who had twins. That PAPP-A levels are not increased at days 15-17 may be consistent with its probable role in facilitating nutrient transport to the placenta via the regulation of IGF's I and II. At this very early period in pregnancy, the syncytiotrophoblast is still rapidly invasive and lacunar lakes, which will form the future vascular networks, have only started to form (Moore, 1988). Perhaps at this stage, the placenta is still too immature for the IGF system to be fully functional. Whilst we cannot

rule out local increases in PAPP-A production, the fact that we failed to detect a rise at days 15-17 does not support a role of this hormone in implantation.

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Table 1. Baseline statistics.

Days gestation	Number	Age (Mean std dev)	Cycle numbers centiles: 95% CI:	Fresh/Frozen ET
15-17				
MIC-1	308	34.6 (4.6)	3 (3-4)	169 (54.9%)/ 139 (45.1%)
PAPP-A	139	34.1 (4.7)	3 (3-4)	81 (58.3%)/ 58 (41.7%)
		p=0.30	*p=0.70*	
22-24	79	33.8 (4.5)	3 (3-4)	54 (68.4%)/ 25 (31.6%)
32-35	45	33.6(4.8)	3 (2-3)	36 (80.0%)/ 9 (20.0%)

At days 15-17, there were no differences between the MIC-1 and PAPP-A groups with respect to age (p=0.30; T-test), cycle numbers (p=0.70, Mann-Whitney U) and types of ART (p=0.50, χ^2).

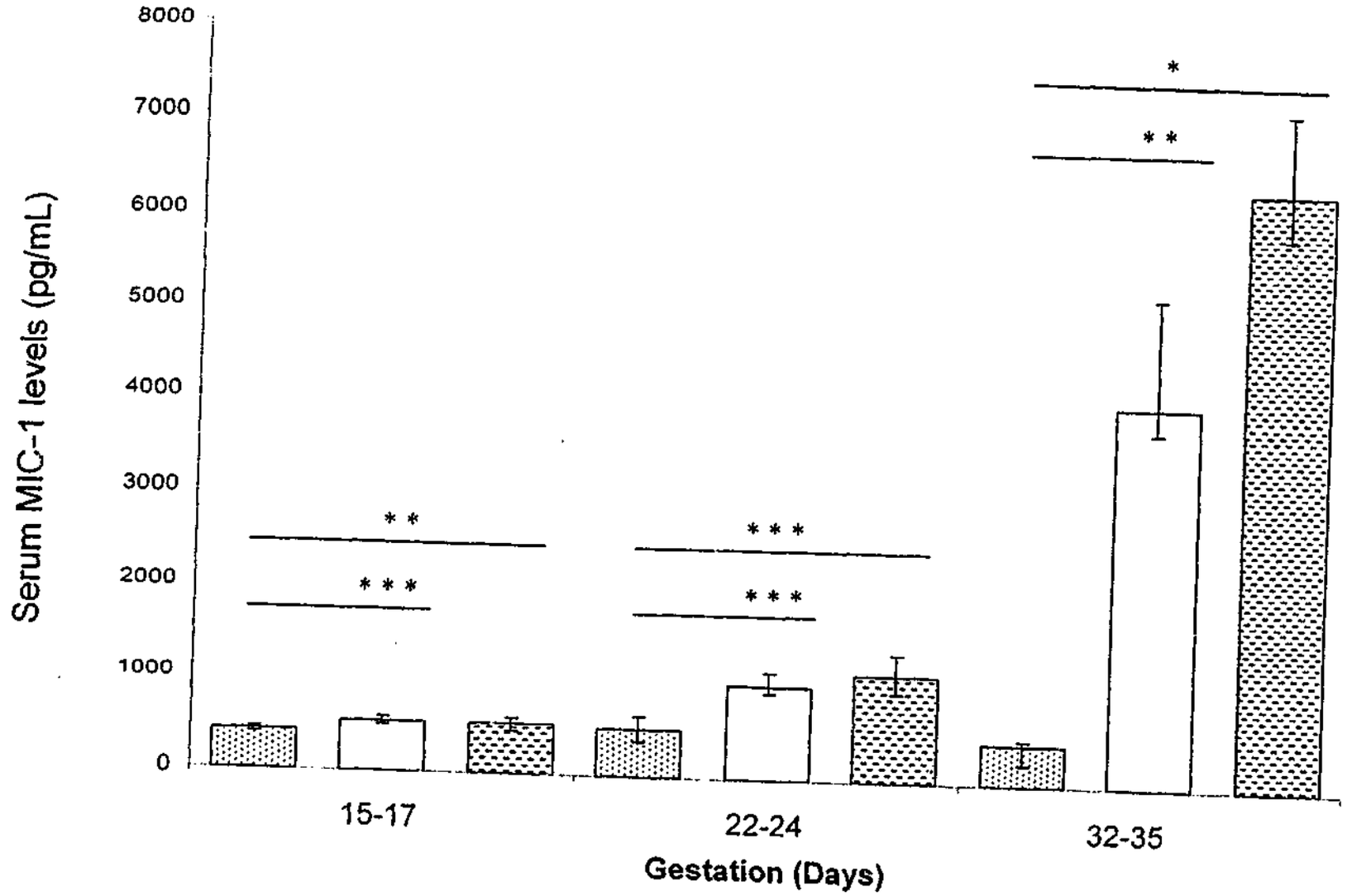

FIGURE 1 : Serum MIC-1 levels (Median and 95% CI) according to gestation at sample collection. No clinical pregnancy (▨), singleton gestation (□), twins (▨). * $p<0.05$; ** $p<0.005$, *** $p<0.0001$.

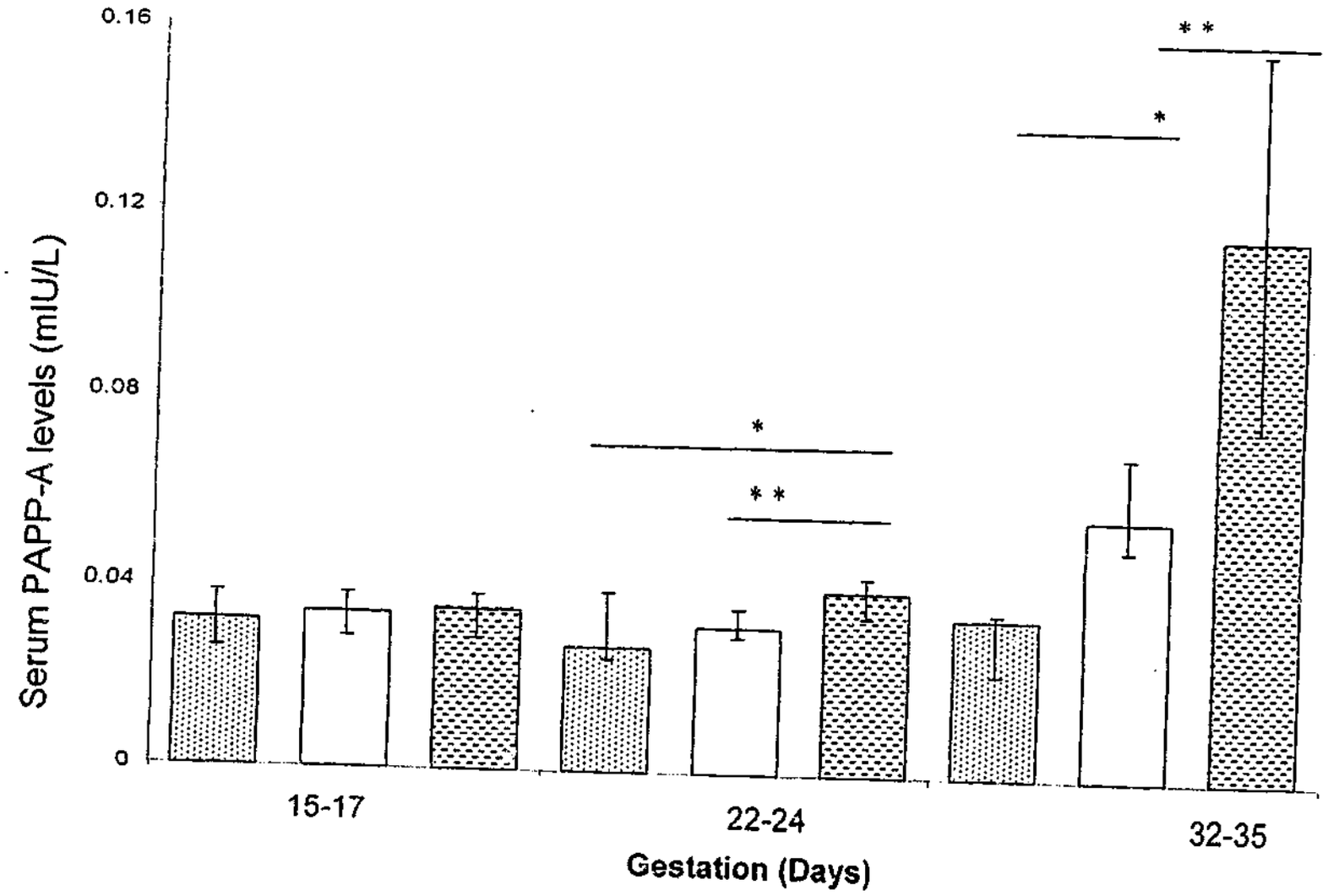

FIGURE 2: Serum PAPP-A-1 levels (Median and 95% CI) according to gestation at sample collection. No clinical pregnancy (▨), singleton gestation (□), twins (▨). * $p<0.05$; ** $p<0.005$, *** $p<0.0001$.

Manuscript 1C:

Increasing risk of clinical miscarriage with decreasing human chorionic gonadotrophin levels at days 15-17 after conception in an IVF cohort

Stephen Tong, Luk Rombauts, and Euan M Wallace.

Declaration regarding the paper entitled:

Increasing risk of clinical miscarriage with decreasing human chorionic gonadotrophin levels at days 15-17 after conception in an IVF cohort (1C)

(submitted)

Contributions to the work involved the following:

Name	% contribution	Nature of contribution
Stephen Tong	60%	Conceived and designed the study, statistically analysed data, and was a major contributor in the writing of the report
Euan M Wallace	20%	Supervised study and contributed to the writing of the report.
Luk Rombauts	20%	Collected clinical details and contributed to the writing of the report.

Declaration by co-authors

The undersigned hereby certify that:

(11) they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;
(12) they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
(13) there are no other authors of the publication according to these criteria;
(14) potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journals or other publications, and (c) the head of the responsible academic unit; and
(15) the original data are stored at the following location(s) and will be held for at least five years from the date indicated below:

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Increasing risk of clinical miscarriage with decreasing human chorionic gonadotrophin levels at days 15-17 after conception in an IVF cohort

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[redacted]

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Abstract

Objectives: To investigate a possible association between low human chorionic gonadotrophin levels (hCG) at the end of the first week after implantation and miscarriages occurring after clinical recognition.

Study design: Observational retrospective study of 1562 women with an ultrasound confirmed singleton pregnancy after *in-vitro* fertilisation. The incidence of miscarriage diagnosed after 8-20 weeks was determined according to relative hCG levels corresponding to days 15-17 after conception.

Results: The overall risk of miscarriage was 10.6% and the mean (std dev) gestational at diagnosis was 12.7 (2.4) weeks. There was an increasing risk of miscarriage associated with decreasing hCG levels (7.7% >75th centile; 9.0% at 25-75th centile; 16.8% <25th centile; $p<0.0001$, $\chi 2$ test for trend).

Conclusions: Low days 15-17 hCG levels in early pregnancy are associated with increased risk of miscarriage well after clinical recognition. The mechanisms underlying late first trimester and second trimester miscarriages may begin very early in pregnancy.

Keywords: Miscarriage; Human chorionic gonadotrophin; in vitro fertilisation; aetiology; early pregnancy ultrasound

Introduction

Human reproduction is extremely inefficient. Preclinical early pregnancy loss has been recognised to be a common phenomenon where pregnancy ends prior to a missed period. It has an estimated incidence of about 30%[1] and has been shown to occur after both spontaneous conception [2,3] and *in vitro* fertilisation (IVF) [4-8].

Once a pregnancy reaches clinical recognition, there is still a 9-15% risk that it will miscarry[3, 9-11], a risk that progressively decreases as gestation advances[12,13]. Many of these clinical miscarriages are likely to be a deliberate expulsion of conceptuses containing chromosomal errors which most commonly results from a failure of the proper separation of chromosomes at the first meiotic division during gametogenesis[1]. Cytogenetic studies of miscarriage specimens have reported an incidence of genetic abnormalities in spontaneous pregnancies as high as 55%[1] to 72%[14].

For these pregnancies that reach clinical viability only to then miscarry, it has not been precisely determined how early pathogenic events tipping the pregnancy towards failure might begin. An appreciation of this would be useful in advancing the fundamental understanding of early pregnancy biology but also for the development of potential preventative therapeutics, since it might help determine the best to time for intervention. Whilst it is clear that low levels of hCG around days 12-16 after conception (4th week of gestation) in an *in-vitro* fertilisation (IVF) are associated with preclinical early pregnancy loss[15], the precise relationship between early hCG levels and later miscarriage occurring after clinical recognition remains uncertain. Previous studies in IVF cohorts have generally grouped clinical miscarriages with early pregnancy loss[7] and ectopic

pregnancies [5,6,16,17] as a single outcome. Furthermore, as these studies did not set out to specifically investigate clinical miscarriage, they have not accounted for important factors, such as documenting proof of clinical viability with ultrasound and separating twins from the analysis. The inclusion of twins would introduce a significant bias since they are associated with higher early hCG levels[6,16] and their rate of loss after clinical recognition may be different to singletons[13,18].

We therefore undertook a large observational study investigating whether low hCG levels at day 15-17 after conception were associated with an increased risk of clinical miscarriages diagnosed after the 8th week of gestation. Importantly, we only included cases where there was early ultrasound evidence confirming the presence of a viable singleton pregnancy with normal cardiac activity. We hypothesised that a low but positive hCG measured during the first week of implantation might reflect lower embryo or implantation quality placing the pregnancy at increased risk of miscarriage even after progressing to clinical recognition.

Materials and methods

A retrospective observational study of an *in vitro* fertilisation (IVF) cohort was undertaken, exploring the relationship between clinical miscarriage and maternal serum levels of hCG at 13-14 days after embryo transfer (ET). This corresponds to days 15-17 after conception, or the 4th week of gestation, since the two weeks of the proliferative phase prior to ovulation is included by convention.

In Australia, IVF centres are mandated to collect and report clinical outcomes to a national government body. We used the data collected for this purpose to undertake the study. This project fulfilled the guidelines of a retrospective audit as defined by The National Health and Medical Research Council of Australia, making it exempt from requiring formal ethics committee approval.

For our primary outcome, we determined the incidence of miscarriage in the groups where day 15-17 hCG levels were <25th, >75th or between 25-75th centiles. We also obtained information regarding at which gestation the miscarriage was diagnosed. hCG levels were measured using an immunometric assay and performed on an automated system (VitrosECi /OrthoDiagnostics/ Rochester, New York)). The intra and inter assay coefficient of variation was <10%.

Baseline data was collected on age, Body Mass Index (BMI), the type of assisted reproductive technology (ART), previous number of deliveries (none vs one or more), the type of infertility and the number of embryos transferred.

We only included women who had undergone IVF and had a clinically recognised, viable singleton pregnancy with normal fetal cardiac activity confirmed on ultrasound. An early pregnancy ultrasound is routinely performed, usually timed to occur during the 6th week of gestation, in all women who undergo IVF treatment and may be pregnant. We restricted our analysis to pregnancies reaching the 8th week of gestation since we were concerned that including miscarriages occurring at 6-7 weeks gestation might introduce a bias where those who miscarry around this time may or may not be included in our cohort depending on whether they had attended their ultrasound examination. We anticipated that the early pregnancy ultrasound would have been done in almost all cases by the 8th week of gestation. Our final definition of a clinical miscarriage for the purposes of our study was a pregnancy loss diagnosed between 8-19 (+6 days) weeks of gestation, whilst an ongoing pregnancy was one which had reached at least 20 weeks.

There is an almost exponential increase of miscarriage risk once women reach the age of 40 [19]. Accordingly, it was decided during study design to exclude women over 40. We also excluded 21 women who elected to terminate their pregnancies and did not include multiple pregnancies diagnosed at the early pregnancy scan to avoid the uncertain contribution of 'vanishing twin' to the overall clinical miscarriage risk [13,18].

Results:

We identified 1562 women with a singleton IVF pregnancy who reached the 8th week of gestation and had ultrasound confirmation of fetal viability. Table I lists their baseline details. Those women who miscarried were significantly older than those that did not ($P<0.0001$) and there were minor differences in the aetiology of infertility between the groups ($P=0.017$) There were no other significant differences between the two groups.

The overall miscarriage rate was 10.6% (n=165). There was a significant trend ($\chi 2$ test for trend; $p<0.0001$) towards an increasing risk of clinical miscarriage with lower early hCG levels (7.7% >75th centile; 9.0% at 25-75th centile; 16.8% <25th centile; table II). The median (95%CI) day 15-17 hCG level in the ongoing pregnancy group was 198 mIU/mL (189-208), significantly higher than those who had miscarried (165 mIU/mL (126-186); $p<0.0001$ Kruskal-Wallis).

The figure graphs the number of women diagnosed with a miscarriage at each gestational age from 8-19 weeks of gestation. The mean (std dev) gestational age at which women where diagnosed with their miscarriage was 12.7 weeks (2.4). The mean gestational age at diagnosis did not differ according to relative day 15-17 hCG levels ($p=0.58$; table 2).

Since women in the miscarriage cohort were significantly older, we undertook a post hoc analysis correlating maternal age and day 15-17 serum hCG levels. In both all women and those who miscarried there was a significant but weak correlation between maternal age and hCG. ($r^2=0.003$, $p=0.017$ and $r^2=0.03$, $p=0.04$, respectively).

Comment

We have found a highly significant trend between decreasing day 15-17 maternal serum hCG levels and increasing risks of clinical miscarriage in a cohort of women undergoing IVF. This observation suggests that the mechanisms leading to miscarriage well after clinical recognition may, at least in some pregnancies, begin as early as the first week of implantation. This has potential relevance to understanding those mechanisms and for the development of novel therapies.

Whilst an association between low hCG and an increased risk of early pregnancy failure in IVF pregnancies has been previously reported [5,6,16,17], the relationship in confirmed 'clinical' pregnancy has remained, until now, obscure because prior reports have generally grouped preclinical loss, clinical pregnancy loss and ectopic pregnancies as a single outcome, [4-7 17]. Poikkeus *et al*[8] did separately identify a cohort defined as having a clinical miscarriage and found an association between low day 12 hCG and an increased risk. However, there were important differences between that study and the current one. Poikkeus *et al*[8] did not confirm a live pregnancy on ultrasound prior to fetal demise and twin pregnancies were included in their analysis. Similarly, while Frishman *et al*[20] reported an increased risk of miscarriage in a small number of women who had a low hCG level (≤ 20 mIU/mL) and a singleton intrauterine gestational sac on early ultrasound at 4 weeks gestation, there was no documented fetal cardiac activity. Nonetheless, the results of all of these previous studies are supported and extended by the current study.

Importantly, the observed miscarriage rate in the current study was 10.6%. This is in agreement with the rates of 10.3 and 12% observed by others after a 6 week viability

gestation scan in a spontaneous [12] and IVF singleton cohort [13] respectively. This suggests that our findings may have broader application beyond an IVF cohort.

As expected, we found a significant relationship between miscarriage and maternal age[19]. However, our post-hoc analysis demonstrating a weak and positive correlation (r^2 =0.003) between age and hCG excluded increased maternal age as the cause of low hCG in our cohort.

While the mean gestational age at diagnosis of miscarriage was at 12 weeks, we did not undertake serial ultrasound examinations to allow us to precisely define when fetal demise actually occurred. It is therefore possible, indeed likely, that in many women fetal demise occurred earlier than the gestation at diagnosis but were only subsequently diagnosed as missed losses. Nonetheless, it is certain that in all women there was a live singleton fetus present at 6 weeks gestation and perhaps likely that a number of miscarriages diagnosed during the second trimester were in fact late miscarriages.

The main implications of our finding is that a significant proportion of clinical miscarriage have their origins by very early pregnancy. We can only speculate as to what underlying pathology that low hCG might reflect, but it is likely to indicate poorer implantation. Weinberg *et al* [2] found that the optimum window period for an embryo to implant is 8-10 days after conception in a cohort of women with a spontaneous pregnancy and that cases where the first positive hCG levels were detected after this time incurred a significant increase in their risk of preclinical pregnancy loss. Although the authors had insufficient numbers to conclude the same for clinical pregnancy loss, it is likely that preclinical and clinical miscarriages are a continuum and may share largely common

aetiologies. It is possible therefore that low day 15-17 hCG in our cohort reflects late implantation, where secretion from trophoblasts begins later day 16 so that by day 15-17, serum hCG levels are lower than pregnancies which had implanted earlier. It has also been recently shown in the same study population that inadequate hCG doubling times during the first week of implantation was associated with lower progesterone secretion from the corpus luteum and an increased risk of miscarriage[21]. This would suggest that the aetiology may not lie with the timing of implantation, but rather with embryo/trophoblast quality resulting in a decreased level of hCG secretion. A complex interaction between fetal and maternal cytokines and immunomodulation[22] is believed to be occur at the gestation when the samples were taken.

The current observation that very early low levels of hCG levels are associated with an increased risk of miscarriage is important for the timing of potential therapeutic intervention. Previous trials of progesterone therapy to prevent spontaneous miscarriage in the general population[23] and the use of hCG[24] and immunotherapies[25] to prevent recurrent miscarriage have often commenced treatments at a time when a clinical pregnancy has been confirmed which would be the fourth week of gestation at its earliest. Our data would suggest that, if such therapies have any merit they are likely to have a greater likelihood of success if they are administered earlier. Of course, we did not specifically investigate a population of women with recurrent miscarriage, where the incidence of euploid losses has recently been reported to be much higher[26] and for whom preventative treatments have been avidly sought. If the observations made here also exist in women with recurrent miscarriage then the cascade of events leading to recurrent miscarriage may also begin early, and it may just be possible that testing for a positive

hCG around the time immediately after implantation may direct treatment more effectively and afford improved treatment efficacy.

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Table I: Baseline details of the 1562 entire cohort, groups according to pregnancy outcome.

Variable		Miscarriage (n=165)	Ongoing pregnancy (n=1397)	P value
Age in years:				
	Mean (std dev)	34.7 (3.6) years	33.5 (3.7)	<0.000 i (T-Test)
Body mass index :				
	Median (95% CI)	23.9 (22.8-24.6)*	23.1 (22.0-23.4)**	0.366 (Mann-Whitney U)
Type of embryo transferred:				
	Fresh	75.2% (124)	75.7% (1055)	0.83
	Frozen	24.8% (41)	24.3% (339)	(χ2)
	Unknown	(0)	(3)	
Previous deliveries:				
	0	83.0 % (137)	83% (1160)	1.00
	≥1	17.0 % (28)	17.0% (237)	(χ2)

Table I: Baseline details – continued:

Aetiology of unfertility:			
Unexplained or unknown	47% (78)	51% (706)	0.017
Other	11% (18)	12% (170)	(χ2)
Tubal	12% (20)	18% (247)	
Endometriosis	13% (22)	10% (146)	
Multiple (any 2 of other/tubal/endo)	16% (27)	9% (128)	
Number of embryos transferred			
1		9.0% (126)	0.77
2		67.1% (937)	(χ2)
3		23.9% (334)	
Gestation at delivery for the ongoing pregnancy cohort			
<37 weeks	-	10.8% (151)	-
>37 weeks	-	89.2% (1246)	
Day 16 β-hCG:			
Median (95% CI)	165 (126-186)	198 (189-208)	0.0001 (Mann-Whitney U)

Data on BMI for 20* and 166** cases were not available.

Table II: The incidence of miscarriage according to relative β-hCG levels at day 15-17.

hCG level (centiles)	Absolute hCG levels (mIU/mL)	Risk of clinical miscarriage % (n)	Mean gestational age (weeks) at diagnosis of miscarriage Mean (SD)
<25th	0 – 114	16.8% (66/392)	12.5 (2.3) wks
25-75th	115 – 321	9.0% (70/778)	12.9 (2.3) wks
>75th	323 – 4640	7.7% (29/392)	12.7 (2.4) wks
P value	-	<0.0001 (χ2 for trend)	0.58 (ANOVA)

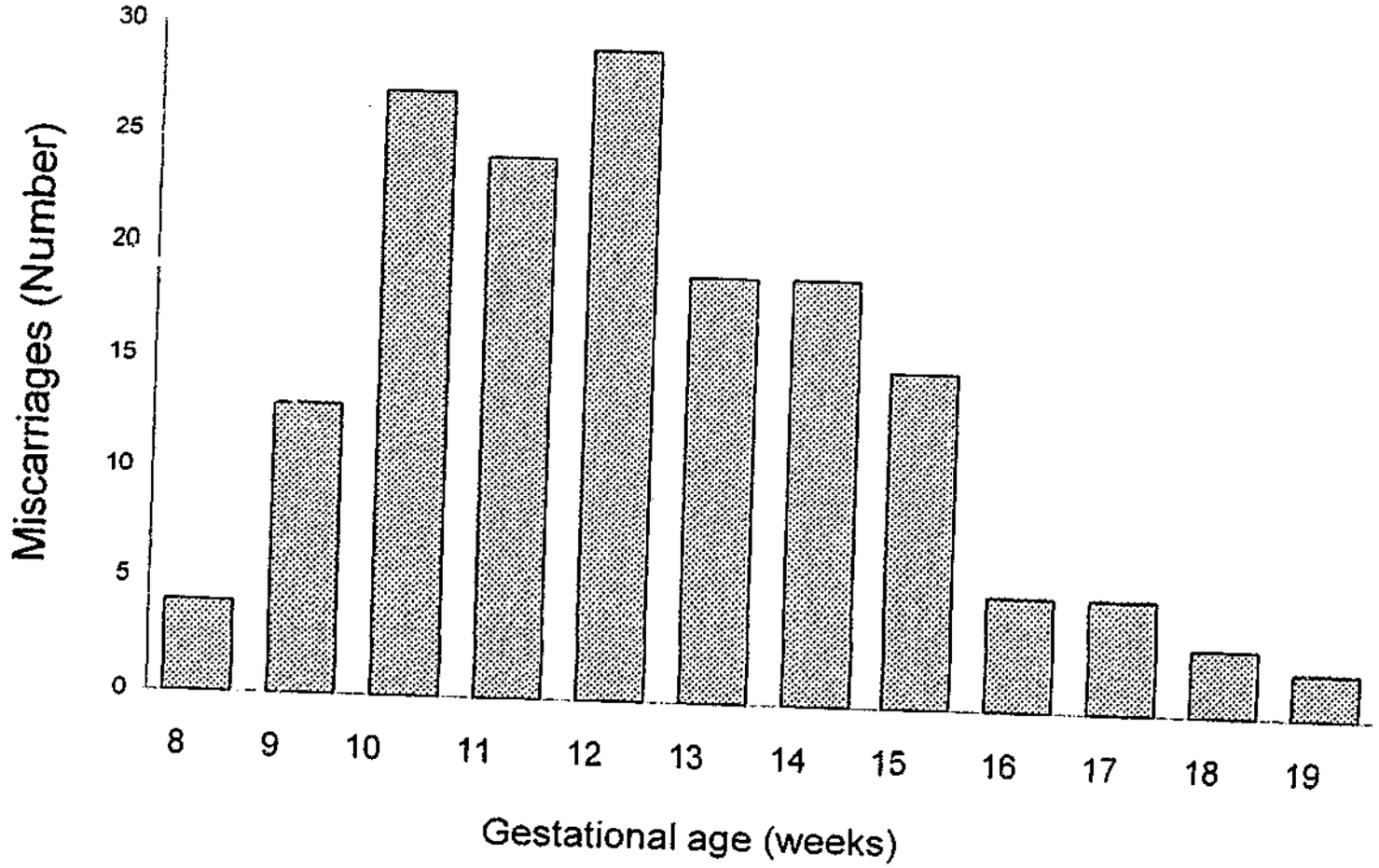

Figure: Gestational ages at which miscarriages were diagnosed

Manuscript 1D:

Increased risk of low birthweight with three embryo transfer and low human chorionic gonadotrophin levels in early pregnancy

Stephen Tong, Luk Rombauts, Joseph L Onwude and Euan M Wallace.

Declaration regarding the paper entitled:

Increased risk of low birthweight with three embryo transfer and low human chorionic gonadotrophin levels in early pregnancy (1D)

(submitted)

Contributions to the work involved the following:

Name	% contribution	Nature of contribution
[1]Stephen Tong	50%	Conceived and designed study, statistically analysed data, and was a major contributor to the writing of the report.
[1,2]Luk Rombauts	15%	Helped in study design, implanted the study, helped collect clinical details, and contributed to the writing of the report.
[3]Joseph Onwude	15%	Performed most of the statistical data analysis, contributed to the writing of the report.
[1]Euan M Wallace	20%	Supervised study, helped in study design, and was a major contributor to the writing of the report.

Declaration by co-authors

The undersigned hereby certify that:

(16) they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;
(17) they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
(18) there are no other authors of the publication according to these criteria;
(19) potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journals or other publications, and (c) the head of the responsible academic unit; and
(20) the original data are stored at the following location(s) and will be held for at least five years from the date indicated below:

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Increased risk of low birthweight with three embryo transfer and low human chorionic gonadotrophin levels in early pregnancy

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In vitro fertilisation, Assisted Reproductive Technologies, human chorionic gonadotrophin, embryo transfer, birthweight

Abstract:

Background: Singletons conceived from assisted reproductive technologies (ART) are at increased risk of low birthweight, an outcome associated with increased perinatal morbidity and common chronic adult diseases. We investigated a possible association between low birthweight and maternal serum human chorionic gonadotrophin (hCG) levels at the end of the first week of implantation, and the number of embryos transferred.

Methods: Using the database at our centre, we investigated 1206 women aged under 40 who had one (n=101), two (n=776) or three embryos (n=329) transferred, conceived a singleton pregnancy and reached 24 weeks of gestation.

Results: The incidence of low birthweight (<10^{th} centile for gestation) was 8.6%. At 15-17 days after *in-vitro* fertilization, hCG levels were lower with more embryos transferred ($p<0.0001$). There was an increased risk of low birthweight with three embryos transferred (vs one and two combined; crude odds ratio 1.76 (1.15-2.66); p=0.008), or a day 15-17 hCG <25^{th} centile (vs ≥ 25^{th} centile; crude OR 2.16 (1.39-3.36); p=0.001). After adjusting for age, previous deliveries and fresh vs frozen transfer, both three embryo transfer and hCG <25^{th} centile remained independently associated with low birthweight, adjusted OR 1.55 (1.00-2.39); p=0.05 and 1.99 (1.27-3.11); p=0.003, respectively. Women with both these parameters in same ART cycle had a 25% absolute risk of conceiving a low birthweight baby.

Conclusions: The mechanisms underlying low birthweight with ART may begin soon after implantation. Restricting the number of embryos transferred to less than three may decrease the overall incidence of low birthweight babies arising from ART.

Introduction:

The consequences of low birthweight may persist lifelong. It is the strongest predictor of perinatal mortality[1] and morbidity, strongly associated with cerebral palsy[2], and has also been linked with an increased risk of common chronic adult diseases, such as hypertension, stroke and diabetes[3]. The risks of adult disease are not only present with the very extremes of low birthweight but increase progressively with decreasing birthweight[3-5].

Singletons conceived with the assistance of assisted reproductive technologies (ART), such as *in-vitro* fertilization, are at two to three times increased risk of low birthweight compared to the general population [6-8]. Recently it was shown[6] that this increase is not explained by pregnancies originating as twin gestations which reduced to a singleton either spontaneously or medically, and that these risks persisted even in women unlikely to have had underlying fertility related disease. These observations suggest that the mechanism underlying low birthweight in assisted conception pregnancies may be directly related to the ART treatment. In light of this, it would be important to further explore this relationship with the eventual aim of identifying any avoidable factors which could decrease the incidence of low birthweight in association with ART without significantly impacting on the couples' chances of having a baby.

Determining how early the mechanisms causing low birthweight in ART pregnancies begins may be important in understanding this link and may also have wider relevance to the understanding of the biology of growth restriction in spontaneous

pregnancies. Therefore, we investigated a possible relationship between low birthweight and both early low maternal serum human chorionic gonoadotrophin (hCG) levels and the number of embryos transferred in singleton pregnancies conceived with the assistance of ART. We hypothesised that low hCG measured at 15-17 days, which corresponds to the end of the first week after implantation[9], may reflect initial difficulties in implantation which in turn confers upon the embryo an increased risk of low birthweight. We included the number of embryos transferred in the analysis since we originally anticipated the need to account for an increase in day 15-17 hCG levels with the more number of embryos transferred.

Methods:

Subjects

We accessed electronically stored information routinely collected from our three ART centres for all embryo transfers that occurred between February 1996 to December 2001. During this period, standard embryo culture media was used, irrespective of the number of embryos transferred. The number of embryos transferred was the decision of the attending clinician.

For this study we only included women who had undergone IVF and had a clinically recognised viable singleton pregnancy with fetal cardiac activity confirmed on early gestational ultrasound and who subsequently progressed to at least 24 weeks of gestation. Baseline characteristics were collected including age, body mass index, whether the embryo(s) transferred was fresh or frozen, whether the women had had a previous delivery or not, and the gestational age at delivery. Pregnancy outcome data collected included the following outcomes: low birthweight (defined birthweight $<10^{th}$ centile for the gestation at which the baby was born, according to previously published national data[10]); significant preterm delivery (<34 completed weeks gestation) and gestational diabetes (a positive two hour oral glucose tolerance test) [11].

These outcomes were then stratified according to the number of embryos transferred and the hCG level at days 15-17 after egg pick-up and fertilisation *in-vitro*. This corresponds to the same number of days after conception, or 4 weeks gestation by convention.

We excluded women aged over 40 since they are at significantly higher risks of pregnancy complications and have a decreased probability of conceiving after ART[12] . It is therefore possible that their uterine biology may be considerably different from those who are younger. We felt that excluding this group would make our results potentially more generalisable.

Approval to undertake the study was obtained from our Human Research and Ethics Committee at Monash IVF.

Statistical analysis:

The baseline and outcome variables were compared using parametric, non-parametric or chi-square tests as appropriate. Multiple logistic regression was used to further investigate associations with low birthweight, adjusting for age, BMI, parity (no previous delivery, versus ≥1), fresh vs frozen embryo transfer, logged hCG levels and the number of embryos transferred.

Results:

Table 1 summarises the baseline details of the entire cohort and the subgroups divided according to whether one, two or three embryos were transferred. There was a significant decrease in hCG levels with increasing number of embryos transferred. The mean (std dev) age in the total cohort was 33.3 (3.8) years and there were slight differences in age, but not body mass index amongst the subgroups. The more embryos transferred, the more likely that fresh embryos were transferred rather than frozen-thawed, and more likely that women had not had a previous successful delivery.

The total incidence of low birthweight was 8.6%. Table 2 shows the incidence of low birthweight, gestational diabetes and delivery <34 weeks depending on the number of embryos transferred. Those who had three embryos transferred had a significantly increased risk ($p=0.027$; χ^2) of having a low birthweight baby (12.2%) compared to those who had one (6.9%) or two (7.3%) embryos transferred (table 2). The total incidence of significant preterm delivery (<34 weeks) in our cohort was 3.1%. Having three embryos transferred conferred a 5.2% risk of delivery before 34 weeks gestation, significantly increased ($p=0.033$; χ^2) compared to two (2.3%) or one (2.0%) ET. The incidence of gestational diabetes was 6.6%. This rate did not vary according to the number of embryos transferred ($p=0.37$; χ^2).

Table 3 shows the incidence of the same obstetric complications grouped according to whether day 15-17 hCG levels were <25th centile, between 25th-75th centile, or >75th centile. Low levels of hCG <25th centile were associated with a significantly increased

risk (p=0.035; χ^2) of low birthweight (12.2%) compared to those who had levels within the 25-75th centile (7.2%), or >75th centile (7.9%; see table 3). The incidence of either gestational diabetes or delivery before 34 weeks was unrelated to relative day 15-17 hCG levels.

Table 4 shows the unadjusted risks of low birthweight in the cohort stratified to both the relative hCG levels and the number of embryos transferred. Having low hCG after three embryo transfer was associated with a 25% chance of having a low birthweight baby, significantly higher (p<0.0001; χ^2) than having two (6.7%) or one (12%) embryos transferred.

Since birthweights were similar in the 1 vs 2 embryo subgroups, these two groups were combined in order to undertake a logistic regression analysis. As birthweights were also similar in the pregnancies with day 15-17 hCG levels between the 25-75th centile and >75th centile, these subgroups were also combined for further analysis. Table 5 shows the results of the crude and adjusted odds of having a low birthweight baby. Maternal age, whether or not the women had had a previous delivery, and whether the embryo was fresh or frozen were not associated with the risk of low birthweight. A low day 15-17 hCG level (<25th centile) remained independently associated with an increased odds of having a low birthweight baby after adjusting for all other baseline variables, including the number of embryos transferred (adjusted OR 1.99 (95%CI 1.27-3.11) p=0.003). Three ET was strongly associated with low birthweight on univariate analysis (crude OR 1.76 (95%CI 1.15-2.66); p=0.008), and remained significant (adjusted OR 1.55 (95%CI 1.00-2.39) p=0.05) after correction for hCG levels.

Discussion:

We have found that a woman's risk of giving birth to a low birthweight baby after ART is significantly increased if she had three embryos transferred compared to women having either one or two embryos transferred. Low early hCG levels was also independently associated with low birthweight suggesting possible very early origins of low birthweight. These observations have implications for current ART practice and offer insights into possible mechanisms of low birthweight.

The total incidence of low birthweight (<10th centile) we observed was 8.6%, the same as expected in a general obstetric population. That we did not see a higher incidence of low birthweight in our ART cohort, as reported by others [6-8] may be explained by the fact that we only included cases where a singleton was confirmed to be present from early pregnancy and that we excluded women over 40. Also, we cannot exclude the possibility that an unidentified factor amongst the three ET cohort was the culprit causing the increase in low birthweight. However, the fact that three ET was associated with lower early hCG compared with transferring less embryos, and that low hCG was independently associated with low birthweight lends strong support to the possibility that having three ET rather than less is somehow directly associated with the mechanism causing low birthweight.

Schieve et al[6] used evidence taken from early pregnancy ultrasound to show that the low birthweight seen in association with ART is not due to a reduction of multiple gestations to singletons occurring onwards from the middle of the first trimester. We consider it likely that most pregnancies in the two and three ET cohort were already

singletons by the end of the first week of implantation. This is because hCG levels at days 15-17 were lower in the these groups compared to the single ET cohort, the reverse of what would be expected if multiple placentation had occurred [13, 14].

Although there are obvious differences between ART and spontaneous conceptions, it is still likely that the fundamental biological events leading to low birthweight would be similar. If this is true, then our data provide new information on the possible timing of the mechanisms causing low birthweight. A long held view that the pathogenesis of low birthweight begins in the second trimester has been questioned by recent evidence linking low birthweight to events earlier and earlier in the first trimester. Low maternal serum levels of pregnancy-associated plasma protein-A[15] at 8-12 weeks gestation, or a fetus measuring smaller then expected by first trimester ultrasound [16] (done ≥6 weeks) have both been associated with low birthweight. In a report of a small study, five out of 11 women who had low hCG levels (<10th centile) across days 16-35 gave birth to a low birthweight baby after ART [17]. By investigating maternal serum hCG levels exclusively at days 15-17, we have observed a strong link between low birthweight and the earliest period of gestation yet reported. We speculate that in a number of cases of low birthweight the cause may be somehow related to difficulties during implantation of the early embryo, as evidenced by low hCG levels in early pregnancy. This is supported by the observations that low hCG levels in early pregnancy is strongly associated with early pregnancy loss[18] whilst the higher hCG levels are, the more likely that a successful pregnancy will occur [13, 14].

The reason why three ET may be associated with an increased risk of low birthweight is uncertain. The fact that correcting for low hCG levels significantly affects the

strength of the association suggests that multiple ET might result in a poorer quality of implantation for the surviving embryo. It is possible that competition between increasing numbers of replaced embryos may delay the implantation of the successful embryo beyond 8-10 days, about the optimal period in which to implant[9]. Alternatively, the timing of implantation may not be affected, but resorption of the nonviable embryos may cause an inflammatory processes disrupting the complex cellular and cytokine interactions occurring around the time of implantation[19]. If this is so then it might have been expected that that transferring two embryos instead of one is also associated with an increased risk of low birthweight. That we did not observe this may be because there were low numbers of women in our cohort who had a single ET. In this regard, it is interesting that a study published over a decade ago described a significant trend between an increased incidence of low birthweight with increasing numbers of embryos transferred[7] and a recent Belgian study found a higher rate of low birthweight babies amongst singletons after single ET compared to two ET [20]. Importantly however, neither of these studies accounted for the possibility that the singleton may have started off as a multiple pregnancy.

With an estimated 35-70 million couples who have used ART to overcome their infertility [21] and an ever increasing number of couples receiving ART each year[12], there has been growing focus not only on the 'take home baby rate', but also on the overall health of the resulting neonates[22]. The transfer of three embryos continue to be popular in the United States. In 2000, there were 65,438 ART treatment cycles for women aged under 40 and the average number of embryos transferred per cycle was 2.9 for women aged under 34 and over three for those aged over 34[12]. Our findings may be an explanation for the recent observation that singletons conceived in the

United States with the assistance of ART are at increased risk of low birthweight[6]. Given that two ET[23], and possibly even single ET [20, 24] can achieve the same pregnancy rates as three ET, restricting the number of embryos transferred may lower the incidence of low birthweight babies, and decrease the future disease burden of the increasing numbers conceived with the assistance of ART[12].

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TABLE 1: BASELINE CHARACTERISTICS

Parameter	Total cohort n= 1206	1 embryo transferred n=101	2 embryos transferred n=776	3 embryos transferred n=329	p=
Median (95% CI) hCG:	193 (183-201)	230 (200-259)	200 (188-217)	161 (148-178)	<0.0001† 1 vs 2: 0.046‡ 1 vs 3: <0.0001‡ 2 vs 3: =0.0001‡
Mean (SD) age:	33.3 (3.8)	34.4 (3.6)	32.9 (3.6)	34.3 (3.7)	p<0.0001*
Median (95% CI) BMI	23.2 (22.9-23.4) n=1071§	22.4 (21.9-23.9) n=85	23.2 (22.8-23.4) n=691	23.4 (22.8-24.1) n=295	0.37‡
ART type:					
IVF	77.1% (930)	54.5% (55)	75.9% (589)	86.9% (286)	<0.0001 ¶
Frozen embryo	22.9% (276)	45.5% (46)	24.1% (187)	13.1% (43)	
Previous deliveries:					
0	82.6% (996)	71.3% (72)	83.0% (644)	85.1% (280)	<0.0001 ¶
≥1	17.4% (210)	28.7% (29)	17.0% (132)	14.9% (49)	
Mean (SD) gestational age at delivery	38.5 (2.2)	38.4 (1.7)	38.6 (2.0)	38.3 (2.8)	0.141*

*Comparison using ANOVA
† Kruskal-Wallis test
‡Dunns multiple comparison test
§ There were 135 cases where information on height was not available in order to calculate the BMI
¶ Chi-square test

TABLE 2: ANTENATAL OUTCOMES STRATIFIED ACCORDING TO THE NUMBER OF EMBRYOS TRANSFERRED.

Antenatal complication	Total cohort n= 1206	1 embryo transferred n=101	2 embryos transferred n=776	3 embryos transferred n=329	p= *
Birthweight <10th centile	8.6% (104/1206)	6.9% (7/101)	7.3% (57/776)	12.2% (40/329)	0.027 1 vs 2: 0.2453 1 vs 3: 0.141 2 vs 3: 0.0097
Gestational Diabetes	6.6% (79/1206)	4.0% (4/101)	7.2% (56/776)	5.8% (19/329)	0.37
Delivery < 34 weeks	3.1% (37/1206)	2.0% (2/101)	2.3% (18/779)	5.2% (17/329)	0.033 1 vs 2: 0.834 1 vs 3: 0.17 2 vs 3: 0.013

* Chi-square tests

TABLE 3: ANTENATAL OUTCOMES STRATIFIED ACCORDING TO THE RELATIVE HCG LEVELS AT DAYS 15-17.

Antenatal complication	hCG<25th centiles (0-117)* n=303	hCG 25-75th centiles (118-317)* n=601	hCG>75th centiles (318-4640)* n=302	p= †
Birthweight <10th centile	12.2% (37/303)	7.2% (43/601)	7.9% (24/302)	0.035 <25th vs 25-75th: 0.012 <25th vs >75th:0.082 25th-75th vs >75th:0.668
Gestational Diabetes	4.6% (14/303)	6.8% (41/601)	7.9% (24/302)	0.233
Delivery < 34 weeks	4.0% (12/303)	2.6% (16/601)	3.0% (9/302)	0.568

* Absolute hCG levels (mIU/L)
† Chi square tests

TABLE 4: RISK OF LOW BIRTHWEIGHT BABIES IN SUBGROUPS STRATIFIED ACCORDING TO BOTH THE NUMBER OF EMBRYOS TRANSFERRED AND RELATIVE DAY 15-17 HCG LEVELS

	1 ET* n=101	2 ET n=776	3 ET n=329	P= †
HCG<25th centile	12% (3/25)	6.7% (13/194)	25.0% (21/84)	<0.0001
Absolute hCG range‡	27-155	0-124	0-92	
25-75th centile	4.0% (2/51)	8.0% (31/388)	6.1% (10/163)	0.48
Absolute hCG range‡	157-319	125-326	94-289	
>75th centile	8.0 % (2/25)	6.7% (13/194)	10.8% (9/83)	0.506
Absolute hCG range‡	326-2450	327-4640	291-1262	

* Embryo transfer
† Chi squared test
‡ (mIU/L)

TABLE 5: MULTIVARIATE ANALYSIS OF THE ASSOCIATION BETWEEN PATIENT PARAMETERS AND THE ODDS OF A LOW BIRTHWEIGHT BABY.

Parameter	Crude OR (95% CI)	P value	Adjusted OR (95% CI)	P value
Age (years)	0.99 (0.93-1.04)	0.74	0.98 (0.93-1.04)	0.58
No previous delivery: vs 1 or more	0.92 (0.55-1.65)	0.87	0.94 (0.54-1.63)	0.84
Fresh ET; vs frozen ET	1.57 (0.92-2.69)	0.09	1.44 (0.84-2.49)	0.19
HCG <25th centile; vs ≥ 25th centile	2.16 (1.39-3.36)	0.001	1.99 (1.27-3.11)	0.003
3 ET; vs 1and 2 ET as a combined group	1.76 (1.15-2.66)	0.008	1.55 (1.00-2.39)	0.05

*Adjusted variables: age, previous delivery, hcg<25 centile vs >25th centile, Fresh vs Frozen embryo transfer, 3 embryo transfer vs one and two embryo transfer as a combined group

Section 2: Biochemical markers of miscarriage at 6-13 weeks' gestation

Manuscript 2A:

Serum concentrations of macrophage inhibitory cytokine 1 (MIC 1) as a predictor of miscarriage

Stephen Tong, Budi Marjono, David A Brown, Sheila Mulvey, Samuel N Breit, Ursula Manuelpillai and Euan M Wallace.

Declaration regarding the paper entitled:

Serum concentrations of macrophage inhibitory cytokine 1 (MIC-1) as a predictor of miscarriage (2A)

Published: Lancet 2004;363:129-130.

Contributions to the work involved the following:

Name	% contribution	Nature of contribution
Stephen Tong	30%	Conceived and designed study, collected and statistically analysed the data and was a major contributor to the writing of the report.
Budi Marjono	20%	Performed the MIC-1 assay and contributed to the writing of the report.
David A Brown	7.5%	Developed the MIC-1 assay, and contributed to the writing of the report.
Sheila Mulvey	7.5%	Collected the clinical notes and samples, and contributed to the writing of the report
Samuel N Breit	7.5%	Developed the MIC-1 assay, and contributed to the writing of the report.
Ursula Manuelpillai	7.5%	Helped conceive and supervise the study, and contributed to the writing of the report.
Euan M Wallace	20%	Helped conceive and supervise the study and was a major contributor to the writing of the report.

Declaration by co-authors

The undersigned hereby certify that:

(21) they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;
(22) they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
(23) there are no other authors of the publication according to these criteria;
(24) potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journals or other publications, and (c) the head of the responsible academic unit; and
(25) the original data are stored at the following location(s) and will be held for at least five years from the date indicated below:

Location(s)

[1]Centre for Women's Health Research, Department of Obstetrics and Gynaecology, Monash University, Monash Medical Centre, Victoria, Australia.
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Serum concentrations of macrophage inhibitory cytokine 1 (MIC-1) as a predictor of miscarriage (2A)

Published: Lancet 2004;363:129-130.

Contributions to the work involved the following:

Name% contributionNature of contribution Stephen Tong30%Conceived and designed study, collected and statistically analysed the data and was a major contributor to the writing of the report.Budi Marjono20%Performed the MIC-1 assay and contributed to the writing of the report.David A Brown7.5%Developed the MIC-1 assay, and contributed to the writing of the report.Sheila Mulvey7.5%Collected the clinical notes and samples, and contributed to the writing of the reportSamuel N Breit7.5%Developed the MIC-1 assay, and contributed to the writing of the report.Ursula Manuelpillai7.5%Helped conceive and supervise the study, and contributed to the writing of the report.Euan M Wallace20%Helped conceive and supervise the study and was a major contributor to the writing of the report.

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(SAM BREIT)

Serum concentrations of macrophage inhibitory cytokine 1 (MIC 1) as a predictor of miscarriage

Stephen Tong, Budi Marjono, David A Brown, Sheila Mulvey, Samuel N Breit, Ursula Manuelpillai, Euan M Wallace

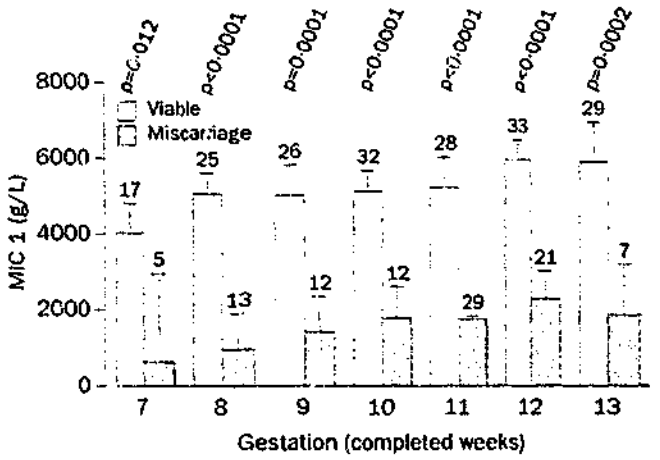
Macrophage inhibitory cytokine 1 (MIC 1) is thought to have immunomodulatory actions favouring fetal viability. We measured serum concentrations of MIC 1 in asymptomatic women at 6–13 weeks' gestation who subsequently miscarried or who had already miscarried. MIC 1 concentrations in the miscarriage cohort (n=100), were a third of those who had ongoing pregnancies (n=197). Multiples of the median for miscarriage was 0·32 (95% CI 0·23–0·32) versus 1·00 (0·93–1·06) for ongoing pregnancies; p<0·0001. Concentrations were just as low 3 weeks before diagnosis as on the day of diagnosis. That MIC 1 serum concentrations seem to be low weeks before miscarriage suggests possible predictive and causative roles, as well as therapeutic potential.

Lancet 2004; **363**: 129–30
See Commentary page 96

10–15% of all clinically recognised pregnancies end in spontaneous loss.[1] Although in many cases the fetus is aneuploid, a large number of pregnancies that are chromosomally normal are still lost. No treatment exists to prevent miscarriage and there is no established predictive marker.

Tight regulation of the maternal immune response to prevent rejection of the fetus is thought to be essential for pregnancy survival. There is evidence that women who have recurrent miscarriages have immune responses to pregnancy that favour the activity of type 1 helper T cells (Th1 facilitates cell-mediated immunity) over type 2 helper T cells (Th2 is associated with humoral immunity).[2]

Macrophage inhibitory cytokine 1 (MIC 1) is a member of the transforming growth factor β superfamily.[3] Since it has Th2-type cytokine actions and is strongly localised to the maternofetal interface,[4] we aimed to investigate whether it plays a part in maintaining pregnancy viability by comparison of concentrations between failed and successful pregnancies.



MIC 1 concentrations in viable pregnancies (n=190) and miscarriage (n=99), grouped by length of gestation
Values are medians. Bars are 95% CI. Numbers in each group are shown above bars. Pregnancies at 6 weeks' completed gestation were excluded because there was only one case of miscarriage

We measured MIC 1 and human chorionic gonadotrophin (β-hCG) concentrations in serum samples from 100 miscarriages and 200 normal control pregnancies obtained at 6–13 weeks' gestation. The samples were retrieved from a serum bank of over 8000 pregnancies, obtained prospectively as part of a Down's syndrome screening study. There was no clinical evidence of pregnancy loss (eg, pain or bleeding) at the time of blood collection in the group of women who miscarried. Gestational age was calculated from a 1st trimester ultrasound scan for control pregnancies and the last menstrual period in the miscarriage group. The healthy controls were matched with miscarriage samples for gestation at blood collection (completed week of pregnancy) and duration of storage by selecting about ten controls from each completed gestational week (6–13 weeks) from the first quarter of each year (1999–2001). We measured the serum MIC 1 concentration with an in-house ELISA, as described previously,[3] and β-hCG with a commercial assay (Dade Behring, Detroit, USA). The slight variations in numbers assayed in each group between the two hormones were the result of varying availability of serum samples.

The figure shows maternal serum MIC 1 concentrations in cases and controls for each completed week of gestation from 7–13 weeks. The concentrations were significantly lower in the miscarriage group than in gestationally-matched controls across all gestations. To allow a comparison between all cases and all controls, gestation-dependent differences were corrected by expressing analyte concentrations as multiples of the normal median for each completed week of pregnancy, derived from the viable control serum samples.

Concentrations of both analytes were significantly lower in miscarriage cases than in controls. For MIC 1, the median (95% CI) multiple of the normal median for the 100 non-viable pregnancies was 0·32 (95% CI 0·23–0·32) compared with 1·00 (0·93–1·06) in the 197 controls. For β-hCG, the corresponding values were 0·35 (0·19–0·36) for the 98 non-viable pregnancies and 1·00 (0·94–1·08) for the 200 controls (p<0·0001 for both hormones). The table shows the results of analyte measurements in the miscarriage cohort, grouped according to the interval between sampling and the date of diagnostic scan. MIC 1 concentrations were equally low in blood taken 3 weeks or longer before diagnosis, or 1–3 weeks before diagnosis compared with those taken on the same day that a miscarriage was diagnosed. By contrast, median concentrations of β-hCG showed a non-significant increase as the time between sampling and diagnosis lengthened (Kruskal-Wallis; p>0·1 for both hormones). We identified a further six cases in which we had confirmation from a late first trimester ultrasound that the fetus was alive at the time of blood collection but later miscarried, mostly in the second trimester. MIC 1 concentrations were significantly lower in these cases (0·70 [95% CI 0·34–1·16], p=0·03) than in the viable group. The mean interval between sampling and diagnosis of miscarriage in this subgroup was 4·8 weeks (SD 1·2). The remaining patients in the miscarriage cohort not included in these groups were those who had miscarried after an unknown length of time after blood collection.

The mean age in the miscarriage group was 31·2 (SD 5·5) years, which was significantly higher than those who had ongoing pregnancies (29·6 years, 4·7; p=0·01). MIC 1 multiples of normal median in the viable pregnancy cohort was not correlated with age (p=0·71).

	Timing of blood sample					
	≥3 weeks before scan (n=12)	p*	<3 but >1 week before scan (n=19)	p*	Same day as scan (n=30)	p*
Hormone						
MIC 1	0·38 (0·21–0·62)	<0·0001	0·37 (0·34–0·51)	<0·0001	0·33 (0·19–0·36)	<0·0001
β-hCG	0·64 (0·36–1·21)	0·045	0·52 (0·36–0·71)	<0·0001	0·39 (0·19–0·49)	<0·0001

Data are multiple of normal median (95% CI). *p value for comparison with viable group.

Miscarriage cohort, group according to the date of sample collection relative to diagnostic ultrasound

We have found that maternal serum MIC 1 is very low in association with miscarriage. Furthermore, our subgroup analyses provide strong evidence that low concentrations of MIC 1 levels precede miscarriage by several weeks. However, our study design prevented us from confirming the presence of a live fetus at the time of sampling in most of the miscarriage cohort. Therefore, whether MIC 1 is a sensitive and specific marker of miscarriage requires confirmation in a prospective study.

In view of the fact that MIC 1 might have actions favouring viability and is strongly localised at the materno-fetal interface, it is tempting to speculate that changed production of MIC 1 in the placenta is part of the mechanism initiating spontaneous pregnancy loss. If a causal link between low MIC 1 and miscarriage is confirmed, then MIC 1, or its synthetic analogues, might be useful in prevention of miscarriage.

Contributors

S Tong helped conceive and design the study, analysed the data, and wrote the report. B Marjono did the MIC 1 ELISAs. D Brown and S Breit developed the MIC 1 ELISA and contributed intellectually to the study. S Mulvey collected the clinical notes and samples. E Wallace and U Manuelpillai helped conceive and supervise the study. All authors helped with the preparation of the report.

Conflict of interest statement

St Vincent's Hospital holds patents for difference aspects of MIC 1 and S Breit and D Brown could benefit financially should any profits arise in the area of MIC 1 and early pregnancy.

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5 Brown DA, Breit SN, Buring J, et al. Plasma concentrations of macrophage inhibitory cytokine-1 (MIC-1) and the risk of cardiovascular events in women. *Lancet* 2002; 359: 2159–63.

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Manuscript 2B:

Very low Pregnancy-Associated Plasma Protein-A in asymptomatic women destined for miscarriage

Stephen Tong, Budi Marjono, Sheila Mulvey and Euan M Wallace.

Declaration regarding the paper entitled:

Very low Pregnancy-Associated Plasma Protein-A in asymptomatic women destined for miscarriage (28)

(Submitted)

Contributions to the work involved the following:

Name	% contribution	Nature of contribution
Stephen Tong	50	Conceived and designed the study, helped in the collection of clinical case notes, statistically analysed the data and was a major contributor to the writing of the report.
Budi Marjono	15	Performed the PAPP-A assay, and contributed to the writing of the report.
Sheila Mulvey	10	Collected the clinical notes and samples, and contributed to the writing of the report.
Euan M Wallace	25	Supervised the study, and was a major contributor to the writing of the report.

Declaration by co-authors

The undersigned hereby certify that:

(26) they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;
(27) they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
(28) there are no other authors of the publication according to these criteria;
(29) potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journals or other publications, and (c) the head of the responsible academic unit; and
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Very low Pregnancy-Associated Plasma Protein-A in asymptomatic women destined for miscarriage

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Abstract

Objectives: Pregnancy Associated Protein-A (PAPP-A) has been recently implicated to have important roles in the placenta and is low in women who present with symptomatic miscarriage. We set out to measure levels in asymptomatic women destined to miscarry.

Design: Retrospective longitudinal observational study.

Setting: Public tertiary hospital.

Patients: 97 asymptomatic women who either had already miscarried or were destined to do so, and 170 controls who had ongoing pregnancy.

Interventions: None

Main outcome measures: Maternal serum PAPP-A and human chorionic gonadotrophin (hCG) levels measured at 7-13 weeks of gestation. Data were expressed as multiples of the normal median (MoM).

Results: PAPP-A was significantly depressed [0.14 MoM, (95% CI 0.11-0.19)] among the miscarriage cohort compared to those with ongoing pregnancies [1.00 MoM (95% CI 0.90-1.09);P<0.0001], with levels just as low 3 weeks before diagnosis [0.11 MoM (95% CI 0.06-0.36); P<0.0001] as those taken on the day of diagnosis [0.19 MoM (0.14-0.43); P<0.0001]. Whilst hCG was also lower in the entire miscarriage cohort, levels increased as the interval between blood collection and diagnosis widened.

Conclusions: PAPP-A levels are very low in association with, and may precede miscarriage. It may play a role in both causation and prediction, and have therapeutic potential.

Keywords: PAPP-A, miscarriage, marker

Introduction

For a long time after its discovery, Pregnancy Associated Placental Protein-A remained a protein of unknown function, but widely recognised by the fact that low levels are associated with Down Syndrome (1). The realisation that PAPP-A is likely to play a critical role in placental function was recently made when Lawrence et al (2) reported it to be a protease of insulin-growth factor binding protein-4 (IGFBP-4). IGFBP-4 binds to, and decreases the amount of bioactive IGFI and II. These are growth factors with active roles in maintaining a healthy placenta, including steroidogenesis (3), glucose and amino acid transport (4) and trophoblast invasion (5). By cleaving IGFBP-4, PAPP-A may be increasing free, and therefore bioactive, IGF I and II allowing favourable actions in the placenta. In further support of the important role of PAPP-A in the placenta, low maternal serum levels of PAPP-A at the end of the first trimester are associated with late obstetric complications related to impaired placentation such as growth restriction (6-9), pregnancy induced hypertension (8, 9). The recent reports describing the actions of PAPP-A have led to a resurgence of interest into PAPP-A biology at the feto-maternal interface (10, 11).

In light of the likely roles that PAPP-A plays in the placenta, it may also be expected that levels might be lower in association with miscarriage. Indeed, it has been shown that maternal serum levels of PAPP-A are depressed in women who have miscarried at the time they present with symptoms (12-14). However, such information may be of limited biological interest, since many other placental hormones are also on the decline at this time (13-16), when the miscarriage is already well advanced. In addition, the diagnostic test at the time of presentation is an ultrasound scan rather than endocrine evaluation.

However, if it can be shown that PAPP-A levels are low prior to a miscarriage then it may imply a possible causative role in both pregnancy maintenance and failure. There is some evidence from a number of case series suggesting that PAPP-A levels might be very low weeks prior to miscarriage during the first trimester (17-19). Here we describe PAPP-A and total human chorionic gonadotrophin levels (hCG) among a large cohort of asymptomatic women who either have already miscarried, or were destined to do so, largely within the first trimester of pregnancy.

Materials and methods

An observational study was undertaken in which PAPP-A and total human chorionic gonadotrophin levels (hCG) levels were measured in the sera of 97 women at 7-13 weeks gestation who had either already miscarried, or were destined to do so. These were compared to levels measured in the sera of 170 women who had a successful pregnancy. All women were public patients at a large tertiary hospital. Blood samples had been collected as part of a Down Syndrome screening study. None of these women had any symptoms of miscarriage at the time when the blood was taken. Gestational age on the day of blood collection was calculated from a 1st trimester ultrasound scan for control pregnancies and from the last menstrual period among the miscarriage group. For the control pregnancies, gestational age did not differ if calculated by ultrasound or last menstrual period ($p=0.17$; n=134 of ongoing pregnancy group where LMP was known). We did not have ultrasound confirmation of fetal viability at the time of blood sampling but were able to identify subgroups within the miscarriage cohort whom had blood sampling at different intervals before their diagnosis of miscarriage (table 1). Of the remaining women in the miscarriage cohort (n=41), we were unsure of the interval between blood collection and ultrasound diagnosis of miscarriage.

Data which grouped observations across different gestations were expressed as a multiple of the normal (viable pregnancy) median (MoM).

These samples constituted sera in a tissue bank. The data on hCG forms part of a slightly larger cohort previously reported (20). We have described hCG, a clinically used marker in early pregnancy, for the sole purpose of comparing its performance with PAPP-A.

We had obtained ethics committee approval for this study as part of a larger Down Syndrome Screening study for which written informed consent to store excess sera from women for further research was obtained.

ELISA for PAPP-A and total intact HCG.

All samples were spun at 3500rpm for 10 minutes, the sera collected and stored at -20 °C until further assay. Both hormones were measured using commercially available ELISA kits (PAPP-A: Diagnostic Systems Laboratory, Texas, USA. HCG: Dade Behring, Detroit, USA) according to the manufacturer's instructions. The limit of sensitivity for PAPP-A is 0.013 mIU/L and 1 IU for hCG. Inter and intra-assay coefficient of variations was <5% for both assays.

Statistical analysis

Hormone levels were expressed as the median and 95% confidence interval. Comparisons between 2 groups were made using the Mann-Witney U test and 3 or more groups using the Kruskal-Wallis test.

Results

The mean (std dev) age of the women in the viable cohort was 29.6 (4.8) years, significantly lower than those who were in the miscarriage group; 32 (5.5) years, p=0.008.

Figure 1 shows absolute PAPP-A levels across 7-13 weeks of gestation in both the miscarriage and the controls. PAPP-A in the ongoing pregnancy cohort increases as gestation advances ($p<0.0001$; Kruskal-Wallis). PAPP-A was significantly depressed in the miscarriage group at all gestations studied for all comparisons (see figure 1). Women who had miscarried also had significantly lower hCG levels compared to ongoing pregnancies at every week of gestation studied (see figure 2).

PAPP-A levels in the entire miscarriage cohort were significantly depressed to 14% of normal levels, median (95%CI) MoM 0.14 (0.11-0.19) vs 1.00 (0.90-1.09), respectively; $p<0.0001$; Mann-Whitney U. In comparison, hCG in the miscarriage group were 38% of normal levels, median (95%CI) MoM 0.38 (0.22-0.47) vs 1.00 (0.92-1.07), respectively; $p<0.0001$.

Table 1 shows the results of PAPP-A and hCG levels amongst the miscarriage cohort, grouped according to the interval between sampling and date of diagnostic scan. PAPP-A levels were as low in blood taken 3 weeks before, or 1-3 weeks before diagnosis compared to those taken on the same day that a miscarriage was diagnosed. In contrast, median hCG MoM levels trended upwards as the time between sampling and diagnosis lengthened, although this did not reach statistical significance (Kruskal-Wallis; $p>0.1$ for both hormones).

Discussion:

PAPP-A levels are very low amongst asymptomatic women who are either destined to miscarry, or had already done so within the first trimester of pregnancy. To our knowledge, the PAPP-A levels we describe represent the lowest of any hormone measured amongst such a cohort relative to ongoing pregnancies.

In most previous published studies investigators reporting PAPP-A levels in relation to miscarriage assayed sera taken at a time when the women presented with symptoms of pregnancy loss when the miscarriage process would have already been well advanced (12-14). There have been two large studies reporting the association between PAPP-A levels in a presumably asymptomatic cohort at 10-13 weeks and subsequent miscarriage (8,9). In both studies, a fetal heart beat was confirmed by an ultrasound scan done at the same time to assess the risk of Down Syndrome. Whilst they both show that low PAPP-A levels at the end of the first trimester are indeed associated with a future miscarriage, levels were only very modestly depressed. However, the women in both these studies would have had their miscarriage either very late in the first trimester, or at a variable time during the second trimester. It is known that by this time the majority of miscarriages in the general population would have already occurred. This is reflected by the fact that a study by Ong et al (9), the miscarriage rate was only 1.8%. In contrast, the overall incidence of miscarriage in clinically recognised pregnancies is 10-15% (21, 22). The possibility therefore remained that PAPP-A might be a sensitive predictor of miscarriage earlier during pregnancy when the majority of pregnancy failures are occurring. Although our study was also primarily taken from a cohort presenting for Down Syndrome screening, our study was quite different from previous reports (8,9) in that a significant proportion of bloods were taken earlier during the first trimester when most miscarriages are occurring.

Unfortunately, the design of our study did not allow us to confirm fetal viability at the time of blood sampling. Nonetheless, we found that PAPP-A levels were very low irrespective of the length of time between blood sampling and the diagnostic scan, strongly suggesting that low levels of PAPP-A may precede miscarriage. These data, when combined with that from three small longitudinal studies published over a decade ago (17, 18, 23), collectively provide convincing evidence that low PAPP-A not only precedes miscarriage, but may prove to be a sensitive marker of 1st trimester pregnancy loss. In these previous studies, a total of fourteen cases are reported where PAPP-A levels were very significantly depressed weeks prior to a spontaneous miscarriage, mostly during the first trimester. Importantly, fetal viability was confirmed at the time when venous samples were drawn weekly. Compared with viable pregnancies, these reports found PAPP-A levels to be substantially lower in association with miscarriage compared to other markers including hCG (17, 23), SP1 (17), CA 125 (18), oestradiol (23) and progesterone (23). Interestingly, there was one case (17) where PAPP-A levels remained well under the 10th centile from 7 weeks until a spontaneous miscarriage occurred at 17 weeks of gestation, with ultrasound evidence of viability obtained up until 48 hours before fetal demise.

A predictive marker for miscarriage would be particularly valuable for clinical trials designed to assess potential therapies. To date such trials have been necessarily limited to women with recurrent miscarriage but a sensitive marker of miscarriage would allow the extension of such trials to women in the general pregnant population who were identified as having a high probability of pregnancy failure. Indeed, a particular strength of our study was that the women studied were unselected public hospital patients, and likely to be representative of the general pregnant population.

If PAPP-A is consistently depressed ahead of clinical pregnancy failure, then it raises exciting novel therapeutic possibilities. However, whether low PAPP-A does in fact consistently precede miscarriage and is a sensitive predictor of pregnancy loss remains unproven at present, requiring a definitive prospective study of large numbers of pregnancies specifically designed to answer this question.

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Table 1: Miscarriage cohort, group according to the date of sample collection relative to diagnostic ultrasound.

Hormone	**Timing of blood sample**		
	3 weeks before scan	**<3 but >1 wk before scan**	**Same day as scan**
	(n=11)	**(n=17)**	**(n=30)**
PAPP-A – MoM +/- 95% C.I.	0.11 (0.06-0.36)	0.16 (0.08-0.29)	0.19 (0.14-0.43)
P value : vs viable	<0.0001	<0.0001	<0.0001
hCG – MoM +/- 95% C.I.	0.64 (0.27-1.21)	0.55 (0.41-0.87)	0.41 (0.22-0.57)
P value: vs viable	0.065	=0.0002	<0.0001

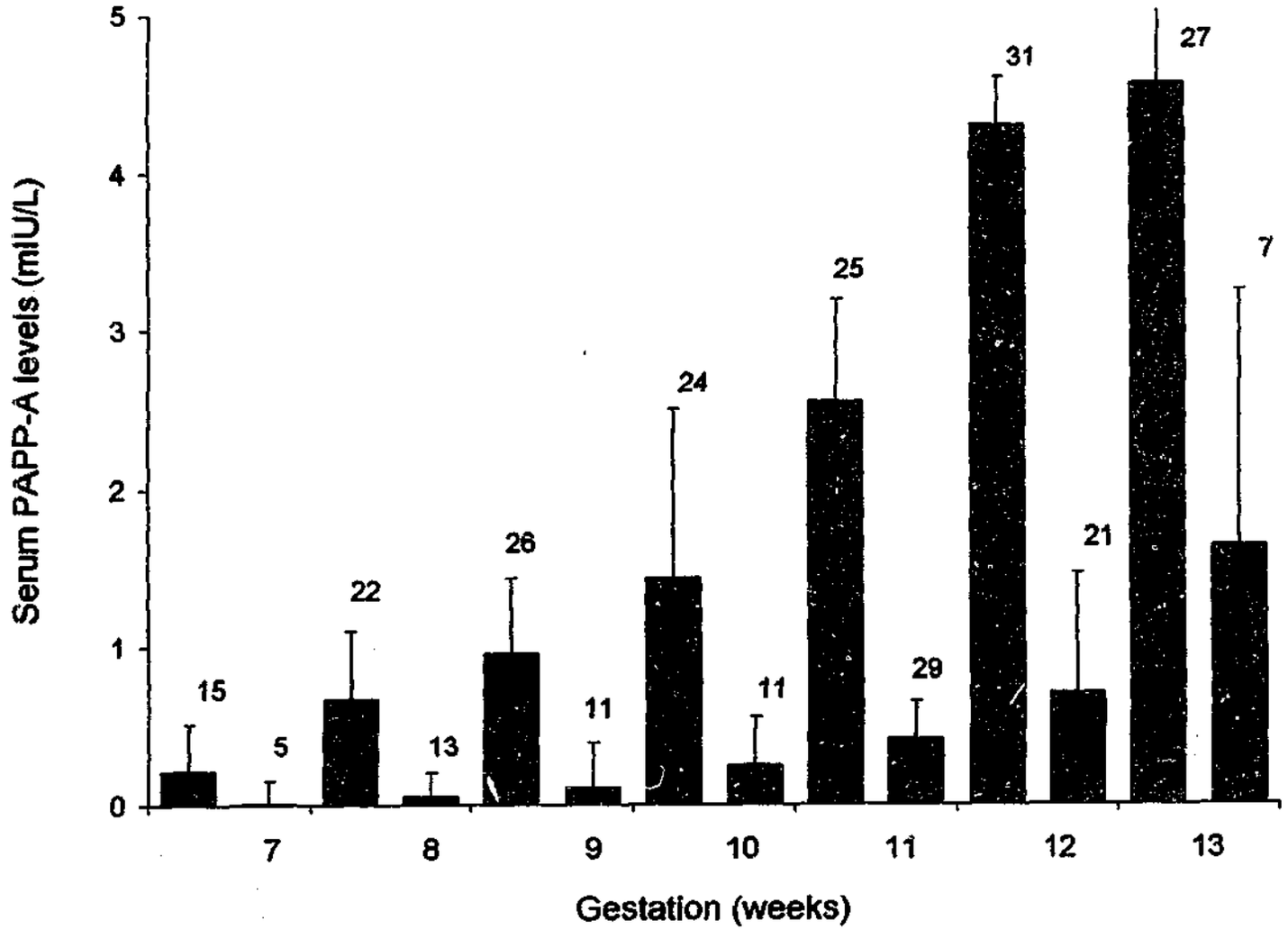

Figure 1: Median (±95% CI) PAPP-A levels across gestation in the viable ( ) and miscarriage ( ) groups. All comparisons at each gestation $p<0.007$ (Mann-Whitney U). Numbers in each group shown above bars. Upper 95% CI in the 13 week's gestation viable group beyond upper limit of graph.

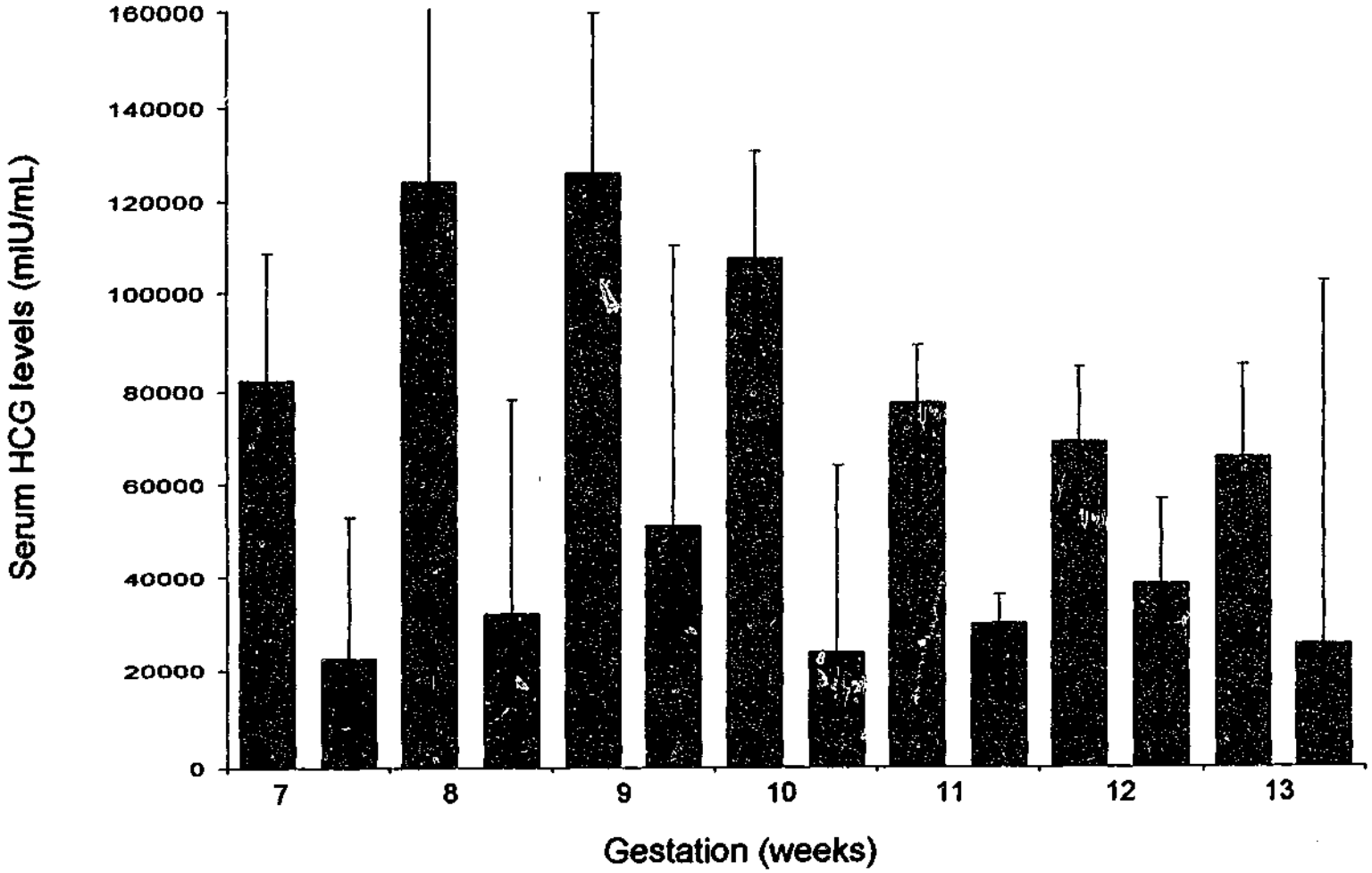

Figure 2: hCG (±95% CI) levels across gestation in the viable ( ) and miscarriage ( ) groups. All comparisons at each gestation $p<0.006$ (Mann Whitney U). Numbers in each group are the same as that shown in figure 1. Upper 95% CI in the 8 week's gestation viable group beyond upper limit of graph.

Manuscript 2C:

First trimester levels of inhibins and activin A in normal and failing pregnancies

Euan M Wallace, Budi Marjono, Katrina Tyzack, and Stephen Tong.

Declaration regarding the paper entitled:

First trimester levels of inhibins and activin A in normal and failing pregnancies (2C)

(Submitted)

Contributions to the work involved the following:

Name	% contribution	Nature of contribution
Euan M Wallace	37.5%	Supervised study, statistically analysed data and was a major contributor to the writing of the report.
Budi Marjono	12.5%	Helped with the pro αC assay, and contributed to the writing of the report
Katrina Tyzack	12.5%	Helped conceive the study and the collection of clinical samples, and contributed to the writing of the report
Stephen Tong	37.5%	Conceived the study, collected clinical samples, performed the inhibin A and activin A assays, and contributed to the writing of the report

Declaration by co-authors

The undersigned hereby certify that:

(31) they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;
(32) they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
(33) there are no other authors of the publication according to these criteria;
(34) potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journals or other publications, and (c) the head of the responsible academic unit; and
(35) the original data are stored at the following location(s) and will be held for at least five years from the date indicated below:

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[Please note that the location(s) must be institutional in nature, and should be indicated here as a department, centre or institute, with specific campus identification where relevant.]

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First trimester levels of inhibins and activin A in normal and failing pregnancies

Abreviated title: Inhibins and activin in miscarriage

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Key words: pregnancy, miscarriage, inhibin, activin, human chorionic gonadotrophin

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Summary

Objective Miscarriage is the commonest complication of human pregnancy. We undertook this study to assess whether inhibin A, pro-αC inhibin and/or activin A, products of the corpus luteum and placenta, might be useful in either the prediction or diagnosis of miscarriage.

Design Case control study.

Patients 98 asymptomatic women at 6-13 weeks gestation who subsequently had a miscarriage and 198 gestation matched women with a normal singleton pregnancy.

Measurements Maternal serum levels of inhibin A, pro-αC inhibin, activin A and human chorionic gonadotrophin (hCG) were measured.

Results Inhibin A, pro-αC and hCG, expressed as multiples of the normal median, MoM, (±95% confidence intervals), in the miscarriage cases were significantly lower than in the viable controls, 0.56 (0.48-0.69) MoM vs 1.00 (0.98-1.13) MoM, 0.55 (0.51-0.84) MoM vs 1.0 (0.86-1.22) MoM and 0.34 (0.23-0.36) MoM vs 1.00 (0.94-1.08) MoM, respectively ($P<0.0001$ for all). Of the three analytes, hCG was the most discriminating between cases and controls. Levels of activin A in the miscarriage cases were not significantly different from controls, 0.96 (0.86-1.07) vs 1.0 (0.95-1.08).

Conclusions These data suggest that inhibin A, pro-αC inhibin and activin A will not be useful in either the prediction or diagnosis of early pregnancy miscarriage.

Introduction

In human pregnancy, the placenta is the major source of inhibins and activins in the maternal circulation (reviewed by Tong et al. 2003), although the corpus luteum is a significant source of both pro-αC inhibin (Illingworth et al. 1996, Rombauts et al. 1996, Lahiri et al. 2003) and inhibin A (Illingworth et al. 1996, Treetampinich et al. 2000) prior to the luteal-placental shift. While the functions of these proteins in pregnancy remain uncertain, we and others have previously shown that maternal levels of inhibin A, pro-aC inhibin and activin A, the major circulating forms of inhibins and activins in pregnancy (Fowler et al. 1998, Thirunavukarasu et al. 2001), are altered in conditions associated with abnormal placental function such as fetal Down syndrome (Wallace et al. 1995, Aitken et al. 1996), pre-eclampsia (Petraglia et al. 1995, Muttukrishna et al. 1997, Manuelpillai et al. 2001) and fetal growth restriction (Wallace et al. 2003). Indeed, there are a large number of published studies exploring the biology of inhibins and activins in normal and pathological pregnancy, particularly with the aim of developing diagnostic or predictive tests for clinical application (Ledger 2001, Tong et al. 2003).

However, the most common pregnancy complication of all is spontaneous miscarriage, affecting about 10-15% of all pregnancies (Wilcox et al. 1988). Therefore, it is perhaps surprising that to date there have been only limited published data on inhibins in association with the early failure of spontaneous pregnancies (Illingworth et al. 1996, Phipps et al. 2000, Muttukrishna et al 2002). In each of these three studies maternal serum inhibin A levels in women presenting with signs and symptoms of a miscarriage (abdominal pain and/or vaginal bleeding) were significantly lower in those who had a miscarriage confirmed, whether complete or

incomplete, compared to those women who had an on-going viable pregnancy. Illingworth *et al* (1996) also observed that pro-αC inhibin was similarly decreased while Muttukrishna *et al* (2002) reported that activin A levels were not altered. However, these latter two studies were of extremely limited numbers of pregnancies and so did not afford an assessment of the either utility of inhibin A or pro-αC inhibin as clinical markers of miscarriage. In contrast, the study by Phipps and her colleagues (2000) was much larger, involving 98 viable and 122 non-viable pregnancies. The authors demonstrated that while inhibin A levels in women with a miscarriage were, on average, decreased by approximately 80% compared to viable pregnancies, inhibin A added nothing to the measurement of progesterone and human chorionic gonadotrophin (hCG) and as such did not have sufficient diagnostic utility. Levels of pro-αC were not reported.

A critical aspect of these three studies is that all of the women studied were recruited subsequent to their presentation with symptoms of miscarriage. To date there has been no study of the utility of inhibins and activins as predictors of early pregnancy loss in women with apparently normal pregnancies. We undertook this work to explore whether levels of inhibin A, pro-αC inhibin and activin A were altered in women with a spontaneous pregnancy who subsequently had a miscarriage, exploring whether these proteins may have utility in the prediction and/or diagnosis of spontaneous early pregnancy loss.

Methods

Stored sera from 98 women who had a first trimester miscarriage (cases) and 198 women with a normal singleton pregnancy (controls), which had been prospectively collected between 1999 and 2001 as part of a Down syndrome screening program, were retrieved for the measurement of inhibin A, pro-αC inhibin, activin A and hCG. All women attending our institution are offered screening for Down syndrome and so the women from whom the samples were collected are representative of our general obstetric population. At the time of collection all samples were centrifuged at 3500 rpm for 15 minutes at 4°C. The serum was then stored at –20°C until assayed. Gestational age on the day of blood collection was calculated from a 1st trimester ultrasound scan for control pregnancies and from the last menstrual period among the miscarriage group. (For the control pregnancies, gestational age did not differ if calculated by ultrasound or last menstrual period). Importantly, there was no clinical evidence of miscarriage (eg vaginal bleeding, abdominal pain) at the time of blood collection in any of the miscarriage cases. The controls were matched with the miscarriage cases for gestation at blood collection (completed week of pregnancy) and duration of storage by selecting approximately 10 controls from each completed gestational week (6-13 weeks) from the first quarter of each year (1999-2001).

Three subgroups of miscarriage samples were also identified: 12 women who had had blood taken at least 3 weeks before an ultrasound scan diagnosed a non-viable pregnancy, 17 women who had had blood taken between 1-3 weeks before the diagnostic ultrasound and 31 patients whose blood had been taken on the day of the scan. In the remaining 38 women with a miscarriage, the interval between blood collection and miscarriage was not known.

The Down syndrome screening program, a research program called the FaST Study, was approved by the Monash Medical Centre Human Research and Ethics Committee.

Total activin A, pro-αC inhibin (Oxford Bioinnovations, Oxford, UK) and inhibin A (Diagnostic Systems Laboratories, Texas, USA) were measured using commercial enzyme-linked immunosorbent assays according to the manufacturers' protocols. The limit of sensitivity for activin A was <100 pg/ml, 15.6pg/mL for pro-αC and 3.9 pg/ml for inhibin A. A commercial automated ELISA system (Dimension: Dade Behring, Detroit, USA) was used to measure hCG, which had a limit of sensitivity of <1 mIU/mL. The inter and intraplate coefficients of variation for all four assays were <10%.

Since levels of all four analytes change with gestation, to allow a comparison of analyte levels between miscarriage cases and controls normal median levels of each analyte were calculated for each completed week of pregnancy from the normal controls and multiples of the median (MoM) derived for both controls and cases. In addition, levels of the four analytes are not normally distributed. Correlations between analytes were therefore performed after suitable transformation to approximate Gaussian distribution (logarithmic transformation for inhibin A, activin A and hCG and square root transformation for pro-αC inhibin).

Results

In the normal viable controls median inhibin A, pro-αC inhibin, activin A and hCG levels changed significantly across the eight weeks of gestation studied ($p<0.0001$ for all analytes, Kruskal-Wallis, table 1 and figure 1). Levels of inhibin A, activin A and hCG increased to a peak at nine to ten weeks and fell thereafter. Levels of pro-αC were more variable but generally increased to a broad peak at 8 to 11 weeks, falling thereafter. Figure 1 summarises the levels of the four analytes in cases and controls across all weeks of pregnancy studied. Levels of inhibin A and hCG were consistently lower in the miscarriage samples than in controls. Pro-αC was lower in the miscarriage cohort only at 8 weeks gestation and was significantly higher than controls at 12-13 weeks. There were no differences in activin A levels between the two groups. Grouping all samples across gestations, median (95%CI) MoM levels of inhibin A in the 98 miscarriage cases were significantly lower than in the 198 viable controls, 0.56 (0.48-0.69) vs 1.00 (0.98-1.13), respectively, $p<0.0001$ (Mann-Whitney U test, table 2), as they were for pro-αC, 0.55 (0.51-0.84) vs 1.0 (0.86-1.22) and hCG, 0.34 (0.23-0.36) vs 1.00 (0.94-1.08) ($P<0.0001$ for both, Mann-Whitney U test, table 2). Levels of activin A in the cases were not significantly different from controls, 0.96 (0.86-1.07) vs 1.0 (0.95-1.08), respectively; $p=0.4$ (Mann-Whitney U test, table 2).

Table 2 summarises the inhibin A, pro-αC inhibin, activin A and hCG levels in the miscarriage cohorts, according to the interval between sampling and date of the diagnostic scan. As the interval between sample collection and diagnosis of miscarriage increased the median inhibin A MoM increased, becoming similar to control levels 3 weeks distant from diagnosis. Levels of pro-αC inhibin were lowest in the miscarriage group sampled 3 or more weeks prior to the diagnosis. Activin A in

the miscarriage groups were similar to controls irrespective of the interval between sampling and diagnosis. Levels of hCG were significantly lower than the viable controls in all groups with levels trending upwards as the interval between sampling and diagnosis increased.

Table 3 describes the correlations between the four analytes in the viable controls and the miscarriage cases. Only samples that had had all four analytes measured were included in this analysis. In general inhibin A, activin A and hCG were significantly correlated in both cases and controls. Pro-αC inhibin was only weakly but significantly correlated with inhibin A in the viable controls but with inhibin A and hCG in the miscarriages. Correlations within each of the miscarriages sub-cohorts were similar (data not shown).

Table 4 shows the number of miscarriages and viable pregnancies that would be detected by inhibin A, pro-αC inhibin or hCG at different MoM thresholds. As a single marker, hCG afforded the best discrimination between failing and viable pregnancies

Discussion

The ontogeny of hCG, inhibin A, pro-αC inhibin and activin A across the narrow window of six to thirteen weeks in the normal viable pregnancies in this study confirms previous reports (Tovanabutra et al. 1993, Muttukrishna et al. 1995, Birdsall et al. 1997, Wallace et al. 1997, Fowler et al. 1998, Tong et al. 2003). More importantly, in this study we have demonstrated that maternal serum levels of inhibin A and pro-αC, but not activin A, are significantly decreased in association with early spontaneous miscarriage. That levels of hCG were decreased to a greater extent than inhibin A or pro-αC argues against either of these proteins finding a role as a useful marker of early pregnancy outcome. Nonetheless, the changes in these proteins in relation to the timing of the miscarriage and the relationships between the proteins do offer further and useful insights into their biology in early pregnancy.

This is the largest study of inhibins in early pregnancy failure yet described. However, we believe that the major strength of the study is that all blood samples were collected from asymptomatic women at varying times relative to the miscarriage. This affords, for the first time, an assessment of the potential value of inhibins both in the prediction and diagnosis of spontaneous pregnancy miscarriage. On the other hand, both a limitation, and a strength, of our study design is that there was no evidence of a live pregnancy at the time of sampling in those women who were sampled more than week before the ultrasound diagnosis of miscarriage. This is a weakness because presumably some of these women had already a non-viable pregnancy when blood was collected but they had not yet experienced any bleeding or pain. Equally, some of these women are likely to have had a live fetus at the time of sampling, particularly those in whom blood was collected three or more weeks prior to the ultrasound. This

design is also a strength because it reflects actual clinical practice in that women present for confirmation of their pregnancy before an ultrasound has been undertaken and so allows evaluation of the predictive value of inhibin. Further, the women who were sampled on the day of diagnosis afford an evaluation of the diagnostic accuracy of inhibins in asymptomatic women.

While this is the first such study in normal women with a spontaneous pregnancy, others have previously reported levels of inhibins in women undergoing assisted reproduction, exploring the utility of inhibin as an early predictive marker of success (Norman et al. 1993, Lockwood et al. 1997, Treetampinich et al. 2000). Immunoreactive inhibin (ir-inhibin) levels were found to be lower, on average, in women who conceived after ovarian stimulation but who subsequently miscarried compared to similar women who had a successful pregnancy. However, the wide variation in ir-inhibin levels between individuals was such that inhibin was not sufficiently predictive of pregnancy outcome (Norman et al. 1993). In women undergoing *in vitro* fertilisation and embryo transfer (IVF-ET) inhibin A levels are also significantly lower in those who have a preclinical biochemical pregnancy compared to those with ultrasound evidence of an intrauterine pregnancy (Lockwood et al. 1997, Treetampinich et al. 2000). Neither of these studies though were large enough to provide estimates of the utility of inhibin A as predictive marker, despite the suggestion that inhibin A may be an earlier predictor of an unsuccessful biochemical pregnancy than hCG (Lockwood et al. 1997). In addition, while it was reported in one study that inhibin A levels were lower in women who have an intrauterine pregnancy confirmed following IVF-ET but who subsequently miscarry compared to similar women whose pregnancy is successful (Lockwood et al. 1997), this finding was not supported in a subsequent study (Treetampinich et al. 2000). Our

data suggest that inhibin A levels are indeed lower overall in women who have failed intrauterine pregnancy but that this is mainly due to women who were sampled at the time of the ultrasound diagnosis, consistent with previous findings in women with a symptomatic miscarriage (Illingworth et al. 1996, Phipps et al. 2000, Muttukrishna et al. 2002). In contrast, levels of inhibin A are not decreased in women who were sampled three or more weeks before a miscarriage. Thus, while inhibin A may be of some diagnostic value our results suggest that it will not usefully predict subsequent miscarriage in a currently viable pregnancy. This supports a previous study of ir-inhibin levels in early pregnancy in a cohort of women with recurrent miscarriage (France et al. 1996). Nonetheless, more recently it was reported that inhibin A, rather than total ir-inhibin, is decreased at 6-7 weeks gestation in a small cohort (n=9) of women with recurrent miscarriage who miscarried again compared to similar women who subsequently had a live birth (Muttukrishna et al. 2002). In that study levels of inhibin A were not different between the two groups of women at 8-12 weeks gestation. While the gestation at which the miscarriage occurred was not detailed the blood samples taken at 8-12 weeks were obviously collected more proximate to the miscarriage than the 6-7 week samples. That inhibin A levels were similar to the viable pregnancies at this time but were lower earlier is opposite to our finding that the more proximate the blood collection is to the miscarriage the lower inhibin A levels are. This apparent difference between the studies may arise from the very small number of pregnancies studied by Muttukrishna and her colleagues (2002). It is also possible that women with recurrent miscarriage have different inhibin profiles than women with a normal pregnancy history. Further prospective evaluation of a larger cohort of women with recurrent miscarriage would clarify this but currently the weight of evidence argues against inhibin A being a useful predictor of miscarriage.

With regard the diagnostic value of inhibin A, Phipps and her colleagues (2000) found it to be less useful than either progesterone or hCG and of no additional value to these markers. Our data for inhibin A and hCG are in agreement with this suggesting that inhibin A will not be clinically useful in either the prediction or diagnosis of early pregnancy miscarriage.

Interestingly, some years ago Illingworth and his colleagues (1996) observed that levels of pro- αC inhibin reflected "pregnancy viability more closely than either inhibin A or hCG". Lockwood *et al* (1997) also reported lower pro-αC inhibin levels in women with a miscarriage following IVF-ET compared to those with a viable pregnancy. While we can confirm that pro-αC levels are reduced, by almost 50%, in women with a miscarriage, overall these inhibin forms do not appear to be a better discriminator than hCG and are not likely to add sufficiently to hCG or progesterone to be useful in the diagnosis of miscarriage. However, unlike inhibin A and hCG, levels of pro-αC inhibin were lowest in the women sampled most distant from the ultrasound diagnosis of miscarriage. We believe that this may be related to the likely sources of the different inhibins. Whereas the placenta is the major source of inhibin A and the only source of hCG, most pro-αC circulating in early pregnancy is thought to be derived from the corpus luteum (Illingworth et al. 1996, Rombauts et al. 1996, Lahiri et al. 2003). That pro-αC is not correlated with β-hCG, in either the viable or miscarriage pregnancies, but is weakly correlated with inhibin A, which is partly derived from the corpus luteum (Illingworth et al. 1996, Treetampinich et al. 2000), and has been shown by others to correlate strongly with progesterone (Illingworth et al. 1996), supports the suggestion that pro-αC is mainly a luteal product in early pregnancy. More direct evidence for this is afforded by the rising pro-αC levels

observed following luteal rescue (Illingworth et al. 1996) and the recent finding that in first trimester pregnancy termination, the interruption of luteal steroidogenesis by mifepristone decreases pro-αC levels but not inhibin A (Lahiri et al. 2003). Thus, our observation than pro-αC is profoundly decreased in women who subsequently miscarry but only in those sampled distant to the miscarriage (ie very early in pregnancy) suggests that luteal function may be deficient in these women and that, as is the case for progesterone as a diagnostic indicator of miscarriage (Phipps et al. 2000), markers of luteal function may be the best predictors of subsequent pregnancy outcome. Certainly, future prospective studies of predictors of pregnancy outcome should include assessments of luteal as well as placental function.

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Table 1: *Median (10^{th} – 90^{th} percentile)* maternal serum levels of inhibin A, pro-αC inhibin, activin A and hCG in 198 normal singleton *pregnancies* at 6-13 weeks' gestation.

Gestation	6 weeks	7 weeks	8 weeks	9 weeks	10 weeks	11 weeks	12 weeks	13 weeks
No. of samples	6	17	25	27	30	28	35	30
Inhibin A (pg/mL)	117.2	136.3	315.8	348.6	221.2	224.8	223.6	194.8
	(98.4-514.2)	(83.0-376.8)	(211.2-776.0)	(196.4-630.4)	(135.0-623.0)	(100.9-418.3)	(147.0-400.0)	(105.0-354.0)
Pro-αC* (pg/mL)	181.6	112.2	463.1	267.5	365.0	437.9	378.4	185.8
	(12.9-786.8)	(41.1-237.2)	(163.4-551.7)	(90.1-365.4)	(75.4-520.1)	(147.1-422.8)	(148.1-353.9)	(79.9-247.9)
Activin A (ng/mL)	0.38	0.61	1.08	1.07	1.46	1.29	1.26	1.42
	(0.26-1.57)	(0.38-1.06)	(0.56-1.75)	(0.64-2.06)	(0.66-3.11)	(0.76-1.93)	(0.8-2.4)	(0.8-2.19)
hCG (pg/mL)	70300	82100	121500	133400	98850	75200	68900	62450
	(26868 - 120357)	(32694 – 143832)	(87534 – 254362)	(67100 – 186070)	(57200 – 166711)	(40120 – 141912)	(35800 – 119200)	(30265 – 13826)

*185 control samples were available for pro-αC measurement.

Table 2: Maternal serum levels of inhibin A and activin in 198 viable pregnancies and 98 miscarriages at 6-13 weeks' gestation.

	All viable pregnancies	All miscarriages	Miscarriage cases sampled ≥3weeks before diagnosis	Miscarriage cases sampled <3wks and >1 wk before diagnosis	Miscarriage cases sampled at time of diagnosis
No. of samples	198	98	12	17	31
Inhibin A MoM (95% CI)	1.00 (0.98-1.13)	0.56 (0.48-0.69)	1.05 (0.52-1.64)	0.66 (0.58-1.08)	0.80 (0.47-0.91)
Pro-αC MoM (95% CI)	1.00* (0.86-1.22)	0.55** (0.51-0.84)	0.14 (0.09-1.14)	0.96 (0.48-1.64)	0.79 (0.53-1.20)
Activin A MoM (95% CI)	1.00 (0.95-1.08)	0.96 (0.86-1.07)	0.93 (0.69-1.31)	0.94 (0.71-1.41)	1.2 (1.12-1.31)
hCG MoM	1.00 (0.94 – 1.08)	0.34*** (0.23-0.46)	0.64 (0.36-1.22)	0.51 (0.47-0.66)	0.39 (0.19-0.49)

*185 control and **94 miscarriage samples were available for pro-αC measurement. ***96 miscarriage samples were available for hCG measurement

Table 3: Relationships between inhibin A, pro-αC inhibin, activin A and hCG in 184 normal pregnancies and 92 miscarriages.

	Viable pregnancies		Miscarriages	
Correlation	R value (95%CI)	significance	R value (95%CI)	significance
log(inhibin A MoM) v log(activin A MoM)	0.3 (0.16-0.43)	<0.0001	0.38 (0.19-0.54)	0.0002
log(inhibin A MoM) v sqrt(pro-αC inhibin MoM)	0.19 (0.04-0.32)	0.011	0.27 (0.07-0.45)	0.01
log(inhibin A MoM) v log(hCG MoM)	0.44 (0.32-0.55)	<0.0001	0.63 (0.49-0.74)	<0.0001
sqrt(pro-αC inhibin MoM) v log(activin A MoM)	-0.01 (-0.6-0.13)	0.86	0.14 (-0.06-0.34)	0.17
sqrt(pro-αC inhibin MoM) v log(hCG MoM)	-0.03 (-0.17-0.12)	0.72	0.32 (0.13-0.5)	0.0015
log(activin A MoM) v log(hCG MoM)	0.28 (0.15-0.41)	<0.0001	0.25 (0.05-0.43)	0.016

Table 4: Number (percentage) of viable pregnancies and miscarriages identified with given inhibin A, pro-αC inhibin and hCG MoMs above different arbitrary levels.

MoM	Inhibin A number (%) of viable pregnancies	Inhibin A number (%) of miscarriages	Pro-αC inhibin number (%) of viable pregnancies	Pro-αC inhibin number (%) of miscarriages	hCG number (%) of viable pregnancies	hCG number (%) of miscarriages
< 0.1	0 (0)	3 (3)	35 (19)	14 (15)	0 (0)	19 (20)
< 0.2	0 (0)	11 (11)	47 (25)	28 (30)	0 (0)	35 (36)
< 0.3	1 (0.5)	22 (22)	50 (27)	32 (34)	1 (0.5)	46 (48)
< 0.4	6 (3)	34 (35)	55 (30)	38 (40)	8 (4)	54 (56)
< 0.5	12 (6)	45 (46)	60 (32)	43 (46)	18 (9)	60 (62)
< 0.6	22 (11)	54 (55)	67 (36)	48 (51)	28 (14)	68 (71)
< 0.7	37 (19)	55 (56)	75 (41)	52 (55)	38 (19)	74 (77)
< 0.8	62 (31)	59 (60)	80 (43)	56 (60)	55 (28)	79 (82)
< 0.9	80 (40)	66 (67)	85 (46)	58 (62)	76 (38)	80 (83)
< 1.0	97 (49)	72 (73)	91 (49)	68 (72)	95 (48)	84 (88)

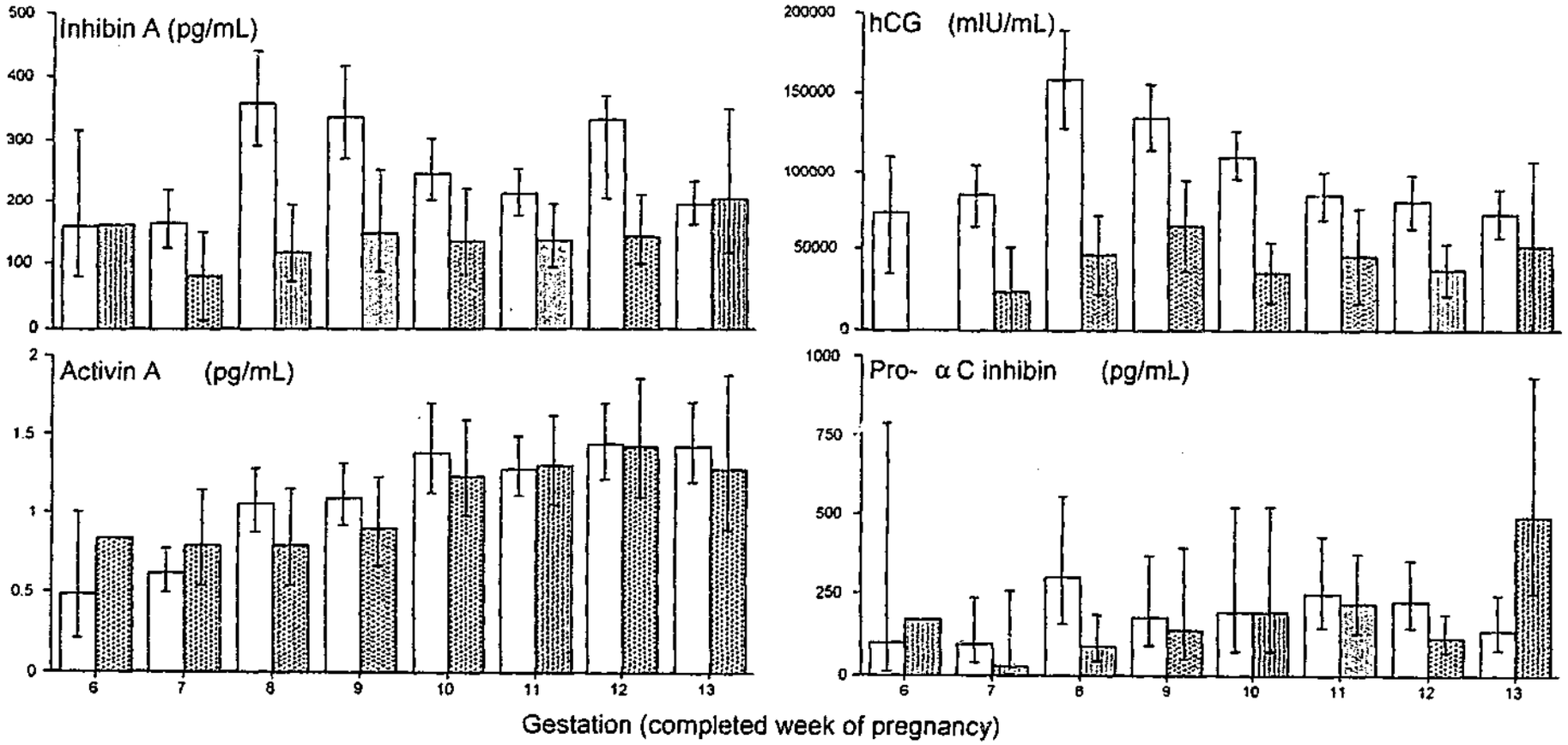

Figure 1. Mean (± 95% CI) maternal serum levels of inhibin A, human chorionic gonadotrophin (hCG), activin A and pro-αC inhibin at 6-13 weeks gestation in 198 women with a normal singleton pregnancy (☐) and 98 women who subsequently had a miscarriage (▒).

Section 3: Investigation of twinning in early pregnancy

Manuscript 3A

Survival of dizygotic twins in early pregnancy

Stephen Tong, Simon Meagher and Beverley Vollenhoven

Declaration regarding the paper entitled:

Sonography: Dizygotic twin survival in early pregnancy (3A)

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Contributions to the work involved the following:

Name	% contribution	Nature of contribution
Stephen Tong	60%	Conceived the study, collected clinical notes, statistically analysed the data, and was a major contributor to the writing of the report.
Simon Meagher	20%	Implemented study, helped collect clinical notes, and was a major contributor to the writing of the report.
Beverley Vollenhoven	20%	Supervised study, and was a major contributor to the writing of the report.

Declaration by co-authors

The undersigned hereby certify that:

(36) they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;
(37) they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
(38) there are no other authors of the publication according to these criteria;
(39) potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journals or other publications, and (c) the head of the responsible academic unit; and
(40) the original data are stored at the following location(s) and will be held for at least five years from the date indicated below:

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Name	Signature	Date
[1]Stephen Tong	[redacted]	22/1/04
[2]Simon Meagher		22/1/04
[1,2]Beverley Vollenhoven		21/1/04

other feedback, coupled with a subject's dynamic learning, can compensate for inaccuracies in the model to provide an easily and voluntarily adjusted control signal. Our results demonstrate that a simple mathematical approach, coupled with a biological system, can provide effective decoding for brain–machine interfacing, which may eventually help to restore function to neurologically impaired humans.

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Sonography

Dizygotic twin survival in early pregnancy

It has been suggested that losses of twin conceptuses in very early pregnancy are high, and that for every liveborn twin pair there are a further 10–12 twin pregnancies that end up as a singleton birth[1]. Here we show that in a group of women who had double-ovulated and conceived, the probability of the second egg also becoming fertilized and developing is 20–30% — which is comparable to the probability of conception and survival of a single conceptus[2]. We conclude that the presence of one embryo does not affect the development of its twin.

So far, no direct measure has been available of the proportion of double ovulations that lead to twins. We have obtained this information by using ultrasound to identify pregnant women with two corpora lutea, and correlating these with the number who had one or two fetuses (dizygotic twins) in early pregnancy. The corpus luteum is an endocrine organ that develops in the ovary at the site at which the egg was released, and can therefore act as an indicator of the number of ovulation events.

As only two small ultrasound studies have identified the corpus luteum in early pregnancy[3,4], we confirmed these findings in a larger series at our centre, where the ovaries of all pregnant women are routinely examined. We scan mainly low-risk pregnancies,

Table 1 Distribution of double-ovulation events

	Both left	*One in each*	*Both right*
Spontaneous	7	14	6
Iatrogenic	2	6	7
Unknown	3	3	0
Total	12	23	13

Forty-eight cases of double ovulation were identified by ultrasound scans of 504 women during early pregnancy. Of these cases, 27 were spontaneous, resulting in 9 sets of twins and 18 singletons; and 15 were induced, resulting in 3 twin pairs.

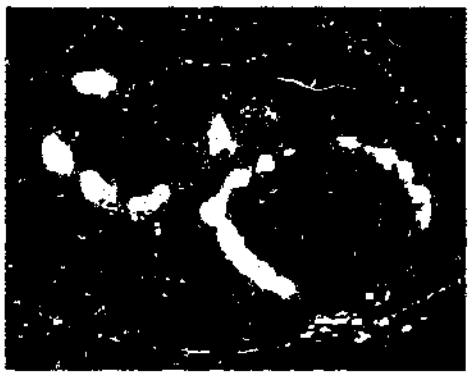

Figure 1 Doppler ultrasound of two corpora lutea in one ovary (signifying dual ovulation). The corpus luteum typically appears as an echo-filled cyst with a ring of peripheral vascularity. Colours reflect variations in blood flow, with yellow as the fastest.

as well as women who had assistance in conceiving. In scans of 504 women where both ovaries were seen in early pregnancy (5–9 weeks gestation), the corpus luteum could be identified in 94.6% of cases. Its mean diameter was 19.6 mm (±5.28 standard deviation). Single ovulations were distributed equally between the two ovaries, occurring on the left in 49.3% of cases.

There were 48 cases of double ovulation identified by these ultrasound scans (Fig. 1). Of these (Table 1), 27 were spontaneous, with 9 among this group conceiving twin pairs (30%) and the remainder singletons. Fifteen double ovulations were induced by clomiphene citrate, and among these there were three sets of twins (20%). We were unable to determine whether the dual ovulation was spontaneous or induced in the remaining six cases, of which three were twin pregnancies. Maternal age among those who had double-ovulated (32.5 years) was not significantly different from those who had had a single ovulation (30.7 years; $P = 0.07$, t-test). All sets of twins were of dichorionic and diamniotic placentation on ultrasound examination, which is consistent with dizygotic twinning.

We conclude that the presumption of huge losses of dizygotic twins in early pregnancy[1] is unfounded, as we would then have seen many more double ovulations with a singleton-pregnancy outcome (signifying an aborted twin). The probability of the second egg also becoming fertilized seems to be similar to that of one egg becoming fertilized in a singleton pregnancy[2]. The presence of one embryo therefore does not impede the development of its twin.

Our study does not, of course, eliminate the possibility that both twins might be lost at a higher rate than singletons. However, we do not believe that this would fit with our finding that the second egg has the same chance of developing as a singleton pregnancy once the first egg is fertilized.

The distribution of spontaneous double ovulations is consistent with a random spread of ovulation between left and right ovaries (Table 1). This suggests that the mechanism responsible for dual ovulation involves signalling from outside the ovary, rather than local intra-ovarian control[5], as we would then have seen more double ovulations from the same ovary.

We have confirmed that the corpus luteum can be readily identified in an early-pregnancy scan, enabling us to characterize a significant number of double ovulations in the human. To our knowledge the last attempt to do this was in 1794, when William Hunter observed after 400 dissections of pregnant uteri: "When there is one child, there is only one corpus luteum; and two in the case of twins. ...In some of these cases, there were two distinct corpus lutea in one ovarium."[6].

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Manuscript 3B

Determining zygosity in early pregnancy by ultrasound

Stephen Tong, Beverley Vollenhoven and Susan Haughey

Declaration regarding the paper entitled:

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Contributions to the work involved the following:

Name	% contribution	Nature of contribution
Stephen Tong	60%	Conceived the study, collected clinical notes, statistically analysed the data, and was a major contributor to the writing of the report.
Beverley Vollenhoven	15%	Helped collect clinical notes, and was a major contributor to the writing of the report.
Simon Meagher	25%	Supervised and implemented study, helped collect clinical notes, and was a major contributor to the writing of the report.

Declaration by co-authors

The undersigned hereby certify that:

(41) they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;
(42) they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
(43) there are no other authors of the publication according to these criteria;
(44) potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journals or other publications, and (c) the head of the responsible academic unit; and
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Determining zygosity in early pregnancy by ultrasound

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KEYWORDS: chorionicity; corpus luteum; twins; ultrasound; zygosity

ABSTRACT

Objectives First-trimester ultrasound can reliably determine chorionicity but not zygosity. We set out to investigate whether it may be possible to determine zygosity using ultrasound by noting the number of corpora lutea (CLs), structures which reflect ovulation. In the presence of a dichorionic twin pregnancy, the identification of one CL would suggest that twins are monozygotic whereas two CLs implies dizygosity.

Methods This was a retrospective analysis of predominantly spontaneous twin pregnancies presenting for an early pregnancy ultrasound at 5–8-completed weeks of gestation. Placentation was correlated with presumed zygosity as predicted by the number of CLs present.

Results Of 33 twin gestations, chorionicity was compatible in all cases with the predicted zygosity. In 15 cases one CL was seen and these were designated monozygotic. Of these, four were of monochorionic placentation and 11 dichorionic. The remaining 18 cases had two CLs and were presumed dizygotic; all were of dichorionic placentation.

Conclusion We propose a novel technique of zygosity determination during very early pregnancy which may have implications both clinically and in genetic research involving twins. However, this study requires further verification by comparing ultrasound results with DNA evidence taken after birth. Copyright © 2003 ISUOG. Published by John Wiley & Sons, Ltd.

INTRODUCTION

Monochorionic twin gestations are at the highest risk of pregnancy complications amongst all types of twinning[1]. For this reason, chorionicity is routinely assessed by ultrasound in twin pregnancies. However, while chorionicity can be determined with near 100% certainty in this way[2], zygosity can be predicted in only 55–65% of twin pregnancies by correlating chorion type with the sex of twins[1]. This is because same-sexed dichorionic twin gestations can be either monozygotic (Mz) or dizygotic (Dz). In contrast, all monochorionic twin gestations are Mz.

The corpus luteum (CL) can be visualized by transvaginal ultrasound in 95–98% of cases between 5 + 0 weeks and 8 + 6 weeks of gestation[3,4]. This high detection rate is not surprising given that it is highly vascular at this early stage in gestation and can be readily identified using power Doppler technology at low velocity settings. We therefore wondered whether zygosity could be determined by noting the number of CLs present. If need be, this could be correlated further against chorionicity. For instance, two CLs suggests Dz. However, if this was associated with monochorionicity (certain Mz twins), this would suggest that the predicted zygosity was incorrect.

We therefore set out to investigate the possibility of using ultrasound to determine zygosity by reviewing ultrasound scans of twin pregnancies and correlating the CL findings with chorionicity.

METHODS

We reviewed the findings of all transvaginal scans of twin pregnancies at 5 + 0 weeks to 8 + 6 weeks of gestation between 1998 and 2001 at our center. The center represents four dedicated obstetric and gynecological ultrasound practices, involving eight ultrasound systems. Scans were performed using an ATL 3000, 3500 or 5000 Ultrasound system (Phillips, Seattle, WA, USA). Once the CLs had been identified, color Doppler examination was used to identify peripheral vascularity and the maximum transverse diameter was recorded to determine CL size.

We then contacted referring doctors to confirm whether the pregnancy was spontaneous or otherwise.

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We excluded all twins conceived by *in-vitro* fertilization, but included twin pregnancies in which clomiphene citrate had been used to induce ovulation. The hospital research and ethics committee approved the study.

RESULTS

We identified 33 twin pregnancies between 5 + 0 and 8 + 6 weeks of gestation. Of these, 23 pregnancies were spontaneous, in six cases clomiphene citrate had been administered, and for the remaining four, the referring doctor was unsure.

Both ovaries were clearly identified in all cases. In 15 of the 33 twin pregnancies one CL was seen, and these were therefore designated as Mz; of these, four were monochorionic and 11 dichorionic. In the remaining 18 twin pregnancies two CLs were noted, and these were all dichorionic.

The mean CL size was 19.0 mm (± 5.73 SD). There was no significant difference in CL size between cases in which one CL was visualized compared with those in which two were visualized ($P > 0.2$; *t*-test).

DISCUSSION

We propose a novel method to determine zygosity in twin pregnancies as early as 5 + 0 weeks to 8 + 6 weeks of gestation. We found that chorionicity was in agreement with the predicted zygosity in all 33 of our cases.

We chose a gestational age of 5 + 0 to 8 + 6 weeks since within this range we have already established it is possible to confidently characterize the CL in 95% of cases[3]. Numbers in our series were quite low since we aimed to include primarily spontaneous twins, a relatively rare event. Our low numbers probably accounts for the fact that dichorionic Mz twins were seen more commonly than were monochorionic Mz twins, the reverse of what should be expected.

Whilst our findings are encouraging, they should be regarded as preliminary since we did not identify any twin pregnancies falsely assigned as Mz when they were in fact Dz, with a second CL not being identified. In addition, a small but unavoidable theoretical error rate (< 0.5%) will always remain with this method, even if ultrasound acquires perfect sensitivity in characterizing the CL. This is because in 3–4/1000 cases of double ovulation (the monozygotic twinning rate), one of the two eggs ovulated will have split, producing Mz twins instead of the predicted Dz twins. Our method clearly requires subsequent verification with a prospective study comparing ultrasound prediction of zygosity with DNA fingerprinting of twins after delivery.

There are many potential applications for our method of determining zygosity. First and foremost, twins have a right to know their zygosity[4–6], and it is likely that they use this information to shape their identities. Placental examination often cannot determine zygosity[7] and DNA fingerprinting is too expensive to be offered routinely[5]. Secondly, the level of obstetric risk of Mz twins who are dichorionic is still uncertain. A recent large epidemiological study attempting to address this question was inconclusive[1], largely because it was not possible to allocate zygosity to dichorionic same-sexed twins. It would be of interest both clinically and biologically to establish chorionicity and zygosity very early on during pregnancy and prospectively follow up these cases. We may discover whether twin genotype or the vascular anastomosis between the siblings is primarily responsible for the increased rate of pregnancy complications amongst monochorionic twins.

Lastly, it is becoming increasingly apparent that chorionicity, which reflects the timing of egg splitting, may be an important consideration in twin studies[5]. For instance, it has recently been found that dichorionic Mz twins split prior to X-chromosome inactivation whereas monochorionic Mz twins divide after this event[8]. The consequence is that dichorionic Mz twins may in fact be genetically more dissimilar to each other when compared with monochorionic Mz twins. Also, it is now apparent that placentation significantly influences fetal environment, as shown by the fact that monochorionic twins are lighter at delivery compared with dichorionic Mz twins[5]. It may therefore be important in future twin research studying nature versus nurture to correct for these significant genetic and environmental biases by distinguishing between the different types of identical twinning. The most practical way to achieve this may be to assign both chorionicity and zygosity at the same time during an early pregnancy ultrasound.

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Section 4: Corpora luteal diameters and miscarriage risk

Manuscript 4A:

Corpora luteal diameters from 5-13 weeks of gestation, and its association with miscarriage risk

Stephen Tong, Boon Ghee Chua, Beverley Vollenhoven, Roshan Shamon, Ruby Biezen, Carol Tong, Kate Tyzack and Simon Meagher.

Declaration regarding the paper entitled:

Corpora luteal diameters from 5 to 13 weeks of gestation, and its association with miscarriage risk (4A)

(submitted)

Contributions to the work involved the following:

Name	% contribution	Nature of contribution
[1,2] Stephen Tong	35%	Conceived, designed and supervised study, statistically analysed data, and was a major contributor to the writing of the report
[1]Boon Ghee Chua	12.5%	Helped collect clinical notes, and contributed to the writing of the report.
[1]Beverley Vollenhoven	10%	Helped design study, and was a major contributor to the writing of the report.
[1]Roshan Shamon	10%	Helped collect clinical case notes, and contributed to the writing of the report.
[1]Ruby Biezen	7.5%	Helped collect clinical case notes, and contributed to the writing of the report.
[3]Carol Tong	5%	Helped collect clinical case notes, and contributed to the writing of the report.
[1]Kate Tyzack	5%	Helped collect clinical case notes, and contributed to the writing of the report.
[1,2] Simon Meagher	15%	Supervised and implemented study, helped collect clinical case notes, and was a major contributor to the writing of the report.

<u>Declaration by co-authors</u>

The undersigned hereby certify that:

(46) they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;
(47) they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
(48) there are no other authors of the publication according to these criteria;
(49) potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journals or other publications, and (c) the head of the responsible academic unit; and

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Corpora luteal diameters from 5 to 13 weeks of gestation, and its association with miscarriage risk

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Key words:

Corpus luteum, miscarriage, ultrasound, measurement

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Abstract:

OBJECTIVE: The diameter of the corpus luteum (CL) may be directly proportional to the level of serum progesterone. The objective of this study was to describe the changes in CL diameters from 5+0 to 13+6 weeks, and to investigate a possible association between CL diameter and subsequent miscarriage.

METHODS: Observational study of 1913 women presenting for an early pregnancy ultrasound between 5+0 to 9+6 weeks, mostly to confirm viability, and 453 women presenting at 10+0 to 13+6 weeks for a nuchal translucency scan. All had a confirmed viable fetus present. We wrote to referring doctors to determine the subsequent miscarriage rate in a total of 409 patients at 5-9 weeks of gestation who had either averaged sized (40-60th centile), very small (<10th centile), or large (>90th centile) CL but an otherwise viable pregnancy confirmed at ultrasound between 5-9 weeks gestation.

RESULTS: The CL varied significantly in diameter across gestation ($p<0.0001$), reaching its maximal diameter of 19.7 mm at 6 weeks' gestation, remaining between 18.8-19.5 mm up until 8 weeks, then gradually decreasing in diameter to become 16.8mm by 13 weeks. There was a significantly increasing risk of miscarriage associated with decreasing CL diameter (5.9% for CL diameter >90th centile; 15.7%, 40-60th centile; 21.9%, <10th centile; $p=0.0089$, $\chi 2$ test for trend).

CONCLUSION: The CL expands to reach its maximal diameter around the time of the luteoplacental shift, then decreases in size from 9 weeks' gestation. Ultrasound assessment of the CL may be of value in stratifying miscarriage risk.

Introduction:

The corpus luteum (CL) is an endocrine gland vital in supporting the developing conceptus[1]. It can be readily identified by Doppler ultrasound as a ring of vascularity[2-5]. It plays a vital role in supporting early pregnancy and bold historical experiments have found that excising this structure before the 7th week of pregnancy always precipitates miscarriage[1]. Pregnancies generally continued however if the lutectomy was performed after the 7th gestational week probably because by this time, the placenta has become large enough to take over the endocrine role of the CL (luteoplacental shift).

The diameter of the corpus luteum (CL) measured by ultrasound may be a useful surrogate marker reflecting the level of luteal endocrine activity since it has been shown to be directly correlated to serum progesterone concentrations in several animal models[6]. Whilst studies of relatively limited numbers have described average CL diameters across early pregnancy pooled gestational age ranges[2,7], none have characterised the progressive changes in CL diameters across early pregnancy in large numbers.

Our centre routinely measures CL diameters using doppler ultrasound for all early pregnancy scans. We therefore set out to describe the variations in CL diameter across the second half of the first trimeste and to investigate whether an association existed between CL size at 5+0 to 8+6 weeks of gestation, and the risk of subsequent miscarriage.

Methods:

Data for this study was collected from four obstetric and gynaecological ultrasound practices which incorporates nine ultrasound systems. Scans were performed using an ATL 3000, 3500 or 5000 Ultrasound system (Phillips, Seattle Washington State, USA). We routinely measure CL diameter in millimeters using power doppler ultrasound in all gynaecological and early pregnancy scans. If the CL is not spherical, we average the diameters measured in two planes. Prior approval from our Human Research and Ethics Committee was obtained before we commenced this study.

Measurements of CL diameters across gestation

Data on CL diameter, gestation and maternal age was retrospectively collected from the records of 1913 pregnancy scans performed at 5+0 weeks to 9+6 weeks of gestation where a viable pregnancy was present. At this gestational age, we[3] and others[2] have previously demonstrated that the CL can be identified in over 95% of cases. The clinical indication for these scans provided by the referring doctor was available in a subgroup of 1053 cases (55%). The gestational sac and embryo were measured in order to determine gestational age. Since we electronically record whether the patient had conceived with the assistance of *in vitro* fertilization, we were able to exclude all such cases in this study.

As we do not routinely collect data on CL diameters from 10 weeks onwards, we undertook a prospective study over 6 months where we measured CL diameters in 453 women at 10+0 weeks to 13+6 weeks of gestation presenting for a nuchal translucency scan to assess their risk of having a Down's Syndrome baby. The accuracy of ultrasound in identifying the CL at this gestational age range has not been

previously reported and we found that we were unable to locate the CL in 96 (24%) of cases where both ovaries were seen. These were excluded from final analysis.

Miscarriage study

We commenced this study after CL diameters had been retrospectively recorded from 1200 viable pregnancies between 5+0 weeks to 8+6 weeks' gestation. We wrote to referring doctors, requesting information regarding subsequent pregnancy outcomes on all patients who had a CL of around average diameter (18-19mm diameter or 40-60th centiles; n=190), a very small CL (≤13mm which is approximately below the 10th centile; n=110), or a large CL (≥25mm, or >90th centile; n=109 patients). Both the investigator preparing the questionnaire and the referring practitioner were blinded as to whether the CL was considered to be large or small.

Results:

Study cohort

The mean age of the entire cohort was 31.2 years (± 5.1 std dev). A review of clinical indications for the 5+0 to 9+6 week ultrasound scans amongst a sample of 1053 cases found that in 78% of cases, there were no clinical concerns written on the referral that the fetus was lost (i.e. viability or dating scan). Of the remainder, there were clinical symptoms leading to the request for a scan to determine viability.

CL diameters

The CL varied significantly ($p<0.0001$ ANOVA) from 5-13 weeks of gestation, reaching its largest diameter of 19.7 mm at 6 weeks of gestation (Table 1). It then ceased to enlarge, remaining at roughly the same diameter until 8 weeks. From 9 weeks onwards, CL diameters decreased with increasing gestation, reaching 16.8mm by 13 weeks of gestation.

Miscarriage study

Outcomes were available in 57% of cases (n=235). Of the remainder, either doctors were unsure (19%) or did not respond (23%). We then excluded a further 14 cases where the women chose to terminate their pregnancy.

There was a significant association between CL size at 5+0 to 8+6 weeks gestation and increasing risk of miscarriage ($p=0.0089$ $\chi 2$ test for trend, see table 2). Those women with a small CL at 5 weeks (+0 days) to 8 (+6 days) weeks of pregnancy had a 3.7 times increased risk of miscarriage ($p=0.0082$) compared to those with a large CL. In pregnancies with a large CL, there was a trend towards a lower miscarriage

rate than those with a CL size around the mean, although this was not significant (p=0.059). The mean gestational age was 6.5 (std dev's ranged from ±0.89 to ±0.97) weeks for all three groups (ANOVA; p=0.98).

Discussion:

In this study, we have reported data from a large number of observations describing the changes in CL diameters from 5-13 weeks of pregnancy. We have also found an increased risk of miscarriage with decreasing CL diameters measured at 5+0 to 8+6 weeks of pregnancy.

A recent prospective study comparing ultrasound CL measurements made with subsequent measurements of the CL obtained surgically afterwards has confirmed the fact that ultrasound can accurately assess CL dimensions[5]. Furthermore, plasma progesterone concentrations have been shown to correlate with luteal diameters in heifers[8], both pregnant and non pregnant llamas[9] and the mare[10]. This suggests that the ultrasound measurements of CL diameters may be a useful surrogate marker of luteal endocrine activity. However, human studies exploring such a correlation between CL volume and progesterone concentrations have so far been inconclusive. Whilst one study[5] failed to find a correlation, another did[11]. A third study by Miyazaki *et al*[12] found a similar pattern of change in CL volume and progesterone concentrations in the luteal phase, although the method of statistical evaluation they used did not show this to be significant. Importantly, all of these studies have been small. Given the weight of animal evidence and the potential applications of using CL diameters as a surrogate marker of biochemical function[13], a proper study sufficiently powered to answer this question would be a worthwhile exercise. This was obviously not possible in the current study which was mainly retrospective in design.

We have observed that the CL during pregnancy attains its largest size at 6 weeks gestation, just prior to the luteoplacental shift[1]. This is interesting given that maternal

serum human chorionic gonadotrophin (hCG) concentrations are known to continue increasing exponentially for another few weeks, reaching peak levels at 10 weeks gestation[14]. It would be interesting to speculate why the CL ceases to expand in spite of the continuing hCG rise. Possibly, the CL is already maximally stimulated by hCG, or there may be an acute trigger around 7 weeks of pregnancy which decreases cellular responsiveness to hCG. The decrease in CL diameter from around 9 weeks onwards coincides with the beginning of the decline in maternal serum hCG levels[14].

We have found that the risk of miscarriage varies greatly with differences in CL diameter measured during the middle of the first trimester. Assuming that the CL is roughly spherical, the volume of large CL's as defined in our study should be just over seven times that of small CL's. Interestingly, we found that pregnancies associated with a large CL diameter might have a better chance of success than those with a CL diameter equal to the mean, although this just failed to reach significance. Those with average CL diameters incurred a miscarriage risk of 15.7%. This is only slightly higher than recently reported miscarriage rates of 10.3% and 12% observed by others after a positive 6 week viability gestation scan in large studies examining spontaneous[15] and IVF singleton cohorts[16] respectively.

A weakness of our miscarriage study was that we were unable to determine pregnancy outcome in a significant number of cases where it was sought. However, this was countered by the fact that doctors from whom we requested information were blinded as to whether we considered the CL of their patients to be large or small.

Frates and colleagues[7] failed to find an association in a small prospective study comparing CL diameters in 50 patients who subsequently miscarried to 151 others who had a successful pregnancy. We had larger numbers and used a different study design, chasing outcomes of only those with a small, average or large CL, taken from a large pool of cases. We anticipated that this approach would accentuate any possible associations between failed pregnancy and CL size.

The reason for the association between CL diameter and miscarriage is unclear. We consider it likely that a small CL reflects a weakened luteotrophic signal due to a faltering embryo/placenta. The alternate explanation is that a small CL may reflect a luteal phase defect which may be the primary pathology initiating pregnancy loss. Should this be true, then the intriguing possibility exists of salvaging some pregnancies by providing progesterone support for women with small CL's. Whether a small CL may be contributing to miscarriage or an epiphenomenon requires further investigation such as correlative studies measuring maternal serum hormones progesterone and hCG, then prospectively determining pregnancy outcomes.

Our data gives rise to the possibility that ultrasound measurements of CL diameter may be a useful predictive marker of miscarriage. The design of the study did not allow us to evaluate its characteristics as a diagnostic marker. However, our current data suggests that whilst it may well prove to have good sensitivity, it is probable that the specificity may be somewhat limited. Even if this is shown to be the case, its performance as a marker might be enhanced by combining it with other ultrasound characteristics. For instance a lower peak systolic velocity in the proximal thecal arteriole at 5-7 weeks of gestation, the main feeding vessel to the CL, has been

associated with an increased risk of miscarriage in a preliminary study of 31 patients[13]. Also, small difference (<5mm) between the mean sac diameter and crown rump length measurement has also been associated with an increased risk of miscarriage[17], although its accuracy as a lone marker may be somewhat limited[18]. Alternatively, CL diameters may be combined with recently proposed biochemical markers of miscarriage[19,20] as a sensitive multimodal marker of future pregnancy loss.

A predictor of miscarriage may be clinically useful for counseling for selected cases including recurrent miscarriages, and in future could be a useful research tool to target potential therapies aimed at averting pregnancy loss[21,22]. Whether CL diameters can in fact accurately predict miscarriage either alone or in combination with other markers will require prospective verification.

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Table 1: Corpora luteal diameters from 5-13 completed weeks of pregnancy. P<0.0001, ANOVA.

Gestation (weeks)	Number of Observations	Mean CL diameter (± std. Dev.)
5	189	18.19 (4.4)
6	668	19.71 (5.4)
7	509	19.12 (4.9)
8	372	19.53 (6.0)
9	175	18.86 (5.4)
10	40	18.82 (4.6)
11	45	18.4 (5.2)
12	217	17.43 (4.4)
13	55	16.76 (3.7)

Table 2: Corpora luteal diameters measured at 5 +0 to 5 +8 weeks' gestation, and the risk of subsequent miscarriage. p= 0.0089, $\chi 2$ test for trend.

Study group	Miscarriage rate - % (Total numbers)	Comparisons of miscarriage rates (P value)
Small CL's (≤13 mm)	21.9% (14/64)	Vs large: 0.0082
CL's around the mean (18 -19mm)	15.7% (14/89)	Vs large: 0.059 Vs small 0.33
Large CL's (≥25mm)	5.9% (4/68)	-

Section 5: Activin A as a marker of later pregnancy complications

Manuscript 5A:

Fetal activin A: associations with labour, umbilical artery pH and neonatal outcome

Stephen Tong, Val Egan and Euan M Wallace.

Declaration regarding the paper entitled:

Fetal activin A: associations with labour, umbilical artery pH and neonatal outcome (5A)

(submitted)

Contributions to the work involved the following:

Name	% contribution	Nature of contribution
Stephen Tong	55%	Helped with study design and the collection of clinical samples, performed the Activin A assay, statistically analysed data and was a major contributor to the writing of the report.
Val Egan	15%	Helped with the collection of clinical samples and clinical case notes, and contributed to the writing of the report.
Euan M Wallace	30%	Conceived the study, helped with study design, and was a major contributor to the writing of the report.

Declaration by co-authors

The undersigned hereby certify that:

(51) they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;
(52) they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
(53) there are no other authors of the publication according to these criteria;
(54) potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journals or other publications, and (c) the head of the responsible academic unit; and
(55) the original data are stored at the following location(s) and will be held for at least five years from the date indicated below:

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Name	Signature	Date
[1]Stephen Tong	[redacted]	22/1/04
[2]Val Egan		22/1/04
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Fetal activin A: associations with labour, umbilical artery pH and neonatal outcome.

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Abstract

Objective: To define the ontogeny of umbilical artery activin A at term and to evaluate activin A as a potential marker of perinatal hypoxia.

Design: A cohort study.

Setting: A university teaching hospital delivery suite.

Population: A convenience sample of 141 term pregnancies.

Methods: At delivery, umbilical artery and vein bloods were collected for blood gas measurements and subsequent measurement of activin A. Activin A levels were correlated with blood gas measurements and with labour and neonatal outcomes.

Results: The median (95% CI) umbilical arterial activin A level at delivery was 1.38 (1.34-1.70) ng/ml. Levels varied significantly across gestation (p=0.03), increasing from 36 to 38 weeks, thereafter decreasing to a nadir at 41 weeks). In 60 matched samples, the median (95%CI) venous and arterial activin A levels were 0.89 (0.81-1.06) ng/ml and 1.38 (1.21-1.61) ng/ml, respectively (p<0.0001). Mean umbilical arterial pH was 7.20 (7.06-7.38; 5-95th centiles) and was not significantly correlated with $\log_{10}$ activin A (r = - 0.01; p=0.68). Compared to healthy controls, there was no difference in arterial activin A in neonates identified as having suffered significant intrapartum asphyxia (p=0.96). Fetal activin A levels were significantly lower in cases delivered by emergency caesarean section for complications during the first stage of labour compared to cases delivered vaginally (p=0.003).

Conclusions: Umbilical artery activin A does not appear to be a sensitive marker of fetal oxygenation or risk of hypoxic ischaemic encephalopathy.

Introduction:

Activin A is a dimeric glycoprotein, of the TGF-β family, secreted by the feto-placental unit into the maternal circulation in increasing amounts as pregnancy advances[1,2]. Maternal serum levels of activin A are increased in pregnancies complicated by pre-eclampsia[3] and fetal growth restriction[4]. Activin A is also detectable in the fetal circulation[5,6], albeit at considerably lower levels than in the mother. Recently, using a pregnant sheep model, it was observed that acute feto-placental hypoxia, induced by clamping of the common internal iliac artery, was associated with about a tenfold increase in fetal activin A levels[7], a response that was sustained throughout the hypoxic insult but that normalised rapidly with the return of normoxia on release of the clamp. There was also a significant inverse relationship between fetal plasma activin A and fetal oxygenation[7]. In support of these experimental data, a significant inverse correlation between human umbilical arterial pH and activin A levels has been reported in a small series of 17 patients[6]. Accordingly, we speculated that umbilical artery activin A collected at the time of delivery might be a useful marker of intrapartum fetal hypoxia and also of subsequent hypoxic ischaemic encephalopathy (HIE). While HIE secondary to perinatal asphyxia is uncommon a number of potential therapies administered in the first few days of life have been proposed [8,9]. The success of such therapies relies upon the ability to target such interventions effectively towards only those infants at high risk of HIE. Accordingly, we undertook this study to define, in detail, the ontogeny of fetal activin A levels at term and the relationships between fetal activin A, fetal pH and neonatal outcome.

Methods:

Samples:

141 arterial umbilical cord samples, and 60 venous samples were prospectively collected for the measurement of activin A. The cohort represented an unselected convenience sample of pregnancies delivering at 36 to 41 completed weeks' of gestation. We excluded cases delivered by elective caesarean section since they would have been unlikely to be associated with acute fetal hypoxia. At delivery, the umbilical cord was double clamped and samples aspirated using 1mL pre-heparinised syringe (PICO 30, Radiometer Medical, Copenhagen, Denmark) within 30 minutes of delivery. Vessel sampling errors occur in approximately 20% of umbilical cord blood collections where either a mixed arterial/venous sample is collected, or the sample was inadvertently taken from the opposite vessel to that intended (ie vein instead of artery)[10,11]. Therefore, only validated samples, using previously proposed criteria[10,11], were utilized in this study. Briefly, samples were considered valid only when umbilical vein pH was >0.02 and CO_2 < - 4mmHg relative to the artery.

Details of all deliveries, including placental and birth weights, duration of labour, mode of delivery, indication for delivery (if relevant) were retrieved from the hospital's computerized birthing outcome database. Previously published criteria[12] were adopted to identify eight cases in which there was clinical suspicion of significant intrapartum hypoxia. The records of these eight cases were reviewed to further define the events during labour, the early neonatal course and whether HIE was subsequently diagnosed.

Approval for this project was granted by the Monash Medical Centre Human Research and Ethics Subcommittee.

Assay for activin A

After collection, all samples were centrifuged at 3500 rpm for 15 minutes at 4°C. The serum was then stored at –20°C until assayed. Total activin A was measured using a commercial enzyme-linked immunosorbent assay according to the manufacturer's protocol (Oxford Bioinnovation, Oxford, UK), as previously described[2,13]. The limit of sensitivity is <100 pg/ml and the inter and intraplate coefficients of variation is less than 10%.

Statistical analyses

Serum activin A is not normally distributed. Therefore, data were expressed as the median (± 95% confidence intervals). Comparisons of activin A levels were made using either the Mann-Whitney U or Kruskal-Wallis tests. Quantitative birthing outcome data were compared between groups using the student's T test. Correlation analyses were performed by log transforming activin A levels and using Pearson's test.

Results:

Overall, the median (95% CI) fetal arterial activin A level at delivery was 1.38 (1.34-1.70) ng/ml. Levels varied slightly but significantly across gestation with activin A increasing between 36 and 38 weeks gestation and falling thereafter to a nadir at 41 weeks. (p=0.03; table 1). In 60 cases, both an arterial and venous sample was taken. The median (95%CI) venous and arterial activin A levels were 0.89 (0.81-1.06) ng/ml and 1.38 (1.21-1.61) ng/ml, respectively (p<0.0001).

Mean arterial pH was 7.20 (7.06-7.38; 5-95th centiles) and was not significantly correlated with log10 activin A (r = - 0.01; p=0.68) (figure 1). There was a weak but significant association between activin A and pO_2 (log_{10} activin A vs pO_2: r^2=0.03, P=0.03) but not with other umbilical arterial blood gas parameters (log_{10} activin A vs pH: r^2=0.001, P=0.69; log_{10} activin A vs pCO_2: r^2=0.001, P=0.90; log_{10} activin A vs HCO_3, r^2=0.001, P=0.90; log_{10} activin A vs base excess: r^2=0.005, P=0.37). Table 2 details the clinical information and umbilical artery activin A levels of the eight cases which fulfilled the criteria for suspected intrapartum asphyxia, two of which were subsequently diagnosed with HIE. Activin A levels, expressed as multiples of the median (MoM) for each completed week of pregnancy to correct for gestation related changes, in all eight cases were similar to that of the 133 remaining cases which did not meet the criteria of significant intrapartum asphyxia, median MoM 1.11 (95%CI 0.54-1.72) vs MoM 1.00 (0.96-1.22); p=0.96, respectively.

Median (95% CI) umbilical arterial activin A levels were similar in 97 neonates delivered by spontaneous vaginal delivery and in 30 undergoing an instrumental (forceps or ventouse) vaginal delivery, 1.00 (1.04-1.38) MoM vs 1.07 (0.72-1.25) MoM, respectively (p= 0.15). However, median (95% CI) umbilical arterial activin A

levels were significantly lower in 14 neonates delivered by emergency caesarean section compared to the 97 spontaneous vaginal deliveries, 0.63 (0.53-0.92) MoM vs 1.00 (1.04-1.38) MoM, respectively (p=0.006), and trended towards lower levels compared to those delivered instrumentally (p=0.08). Of the 14 emergency caesarean section deliveries, three were performed for reasons other than fetal distress or obstructed labour (breech presentation in early labour, placenta praevia in early labour and an oblique lie in early labour). Of the other 11, five had a caesarean section for obstructed labour and six because of cardiotocographic evidence of fetal compromise. The median (95%CI) activin A level in these latter 11 was 0.55 (0.47-0.82) MoM, significantly lower than the combined vaginal and instrumental delivery group (p=0.003).

Arterial activin A MoM levels were not significantly correlated with birthweight, placental weight, or with the duration of first or second stage of labour (data not shown, p>0.62 for all comparisons).

Discussion:

In this study we have described the ontogeny of activin A in the umbilical cord artery and vein in term deliveries and the relationships between fetal activin A levels and fetal pH, duration of labour, mode of delivery and neonatal outcomes. Despite accumulating experimental and clinical data to suggest that activin A may be a useful marker of feto-placental oxygenation and possibly of neonatal outcome[3,4,6,7,14] the observations from this study suggest that umbilical artery activin A is not correlated with fetal pH and only very weakly with oxygenation and thus is unlikely to be a useful predictor of perinatal hypoxia and particularly hypoxic ischaemic encephalopathy in the term infant. Nonetheless, the study has offered other insights into the biology of activin in the fetoplacental unit at term.

This is the largest study of fetal activin A levels yet reported and the first study to describe in detail the ontogeny of fetal activin A during the final month of pregnancy. Previous studies[5,15] have reported umbilical artery levels in term pregnancies but have had insufficient sample numbers to explore whether there were gestational changes in those term cohorts. Our study has shown that activin A levels do change between 36 and 41 weeks, increasing to 38 weeks and then falling to 41 weeks However, both the absolute levels of activin A in the fetal circulation and the magnitude of the change in those levels are much lower than previously observed in the maternal circulation[1,2,5,6]. It is widely accepted that the activin A in the maternal circulation is principally of placental origin (reviewed elsewhere [16,17]) . The observation here that the levels of activin A in the umbilical artery are significantly higher than in the vein, consistent with a previous small study of 16 pregnancies[18], suggests that the fetus is an important source of fetal circulating activin. While this study did not address the likely source of

fetal activin A, a number of fetal tissues express the β_A subunit messenger RNA, including the gonads, kidneys, liver and the adrenal glands[18]. The observation that activin levels are similar in male and female fetuses[15] argues against a gonadal origin. The adrenal gland is the most likely source of activin A detected in the fetal circulation at term[20,21] with the fetal production of activin A potentially linked to adrenal changes associated with stress and/or parturition[20].

In this regard, and in light of previous observational and experimental data[4,6,7,14], we had expected to observe an inverse relationship between umbilical artery pH and activin A. This was not the case. We found no relationship between activin A and either pH and only a very weak association with pO_2. In an experimental model, acute *in utero* fetoplacental hypoxia increased fetal activin A levels such that there was a strong inverse relationship between activin A and oxygen saturation[7,22] . However, this relationship was only apparent when SaO_2 had fallen to below 50%[22] – a degree of hypoxia probably not present in the pregnancies studied here. It is therefore possible that the lack of any apparent relationship between activin A and pH simply reflects that the extent of fetal hypoxia in this study was not sufficient to stimulate activin production. Nonetheless, an inverse relationship between umbilical artery activin A and pH has been reported previously[6]. We cannot easily explain the difference between that report and the current study. Importantly, the previous report was of only 17 pregnancies compared to 141 here. In addition, the arterial samples in the current study were validated according to agreed criteria[10,11] whereas this was not undertaken in the prior report. Accordingly, we believe that the current data are more likely to be truly representative and therefore that umbilical artery activin A is not likely to be useful as a marker of feto-placental oxygenation at the time of delivery. In support of this conclusion is our observation that umbilical arterial activin A levels were not increased

in either our small (n=8) cohort of babies who fulfilled criteria for intrapartum hypoxia or in the two babies who subsequently developed HIE. It should also be noted that the umbilical artery pH in our series is slightly lower than previously reported in much larger series [10,23] . However, that we still observed no relationship between pH and activin in this series is perhaps stronger evidence that umbilical artery activin will not be a useful marker of fetal compromise in the term infant at delivery.

It has been previously reported that umbilical arterial activin A levels are no different in those babies delivered vaginally after a spontaneous labour compared to those delivered by elective caesarean section[5,6]. Such data would not support a role for fetal activin A in the establishment of labour. However, in this study we observed that among those already in established labour, cord artery activin A levels were significantly depressed in those who had an emergency caesarean section for complications arising in the first stage of labour. These data should be interpreted with caution since numbers in the caesarean section group are low and there was no prior hypothesis for this analysis. In addition, in a previous smaller study, no such difference in fetal activin A levels between modes of delivery was observed[6]. Nonetheless, our findings that low fetal activin A levels are associated with both dysfunctional labour and with prolonged pregnancy give rise to the possibility of a causative link. Maturation of the fetal adrenal cortex to secrete cortisol is thought to be important for successful parturition and fetal maturation[20,21,24]. It is also known that activin increases ACTH-stimulated cortisol production by the fetal adrenal definitive zone[20] and possibly induces regression of the adrenal fetal zone[25]. Thus, it is possible that the low fetal activin A levels observed in the pregnancies with a dysfunctional labour and in prolonged pregnancies may reflect incomplete adrenal functional maturation and underlie impaired parturition. Arguing against this would be the observation here that

umbilical artery activin A was not associated with duration of the first stage of labour. Nonetheless, further evaluation of maternal and fetal activin levels in post-term pregnancies and dysfunctional labours may be worthwhile.

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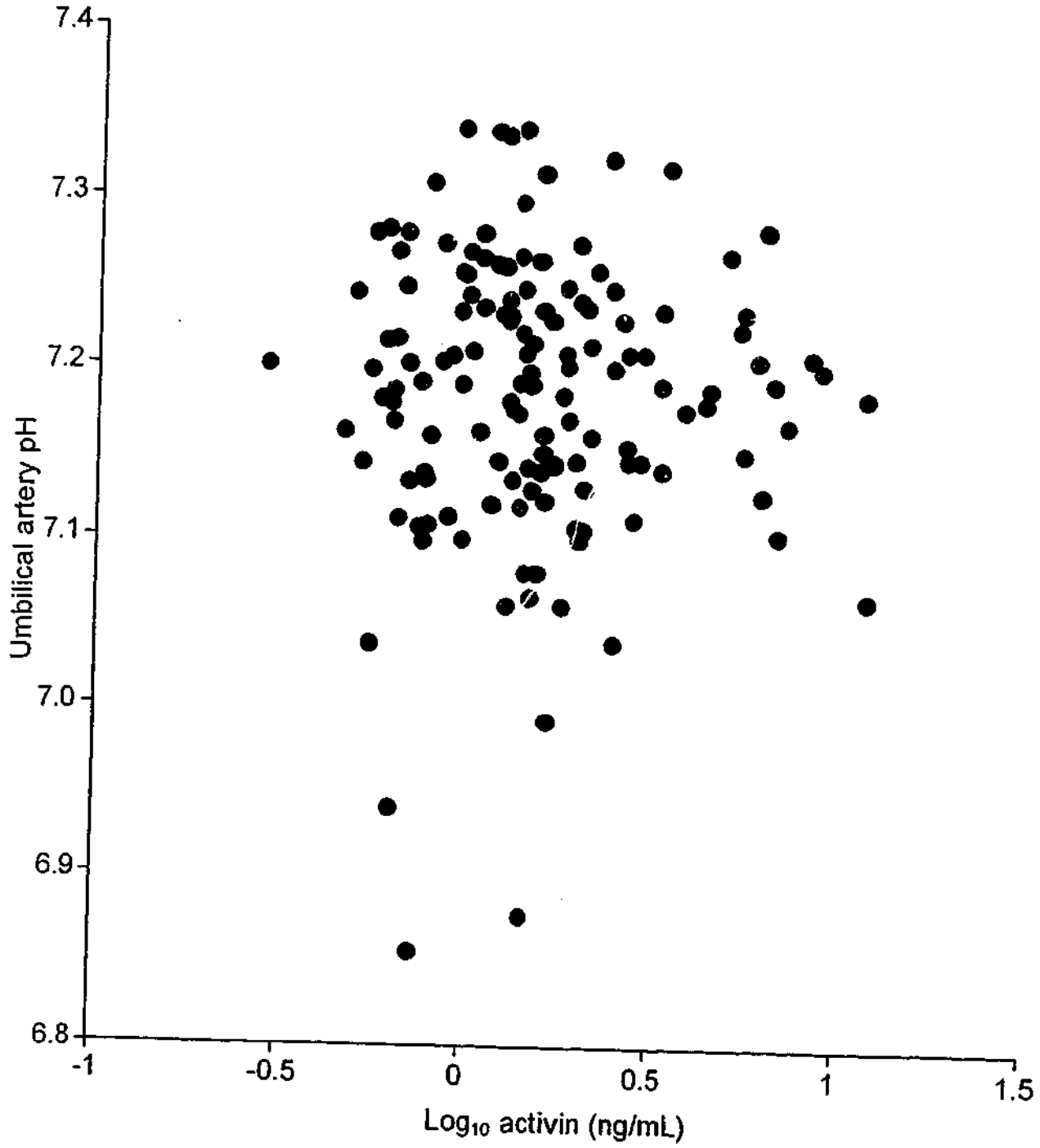

Figure 1. Relationship between umbilical artery activin A and pH in 141 term (36-41 weeks) deliveries.

Manuscript 5B:

Cord sampling at delivery: do we need to always collect from both vessels?

Stephen Tong, Val Egan, Julie Griffin and Euan M Wallace

Declaration regarding the paper entitled:

Cord blood sampling at delivery: do we need to always collect from both vessels? (5B)

British Journal of Obstetrics and Gynecology 2002;109:1175-1177.

Contributions to the work involved the following:

Name	% contribution	Nature of contribution
Stephen Tong	52.5%	Conceived the study, collected and statistically analysed the data, and was a major contributor to the writing of the report.
Val Egan	15%	Helped with data collection and contributed to the writing of the report.
Julie Griffin	12.5%	Helped with data collection and contributed to the writing of the report.
Euan M Wallace	20%	Supervised the study, helped with study design, and was a major contributor to the writing of the report.

Declaration by co-authors

The undersigned hereby certify that:

(56) they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;
(57) they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
(58) there are no other authors of the publication according to these criteria;
(59) potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journals or other publications, and (c) the head of the responsible academic unit; and
(60) the original data are stored at the following location(s) and will be held for at least five years from the date indicated below:

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[2]Val Egan		22/1/04
[3]Julie Griffin		19/1/04
[1,2]Euan M Wallace		20/1/4

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SHORT COMMUNICATION

Cord blood sampling at delivery: do we need to always collect from both vessels?

Stephen Tong[a,*], Val Egan[b], Julie Griffin[b], Euan M. Wallace[a]

The literature suggests that up to 19% of umbilical cord blood samples are invalid. Accordingly, it has been proposed that blood should be universally collected from both vessels. We prospectively collected paired arterial and venous blood to examine whether our centre, where staff were experienced in single vessel collection, was more accurate. Of 289 paired samples, 53 (18.3%) were considered invalid. Despite this significant error rate, we propose that routinely, only arterial sampling is needed and that an additional venous sample need only be taken to validate samples in cases of pH < 7.15, a difficult delivery or a non-vigorous baby.

Introduction

Blood gas analysis is the gold standard in determining intrapartum fetal hypoxia. There are two important reasons for universal cord blood sampling immediately after delivery. Firstly, it provides an objective method for continuous quality improvement where decisions in the labour ward can be judged against the fetal acid–base status[1]. Secondly, although it has been generally accepted that the large majority of cerebral palsy occurs as a result of multifactorial and mostly unpreventable reasons[2], 'damaged baby' awards have continued to rise steadily. It is therefore prudent to have normal blood gases on record for a baby who had a normal delivery but subsequently develops neurologic impairment, or even minor learning disabilities[1].

Authorities[2,3] have suggested that the routine collection of cord blood for blood gas analysis should include sampling from both artery and vein. Westgate *et al.*[3] found that of 1798 paired samples, 350 (19.4%) had unphysiologic pH and carbon dioxide (CO_2) readings (compared with the artery, the oxygenated umbilical vein must have a higher pH and lower CO_2).

It is possible that the error rate found in Westgate *et al.*'s[3] study may be in part attributable to a learning curve since their study essentially introduced cord blood collection to their centre. We have been universally collecting and analysing arterial cord blood (single vessel) for two years and are therefore well practiced in umbilical blood collection. We therefore set out to determine whether our error rate was smaller than that previously reported.

Methods

Cord venous and arterial blood were prospectively taken by labour ward staff from consecutive unselected deliveries at 36 weeks of gestation. The umbilical cord was double clamped at delivery and blood samples were collected using 1 mL heparinised syringes (PICO 30, Radiometer Medical, Copenhagen, Denmark) from both the umbilical artery (thinner vessels) and vein (larger vessel). Members of the staff were asked to decide which was artery and vein and to analyse samples in a dedicated blood gas analyser (AVL Compact 3, Roche Diagnostics, Victoria, Australia) situated in the delivery suite, recording pH and CO_2.

We then determined the proportion of invalid samples using criteria described by Westgate *et al.*[3] and adopted by others[4]. Specifically, for samples to be valid, the pH in the vein should be >0.02 relative to the artery, and $CO_2 < -4$ mmHg. No education was provided to the staff on what parameters to expect in each vessel.

We then attempted to identify the proportion of invalid samples, which were a clean switch of arterial and venous bloods. This type of error would result in the pH and CO_2 being exactly inverse to that expected (i.e. venous − arterial pH being negative, but with a difference of >0.02; and CO_2 difference being positive, but >4 mmHg). The remainder were assumed to be mixed arterial/venous samples.

Results

Of 321 paired samples collected, 32 pairs were excluded because 17 had incomplete pH data and 15 had incomplete

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Table 1. The centiles for pH and CO_2 in the umbilical vein (UV) and artery (UA) among validated samples ($n = 236$).

	Centiles					
	2.5th	5th	50th	95th	97.5th	Skewness
UV pH	7.138	7.195	7.309	7.389	7.417	−0.99
UV CO_2 (mmHg)	29.6	30.4	40.61	54.8	59.3	0.93
UA pH	7.049	7.065	7.202	7.318	7.327	−0.78
UA CO_2 (mmHg)	41.3	43.3	58.2	77.1	82.6	0.76

pCO_2 results. Among the remaining 289 samples, 35 had venous − arterial pH difference <0.02, 18 had CO_2 difference >−4 mmHg (but valid pH readings). This means that 18.3% (53) were invalid samples according to the criteria we adopted[3]. Nine (17%) of the 53 invalid samples, representing 3.1% of all samples collected (9/289) appeared to be a simple switch of arterial and venous samples. The remainder, 83% (44/53) of invalid results, were uninterpretable samples probably representing mixed arterial and venous blood. The pH and CO_2 of valid samples are shown on Table 1.

Discussion

Our labour ward staff are not naïve to the collection of cord blood and we had anticipated that the sampling error rate would be much smaller then that reported in the literature. The 18.3% error we found was therefore surprisingly high. However, this rate is remarkably similar to Westgate *et al.*'s[3] figure of 19.4% (derived from their publication) and considerably worse than Arikan *et al.*'s[4] figure of 6%. It is not clear from that latter study how long their centre had been practicing cord blood sampling. Importantly, the same criteria[3] were used by all three studies to validate samples.

It has never been addressed whether samples deemed erroneous were largely a simple switch between samples or mixed venous and arterial blood. The first mistake could be due to an inadvertent swap of syringes after collection while the second would be the accidental sampling of both vessels in one needle. We found that a majority of invalid samples were in the latter group. Since our study has confirmed that there will continue to be a significant error in universal sampling, we need to determine whether these mixed samples better reflect arterial or venous acid–base balance. How do clinicians, or the law, interpret a pH of 7.09 in a severely neurologically impaired baby, if the sample was shown to be mixed arterial/venous blood? Was the infant in fact severely asphyxiated intrapartum?

It is not known whether careful labour ward education can effectively decrease the error rate. It might be a possibility that the act of collecting blood from the cord with a fine needle is flawed and will therefore always incur a considerably error, irrespective of experience. Anecdotally, our midwives have found that while most cord blood collections are straightforward, there is a minority where the respective vessels are very difficult to differentiate with certainty.

Are we now mandated to sample both vessels at all deliveries? While many authorities support this[2,3], others do not feel that it is absolutely necessary[1].

There are sufficient data from large studies to provide sensible guidance to universal cord blood collection. It is currently believed that newborns are at risk from complications directly resulting from intrapartum asphyxia only if the umbilical arterial pH is <7.00 in combination with low Apgar scores[2,5]. Isolated cord arterial blood acidaemia does not appear to have any clinical significance in the vigorous newborn[1,2]. In addition, the mean pH difference between arterial and venous samples is only about 0.09[3,6] and that less than 10% show a 0.2 or more difference[3]. Taking these facts together, we believe that it should be safe in routine practice for labour wards to collect universally from the umbilical artery exclusively. However, an additional venous sample needs to be collected in situations where the 'arterial' pH is <7.15, where the delivery had been difficult or the baby was non-vigorous at birth. This strategy is similar to a screening test, where a further venous sample is only needed to validate the arterial sample in selected cases.

In such a protocol, if a venous sample was inadvertently taken, but the pH from the single vessel collection is over 7.15, it is exceedingly unlikely that there was in fact a large difference and that a significant acidaemia (<7.00) was missed. Even in the rare instance that this occurs, it is not believed to be significant if the baby was born vigorous. In addition, single vessel collection may significantly decrease the overall rate of invalid sampling by avoiding a clean switch of the two samples, which represented 17% of our total error rate.

This approach that we propose strikes a balance from costly over-investigation of thousands of normal deliveries (with extra burden on staff), while acknowledging the considerable error rate in sampling that we and others[3,4] have now consistently found.

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Manuscript 5C:

Maternal serum activin A and the prediction of intra-uterine growth restriction

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Declaration regarding the paper entitled:

Maternal serum activin A and the prediction of intra-uterine growth restriction (5C)

(submitted)

Contributions to the work involved the following:

Name	%contribution	Nature of contribution
Andrea Barkehall-Thomas	40%	Implemented the study, collected samples and clinical data, and was a major contributor to the writing of the report.
Stephen Tong	20%	Helped with the design of the study, performed the activin A assay, and contributed to the writing of the report.
Lesleigh S Baker	10%	Helped collect clinical samples and clinical data, and contributed to the writing of the report.
Andrew Edwards	10%	Helped collect clinical samples and clinical data, and contributed to the writing of the report.
Euan M Wallace	20%	Supervised, conceived and designed the study, helped with the collection of clinical data, statistically analysed the data, and was a major contributor to the writing of the report.

Declaration by co-authors

The undersigned hereby certify that:

(61) they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;
(62) they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
(63) there are no other authors of the publication according to these criteria;
(64) potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journals or other publications, and (c) the head of the responsible academic unit; and
(65) the original data are stored at the following location(s) and will be held for at least five years from the date indicated below:

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Full Title: Maternal serum activin A and the prediction of intra-uterine growth restriction.

Abbreviated title: Activin A and IUGR

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Key words: activin, fetal growth restriction, preeclampsia

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Summary

Objective Differentiating between the small healthy fetus and the high risk growth restricted fetus remains a significant obstetric challenge. We undertook this study to evaluate maternal serum activin A as a marker of fetal growth restriction.

Design Case control study

Patients 62 women referred for fetal assessment because of a clinical suspicion of a small for gestation fetus. Twenty seven women had a normally grown fetus, twelve had a constitutionally small fetus, eighteen had an IUGR fetus and five had an IUGR fetus with pre-eclampsia.

Measurements Maternal serum levels of activin A.

Results Activin A levels, expressed as median (95% CI) MoMs, were similar in the women with a normal sized fetus and in those with a healthy small for gestational age fetus, 1.14 (95%CI 1.0-1.5) and 1.31 (95% CI 0.8-2.1), respectively (p=0.97). Compared to the women with a normal sized fetus or a healthy small fetus, activin A levels were significantly elevated in the women who had either an IUGR fetus 2.37 (95% CI 1.6-3.7; p=0.007 compared to normal and p= 0.03 compared to healthy small) or who had preeclampsia/IUGR, and 7.99 (95% CI 1.9-26.3; p=0.002 compared to normal, p= 0.006 compared to healthy small).

Conclusions These data confirm that circulating activin A is increased in association with fetal growth restriction. However, a single blood sample for activin A will not efficiently discern between healthy and compromised small fetuses.

Introduction

Intrauterine fetal growth restriction (IUGR) secondary to impaired placentation is associated with increased perinatal mortality and morbidity. However, once IUGR is diagnosed in a given pregnancy it is possible to institute appropriate fetal surveillance and timely delivery thereby improving outcomes (Neilson and Alfirevic, 2002). The difficulty is in making the diagnosis, differentiating the IUGR fetus from the healthy small fetus. Indeed, reaching the diagnosis often requires serial ultrasound assessment of fetal wellbeing, Doppler studies of the feto-placental circulation and fetal biometry. This is both timely and expensive and can engender significant maternal anxiety. However, recently it has been suggested that maternal serum levels of activin A may usefully discriminate between the well small fetus and the growth restricted fetus (Bobrow et al., 2002; Wallace et al., 2003). In those studies, maternal serum activin A was significantly elevated in pregnancies complicated by IUGR whereas levels were normal in pregnancies with small healthy fetuses.

Activin A is a placentally derived growth factor (Qu and Thomas, 1995) of the transforming growth factor β superfamily . Maternal serum levels of activin A increase across pregnancy (Fowler et al., 1998; Schneider-Kolsky et al., 2000) and further elevations have been observed in women whose pregnancy is complicated by abnormal placentation such as in preeclampsia (Muttukrishna et al., 1997; D'Antona et al., 2000; Manuelpillai et al., 2001; Florio et al., 2002) and placental abruption (Florio et al., 2003). While it has been shown that the elevated activin A levels in association with established preeclampsia are secondary to increased placental production (Manuelpillai et al., 2001; Silver et al., 2002) the mechanism underlying that increased production remains to be fully elucidated. In this regard, acute

fetoplacental hypoxia in a pregnant ovine model (Jenkin *et al.*, 2001) has been shown to acutely increase activin A levels suggesting that hypoxia may act as a trigger. In contrast, *in vitro* culture of placental explants in reduced oxygen environment has not been shown to increase activin A production (Blumenstein *et al* 2002; Manuelpillai *et al* 2003).

Whatever the mechanism, if maternal serum activin A can be used to effectively discern between the IUGR and the well small fetus then it may significantly simplify the planning of the care of the woman with a clinically small fetus, reassuring those with a normal small fetus and affording increased surveillance of those with IUGR. In this study we aimed to extend the previous studies by assessing whether the prospective measurement of maternal serum activin A at the time of clinical suspicion of a small for gestational age fetus is useful in the prediction of the potentially compromised fetus with IUGR as opposed to the healthy constitutionally small fetus or normally grown fetus, as determined by ultrasound assessment.

Methods

All English speaking women with a singleton pregnancy referred to the fetal surveillance unit of a tertiary university teaching hospital for fetal assessment following the clinical suspicion of small for gestational age were eligible for inclusion. In all pregnancies gestational age had been established previously by early pregnancy ultrasound and in no pregnancy was there any evidence of a structural fetal anomaly. Cases with a known fetal aneuploidy, structural anomaly or syndromic diagnosis or referral for biometry following an antepartum haemorrhage were excluded. Non-English speaking women were not eligible for the study. Written, informed consent to participate to the study was obtained prior to fetal assessment.

Approval to undertake the study was granted by the institutional human research and ethics committee.

Assessment of fetal wellbeing undertaken at the initial ultrasound scan included fetal biometry, modified biophysical profile (gross body movements, fetal tone and breathing, amniotic fluid index) and Doppler assessment of the feto-placental circulation. All scanning was performed using an ATL HDI 3000 (Philips Medical, North Ryde, NSW, Australia) by one of three operators (ABT, LB, AE). Doppler studies were undertaken with combined grey scale and colour flow imaging, as previously described in detail (Edwards et al., 2002) Further fetal biometry or surveillance was undertaken according to established hospital management protocols.

A venous blood sample for measurement of maternal serum activin A was obtained at the time of the ultrasound scan. After collection, all samples were centrifuged at 3500 rpm for 15 minutes at 4°C. The serum was then stored at –20°C until assayed. Total activin A was measured in duplicate, using a commercially available enzyme-linked immunosorbent assay according to the manufacturer's protocol (Oxford Bioinnovation, Oxford, UK). The limit of sensitivity for activin A was <100 pg/ml and the inter- and intra-plate coefficient of variations were <10%. To correct for changes in activin A associated with gestational age, levels were expressed as multiples of the normal median (MoM), derived from a normal range of 156 normal healthy singleton pregnancies.

Following delivery, maternal (age, indication and mode of delivery, presence of gestational hypertension, smoking status and pre-existing medical conditions) and neonatal (gestation at delivery, birth weight, admission to newborn services, and complicating diagnoses) information was collected by a hand search of the maternal

and neonatal records. The diagnostic criteria for gestational hypertension and pre-eclampsia were according to international criteria (Brown et al., 2000). The gender specific birth weight centile was recorded according to Australian birth weight standards for the number of completed weeks of gestation (Roberts and Lancaster, 1999). A birthweight on or above the 10^{th} percentile was considered normal growth.

On review of the obstetric history and neonatal outcome the pregnancy was independently classified as normal, constitutionally (healthy) small, or growth restricted (IUGR), with or without preeclampsia, by two operators (ABT, EW), blinded to the activin assay results. Disagreements in classification were resolved by discussion between the two clinicians, blinded to activin results.

All statistics were analyzed using Stata Intercooled Basic version 6. Normally distributed data were analyzed using students t-test for continuous data and the Mann-Whitney U test was used for comparison of non-parametric independent continuous data. $P<0.05$ was considered statistically significant.

Results

Sixty nine women were consented and enrolled to the study. One woman was excluded when a clinical diagnosis of a placental abruption was made and one woman was excluded post natally when the infant was diagnosed with gonadal dysgenesis and a mosaic cell line. Serum samples were not available for analysis from five women. Sixty two patients remained for analysis.

Twenty seven patients were classified as having a normally grown fetus, twelve had a constitutionally small well fetus, eighteen had an IUGR fetus and five had an IUGR

fetus and pre-eclampsia. The mean (SD) maternal age was 29.6 (5.5) years and was similar across all four groups (table 1). There were no maternal or perinatal deaths.

The median (range) gestational ages at sampling and delivery were 34 (27-38) and 38 weeks (range 27-40 weeks), respectively (table 1). Women with either isolated IUGR or IUGR and pre-eclampsia were delivered significantly earlier than the normal group ($p = 0.002$ and 0.0008, respectively; table 1). Three (11%; 95% CI 2-29%) of the women with a normally sized fetus underwent either induction of labour or caesarean section for concerns regarding fetal growth or wellbeing compared to six (50%; 95% CI 21-79%) women with a constitutionally small fetus ($p=0.008$, versus normal sized group), 16 (88%; 95% CI 65-99%) women with an IUGR fetus ($p<0.00001$, versus normal sized group) and all five (100%) of the women with preeclampsia/IUGR.

The study was not designed to be of adequate power to determine differences in the obstetric or neonatal morbidity between the groups. Neither the percentage of women who smoked nor the percentage of women with a pre-existing condition that could influence fetal growth (eg hypertension, insulin-dependent diabetes mellitus, chronic renal disease, connective tissue disorders or thrombophilias) differed between the groups (data not shown).

Maternal serum activin A levels, expressed as median (95% CI) MoMs, were similar in the women with a normal sized fetus and in those with a healthy small for gestational age fetus, 1.14 (95%CI 1.0-1.5) and 1.31 (95% CI 0.8-2.1), respectively ($p=0.97$). Compared to the women with a normal sized fetus or a healthy small fetus, activin A levels were significantly elevated in the women who had either an IUGR fetus 2.37 (95% CI 1.6-3.7; $p=0.007$ compared to normal and $p= 0.03$ compared to healthy small) or who had preeclampsia/IUGR, and 7.99 (95% CI 1.9-26.3; $p=0.002$

compared to normal, p= 0.006 compared to healthy small). Figure 1 summarises these data.

Discussion

The efficient identification of the "at risk" small fetus remains a significant clinical challenge. Ultrasound fetal biometry and umbilical artery Doppler flow studies have become established as the principle investigations to firstly confirm the clinical suspicion of a small for gestational age fetus and then to assess whether that fetus is healthy small or growth restricted and at risk (Kiserud and Marsál 2000). However, this approach has a number of potential limitations. It depends upon the efficiency of the clinical examination that triggers the ultrasound assessment, it requires considerable ultrasound hardware/expertise and umbilical artery Doppler studies are often less informative in the growth restricted fetus in late pregnancy (Herschkovitz *et al.*, 2000; McCowan *et al.*, 2000). Therefore, to arrive at a decision regarding the risk allocation of a small fetus often requires serial ultrasound assessment in conjunction with other clinical parameters such as maternal age and medical and obstetric history. The development of an efficient but simple, cheap and non-invasive method of fetal assessment would therefore represent significant progress of considerable clinical value.

The recent observations that maternal serum activin A is significantly elevated in pregnancies complicated by fetal IUGR (Bobrow *et al.*, 2002; Florio *et al.*, 2002; Wallace *et al.*, 2003), particularly in the absence of preeclampsia (Wallace *et al.*, 2003), but not in those with a constitutionally small fetus (Bobrow *et al.*, 2002; Wallace *et al.*, 2003) offered the potential of such a test. In this study, we have extended those previous studies to prospectively assess the utility of maternal serum

activin A in all women referred with a clinical suspicion of a small fetus, whether or not the fetus was small. This is important because the specificity of the clinical determination of the small for gestational age fetus is low (Rosenberg *et al.*, 1982; Secher *et al.*, 1991) and therefore most women being referred for ultrasound assessment actually have a normally grown fetus. A complete evaluation of the utility of activin A in this clinical setting must therefore include all pregnancies where there is a clinical suspicion of IUGR, whether or not the fetus is confirmed as small. In the previous studies activin A was evaluated only in pregnancies where ultrasound biometry had already confirmed a small fetus (Bobrow *et al.*, 2002; Wallace *et al.*, 2003) or where preeclampsia was present (Florio *et al.*, 2002). Such study design does not allow the evaluation of utility undertaken in this current study.

In the current study we have shown that maternal serum activin A levels are not elevated in women with a healthy small fetus compared to those referred with a clinical suspicion of a small fetus but where biometry demonstrated a fetus with biometry $>10^{th}$ centile. In addition, activin A levels in both of these groups of women did not differ significantly from the normal range. These observations are consistent with and extend the previous report that activin A levels were normal in pregnancies with a small healthy fetus (Wallace et al., 2003). Furthermore, this study confirms that activin A levels are significantly increased in association with IUGR, either in isolation or in combination with pre-eclampsia. In women with isolated IUGR levels are increased approximately 2.5 fold compared to normal and in those with IUGR/preeclampsia approximately 8 fold. While these data broadly confirm the previous reports (Bobrow *et al.*, 2002; Florio *et al.*, 2002; Wallace *et al.*, 2003), the differences in activin A levels between the healthy small pregnancies and the IUGR pregnancies were not as profound as previously reported. In the current study there

was considerable overlap in activin A levels between these two groups suggesting that activin A will not be able to efficiently discriminate between them and therefore be of limited value in the initial determination of fetal risk status. These data are more in keeping with the conclusions from another study that reported essentially normal activin A levels in a "growth restricted" pregnancy cohort (Keelan *et al*, 2002).

While maternal serum activin A is increased in association with fetal IUGR this study has not explored what the mechanisms leading to this increase are nor what biological significance, if any, activin has in this setting. The placenta is widely accepted as the principal source of activin A in the maternal circulation in normal pregnancy (reviewed by Tong, Wallace and Burger, 2003) and the elevated levels observed in preeclamptic pregnancy have been shown to be due largely to increased placental production (Manuelpillai *et al.*, 2001; Silver *et al* 2002). It is therefore likely that the increased levels of activin A observed in this and previous studies (Bobrow *et al.*, 2002; Wallace *et al.*, 2003) reflect increased placental production, although no direct evidence for this yet exists. In an ovine model of acute feto-placental hypoxia (Jenkin *et al.*, 2001) activin A secretion increased during hypoxia although whether the activin A was of placental origin was not addressed. In that study, the increased activin A was followed by increasing prostaglandin E_2 and then prostaglandin F suggesting that activin may be stimulating prostaglandins, perhaps to induce vasodilatation and thereby improve perfusion. Indeed, the endothelial cells of the maternal and fetal vasculature itself express activin receptors consistent with a possible direct vascular effect (Schenider-Kolsky *et al.*, 2002). Again, whether such an effect actually arises has not been reported and extrapolation from the ovine model that has different placentation from the human should be made with caution. It is clear that there is still much to learn about the biology of activin in normal and pathological

pregnancy and that insights into the biology of this protein may open new horizons on the regulation of feto-placental health.

In conclusion, while this study confirms that maternal serum activin A is increased in association with fetal growth restriction and not increased in association with either normally grown or healthy small fetuses, the degree of elevation is unlikely to be sufficient to be of clinical utility.

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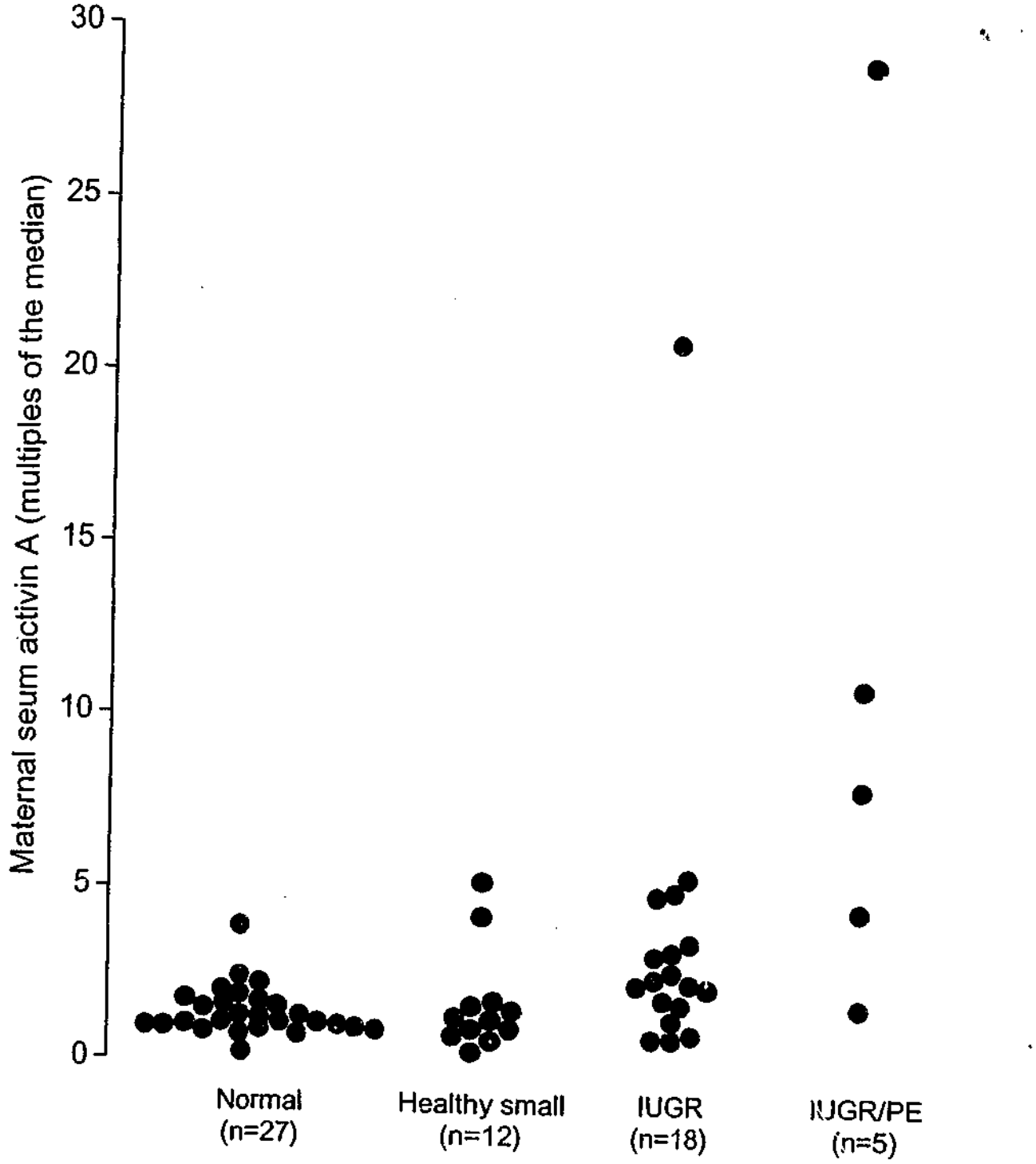
Figure legend

Figure. Maternal serum levels of activin A, expressed as multiples of the normal median (MoM), in 62 singleton pregnancies referred with a clinical suspicion of small for gestational age.

Table 1. Clinical details of 62 pregnancies referred for fetal assessment with a suspicion of a small for gestation fetus.

	Normal	Healthy small	IUGR	IUGR-PE
Mean ±SD maternal age (years)	30.7 ±5.9	29.1 ±5.5	28.7 ±5.2	28 ±3.5
Median(range) gestation at sampling (weeks)	33 (27-38)	36.5 (28-38)	35 (29-39)	31 (27-35)
Median(range) gestation at delivery (weeks)	39 (34-40)	38 (35-40)	37 (29-40)	32 (27-37)
Obstetric intervention rate (95% CI)	11% (2-29%)	50% (21-79%)	89% (65-99%)	100% (48-100%)
Median (range) birth-weight (g)	3090 (2136-3520)	2518 (2022-3000)	2020 (702-2500)	1174 (760-2255)


Maternal seum activin A (multiples of the median)
30
25
20
15
10
5
0
Normal
(n=27)
Healthy small
(n=12)
IUGR
(n=18)
IUGR/PE
(n=5)

Discussion

In this doctorate, several markers of potential clinical utility have been identified and new insights have been generated relating to the biology of early singleton and multiple pregnancy.

Although each manuscript describing original research already contains a complete discussion, the strict requirements of peer-reviewed publications are such that speculation regarding the data should be more limited than that permitted in a doctoral thesis. Therefore, the aim of this section is to highlight the most important findings of the studies reported in this thesis and to build on discussions already made by offering additional speculation on possible implications and the future research steps required. In particular, new concepts relating to reproductive biology will be proposed which draw on different sections of this doctoral thesis. Significant repetition of the considered discussions already made in the preceding manuscripts will be avoided.

A brief summary of the main findings from all five sections of the PhD will be presented first. Next, the clinical markers of most promise will be highlighted and an outline of the studies needed in order to verify their worth. The last section will involve a further discussion of the more important biological concepts which have arisen from the studies undertaken during this PhD.

Summary of Findings

Section 1: Biochemical markers of pregnancy outcome at days 15-17 after IVF

- Early maternal serum inhibin pro αC levels are highly sensitive and specific in predicting whether a clinical pregnancy will result (1A).
- Maternal serum MIC-1 levels are increased by days 15-17 after conception whereas PAPP-A levels are not increased at this time, but begin to rise one week later (1B). However, there is significant overlap in levels seen among women who will become clinically pregnant versus those who will not.
- With decreasing15-17 hCG levels, the risk of clinical miscarriage diagnosed between 8-19th week of gestation increases (1C).
- Low early hCG levels are strongly associated with low birthweight as is three embryo transfer, when compared to having two or one embryos transferred (1D).

Section 2: Biochemical markers of miscarriage at 6-13 weeks gestation

- MIC-1 (2A) and PAPP-A (2B) are highly significantly depressed in association with miscarriage, and levels may be low preceding pregnancy failure.
- Inhibin A and pro-αC are depressed in association with miscarriage. However, levels significantly overlap with those seen in women with ongoing pregnancies. Activin A levels are not decreased in association with miscarriage (2C).

- Among women who double ovulate and become pregnant, the proportion who have twins present at 5-9 weeks gestation is around 25%, the remainder being singletons (3A). Since 25% is similar to the fecundability rate, it appears that the presence of one twin does not impede the development of its co-twin.
- It may be possible to determine the zygosity of spontaneous dizygotic twins by noting the number of corpora lutea at early pregnancy ultrasound (3B).

Section 4: Corpora luteal diameters and miscarriage risk

- The corpus luteum (CL) during early pregnancy reaches its maximal diameter at 6 weeks gestation, remains around the same size until around 8 weeks' gestation, before steadily shrinking until at least 13 weeks' gestation. At 5-9 weeks' gestation, there is an increasing risk of miscarriage with decreasing CL diameter (4A).

Section 5: Activin A as a marker of later pregnancy complications

- Fetal arterial umbilical Activin A levels measured at delivery are not associated with pH or hypoxic ischaemic encephalopathy (5A).
- Errors in routine arterial umbilical cord sampling by labour ward staff may be as high as 19% (5B).

- Although maternal serum activin A levels are increased in association with fetal growth restriction, they are not useful in triaging women during clinical antenatal care (5C).

Potential Markers of Clinical Utility

In this doctorate, several novel prospective markers of promise have been identified. However, for reasons outlined below, all of these will require further study to fully assess their accuracy as a screening or predictive marker prior to clinical application.

1. Inhibin pro αC levels in the prediction of pregnancy success in frozen-thawed IVF cycles without complete ovarian suppression (1A)

Inhibin pro αC levels at days 15-17 after fertilization *in vitro* had 83% sensitivity at 95% specificity in predicting clinical pregnancy success (1A). Whilst highly accurate, it was neither better than, nor helped to enhance the predictive value of human chorionic gonadotrophin (hCG), the existing clinical marker.

A post-hoc analysis of those who had frozen embryo transfer (ET) but did not undergo complete ovarian suppression found that inhibin pro-αC levels displayed 100% sensitivity for 95% specificity in predicting the presence of a clinical pregnancy. If these values are correct, then pro-αC may be potentially better than hCG in the prediction of a clinical pregnancy in patients undergoing this type of IVF regimens (Homan et al., 2000; Sugantha et al., 2000; Urbancsek et al., 2002). An explanation to account for this observation is that with these particular frozen ET IVF regimens, there is smaller amount of luteal tissue present in the ovaries compared to fresh ET cycles. Consequently, there is a narrower range of serum pro-αC concentrations in both the pregnancy and non-pregnant cohorts and less overlap in serum levels seen between these groups.

However, this requires verification primarily because the hypothesis arose from a post-hoc observation. Such a study would be very similar in design to that already reported (1A) but limited only to those who had a frozen ET and did not have a regimen that involved ovarian suppression (See 1A, table 1). In addition, it should include a prior power calculation, based upon the results reported here (1A). Besides reporting the presence of clinical pregnancy as a primary outcome (ie. reaching 6 weeks gestation), the other outcome to consider would be whether the pregnancy progressed to viability (24 weeks gestation). Given the very promising early results (1A) and the fact that a confirmatory study would be fairly similar in design as the present study, it is highly likely that a repeat study will indeed confirm that pro-αC is extremely accurate in predicting pregnancy in this setting.

There is understandably much anxiety faced by couples over IVF and in particular, the first hCG test informing both the clinician and the patients whether the IVF cycle might have succeeded (McNaughton-Cassill et al., 2002). With significant numbers of frozen ET's being performed, any marker which significantly improves on the performance of hCG has good prospects of being adopted clinically.

2. Markers to predict clinical miscarriage; and a possible multiple marker approach (2A; 2B, 4A)

Sensitive markers which can predict miscarriage may have potential use as a research tool to target therapies aimed at averting pregnancy loss. As an example, progesterone support which has not been successful in trials performed amongst the low risk

population (Goldstein et al., 1989), may be more efficacious if targeted specifically towards those at high risk.

Two biochemical markers have been identified in this doctorate which show promise as markers of miscarriage. Median MIC-1(2A) and PAPP-A (2B) were 32% and 14% respectively of levels seen among women who had ongoing pregnancies.

Furthermore, these studies suggest but have not proven, that these proteins may be depressed prior to the miscarriage occurring. The design of these studies meant that for the majority of pregnancies reported, it was not possible to confirm fetal viability at the time of sample collection. For MIC-1, such information was available in a very small number who had miscarried (n=6) and was supportive of the overall findings. Nonetheless, a prospective study is clearly needed in order to confirm these results. This might entail the prospective recruitment of women presenting at 6-10 weeks of gestation for antenatal care. The risk of subsequent miscarriage from this period in gestation is around 6-10% (Mackrydimas et al., 2003), meaning that a reasonable number of cases would be available for proper analysis. Importantly, non-bradycardic fetal cardiac activity would need to be documented as being present at the time of blood sampling and that there were no symptoms of miscarriage. The final numbers needed would depend on a power analysis, although it is likely that such a study would involve at least several hundred study participants.

It was also discovered that the smaller the diameter of the CL at 5-9 weeks' of gestation when measured by ultrasound, the more likely that the pregnancy will miscarry (4A). Unlike biochemical markers which may require specialized assays, the advantage of CL diameters as a marker of miscarriage is that it can be readily measured by anyone familiar with early pregnancy and gynaecological scanning. Beside predicting miscarriage for research purposes, it may also have a clinical role in prognostication among women who present with the symptoms of miscarriage but are shown to have a viable pregnancy at the time of the ultrasound. It is possible that its predictive power might be enhanced if used in a model which takes into account the presence and degree of any clinical signs, such as vaginal bleeding. Like the biochemical predictive markers reported in this doctoral thesis, the data for this study was also obtained retrospectively and whether the CL can reliably predict miscarriage would also require proper prospective evaluation

It is important to note that there have been no reports to our knowledge which suggests that directs relationships exist between MIC-1, PAPP-A or CL diameters. If they are largely independent of each other, it is possible that they could be used in combination as a sensitive multi-model marker of miscarriage. Perhaps added to these might be ultrasound assessment of theca interna blood flow (Guerriero et al., 1999), mean sac diameter minus crown rump length measurements (Bromley et al., 1991); and biochemical markers such as anandamide hydrolase (Maccarrone et al., 2000) progesterone (Phipps et al., 2000) and hCG.

3. Determining zygosity at early pregnancy ultrasound in spontaneous twins (3B)

We have proposed that zygosity may be determined in cases of spontaneous twinning by noting the number of corpora lutea (CL; 3B). Such information might be of use for the obstetrician, but would certainly be of keen interest to the twins (Derom et al., 2001), their parents, and genetic researchers (Monteiro et al., 1998).

So far, the data presented were derived from a small retrospective series which demonstrated that by using this method, the predicted zygosity was compatible with the reported chorionicity in all cases. However it was not possible to follow-up cases in this retrospective study in order to verify zygosity using DNA fingerprinting technology, meaning that dichorionic diamniotic twinning could have been either monozygotic or dizygotic. As such the proposal should be regarded as preliminary and clearly requires verification. This could be achieved by a prospective observational study comparing ultrasound prediction of zygosity with DNA fingerprinting of buccal swabs taken from same sexed twins after delivery. Though opposite-sexed twins could be safely assumed to be dizygotic it might be prudent to all perform DNA testing on monochorionic twins despite the fact that they would almost certainly be monozygotic. This would eliminate any chance of erroneous assignment of chorionicity by ultrasound although this has been reported to be 100% accurate if undertaken sufficiently early in pregnancy (Stenhouse et al., 2002).

It is also important to note that this method to determine zygosity cannot be used for twins conceived by IVF but can only be used for the comparatively rarer case of spontaneous twinning. As such, only 12 spontaneous conceived twin pairs would be available for potential recruitment for every 1000 early pregnancy scans performed (Tong & Short, 1998). Of these cases, about two to three would be expected to spontaneously reduce either to singletons or miscarry altogether after the initial ultrasound scan confirming viability (Tummers et al., 2003). Therefore, a follow-up study would be most realistically achieved if a convenience sample were recruited where women who had spontaneously conceived and had viable twins discovered at an early pregnancy ultrasound would be invited to participate.

Further discussion relating to the biological concepts of potential importance

The fact that only few endocrine markers were investigated in a limited range of clinical situations has given rise to new biological concepts and hypothesis' of potential importance. Many of these have already been discussed in the preceding manuscripts. The purpose of this section of the discussion is to highlight the most important concepts which merit further speculation beyond that permissible in a peer-reviewed manuscript. Subsections 3 and 4 introduce new ideas not yet proposed.

1. The products detected by the inhibin pro-αC assay may play a biological role in the establishment of very early pregnancy

Most early pregnancy hormones, including those from the corpus luteum increase steadily across early pregnancy (Fowler et al., 1998). The fact that luteal derived inhibin pro-αC peaks at around day 16 (Fowler et al., 1998; Illingworth et al., 1996) and that levels measured at this time are highly predictive of pregnancy (1A) strongly implies a possible biological role for these inhibin forms. In further support of this is the fact that the strength of the association between inhibin pro-αC and pregnancy outcome markedly reduces by 6-13 weeks (2C), a time when serum levels have been in decline for some weeks (Fowler et al., 1998; Illingworth et al., 1996). A peak at day 16 may reflect the end of a progressive and important rise in levels across the first week of implantation. This is

a critical period for embryonic survival since hCG levels at days 15-17 are associated with both early pregnancy loss (Macklon et al., 2002), and miscarriage occurring later in pregnancy (1C). Others have found that during this first week, the lack of a progesterone rise from the CL correlates with fetal demise, further highlighting the importance of the complex endocrinology occurring during the implantation period (Baird et al., 2003).

Since dimeric inhibin A levels measured by day 16 do not appear to correlate with pregnancy outcome (Treetampinich et al., 2000) when compared to pro-αC (1A), any biological role that inhibin pro-αC forms may play could also involve the lower molecular weight forms. These smaller forms comprise around 40% of the proteins detectable by the ELISA assay in pregnancy sera (Thirunavukarasu et al., 2001) and they include the intact free α subunit and partially processed inhibin forms which contain the pro and αC epitopes. Undoubtedly, the strongest argument against inhibin pro-αC having any biological role in early pregnancy is the fact that none of these smaller molecular weight have been shown to display any biological activity.

Nonetheless, given the potential use of an agent which facilitates implantation in infertility treatment, a further investigation of any possible biological role of inhibin pro-α in the establishment of pregnancy merits investigation. This would entail a significant undertaking. Firstly the relative abundances of the different inhibin pro-αC forms have not been specifically determined in pregnancy bloods as early as days 15-17 and thus would need to be determined. This could be achieved by following the methods previously used to describe the relative abundances of the different molecular weight

forms for later pregnancy (Thirunavukarasu et al., 2001). If not commercially available, these inhibin pro-αC forms could be cloned, expressed, purified, then used in established models used to investigate early pregnancy, such as the addition to *in vitro* first trimester trophoblast cultures, in vivo injections into animal models (Chaouat et al., 1995), and even their effects on embryonic development after fertilization in vitro (Tsai et al., 1999). Should such studies be consistently negative, it would be compelling evidence to refute any biological role for inhibin pro-αC forms. However, should biological activity be discovered, the importance of this finding would might well extend beyond early pregnancy research since these smaller molecular weight forms have always been regarded as inert.

2. Macrophage inhibitory cytokine-1 and Pregnancy associated protein A analogues may be useful in preventing miscarriages

Treatment of miscarriage remains a distant prospect, but may be worthwhile exploring given that it remains the most common complication of pregnancy and can cause significant distress to those affected (Hori et al., 2002). In particular, it would be desirable to identify an efficacious agent to treat recurrent miscarriage, an area where therapeutic interventions have been avidly sought (Scott & Pattison, 2001; Stricker et al., 2000).

The fact that low levels of MIC-1 (2A) and PAPP-A (2B) may precede miscarriage suggests a role in causation. This possibility is strengthened by the fact that these proteins

are known to have biological properties which would be consistent with pregnancy support (see discussions, 2A; 2B). If low levels may be contributing to miscarriage, then analogues of these proteins may be useful in salvaging some miscarriages. It is important to stress that whether these proteins have any causative and/or therapeutic role in miscarriage is extremely speculative at this stage. Although various roles for both these proteins promoting ongoing pregnancy is suspected, little is actually known regarding their precise biological actions (1C). Therefore, proof as to their therapeutic value will require a major research effort, from firstly further delineating their actions in early pregnancy, in vivo culture studies and experimental animal models. Any possibility of human trials will depend on the outcome of the results of these basic science studies.

If either of these agents can be shown to prevent miscarriage, it might be envisaged some time in the future that women could be offered testing using a multimodal marker of miscarriage one or two weeks after a missed period to assess their risk of miscarriage. Those with blood (2A;2B) and/or ultrasound markers (5A) pointing to a high risk of pregnancy loss could be offered treatment to salvage the pregnancy. There has been active research into the non-invasive assessment of fetal chromosomes via cells obtained either in the maternal blood (Jackson, 2003) or possibly the cervix (Fejgin et al., 2001). Should an accurate test become available, it could be added to initial testing to ensure that a euploid fetus is present before treatment is offered.

3. Monochorionic, but not dichorionic twins, may be responsible for increased twin losses observed during the first half of pregnancy

Landy and Keith's meta-analysis on vanishing twin (Landy & Keith, 1998) appeared to clarify the situation regarding the level of twin losses during the first half of pregnancy, and is still often cited. The review concluded that in as many as 40% of clinically recognised twin gestations, a reduction in either one or both twins occur suggesting that vanishing twin is common phenomenon. However, several concerns exist regarding this estimate. The meta-analysis included a heterogenous group which were a combination of both IVF and spontaneously conceived twins. The review did not take chorionicity into account and included small studies dating back to the late 1970's when ultrasonography was still in its infancy. The authors acknowledge that in these early studies, it is possible that many structures such as a clot or placental tissue may have been initially mistaken as a second twin. This would overestimate the incidence of twin losses.

Perhaps the biggest concern in Landy and Keith's review (Landy & Keith, 1998) was that there was no comparison made with singleton miscarriage rates. To properly conclude that twin gestations are at increased risk of 'vanishing', each twin should be considered a discrete gestation sac and their individual level of risk compared to that incurred by singleton gestations sampled from the same population. A study which finally did this was published only recently by Tummers *et al* (Tummers et al., 2003). Whilst their study did not consider chorionicity, most of the twins would have been dizygotic (or dichorionic) since the report was based on an IVF cohort. They presented the surprising

finding that in cases which had fetal cardiac activity confirmed on early ultrasound, a sac belonging to a singleton pregnancy had a 12.2% risk of subsequent miscarriage, whilst a twin gestational sac had a significantly lower risk of miscarriage (7.3%). These findings call into question whether dizygotic twins reaching six weeks gestation do in fact have a significantly increased risk of pregnancy loss afterwards, when compared to singletons.

Prior to six weeks, mathematical extrapolations proposed by others suggested that significant wastages in twin conceptions occurs such that only 1 in 50 twin conceptions survive and that 12-15% of all singletons began as a twin gestation (Boklage, 1995). We found (4A) that of those who double ovulate and become pregnant, the probability of fertilization and development of the second egg until at least 5-9 weeks of gestation was in fact very similar to that of the singleton fecundability rate (Wang et al., 2003; Wilcox et al., 1988; Zinaman et al., 1996). This is at odds with any suggestion that large losses of twins occur in early pregnancy. However, our data could not rule out the possibility that both twins may be lost at a greater rate. This could be investigated indirectly by determining the dual ovulation rate in a non-pregnant cohort by ultrasound studies using the CL as a surrogate marker of ovulation. By assuming that human fecundability is around 18-30% (Wang et al., 2003; Wilcox et al., 1988; Zinaman et al., 1996), and knowing that the dizygotic twinning rate is about 6-10/1000 (0.6-1.0%) in Caucasians (Bulmer, 1970), the expected dual ovulation rate would be around 3-5% if twin losses are not significantly increased. However, if significant wastage in dizygotic twinning was occurring, then the dual ovulation rate would be much higher.

The evidence discussed above suggest that dizygotic twins may not be at an increased risk of pregnancy failure compared to singletons. In contrast, evidence exists suggesting that monochorionic (monozygotic) twins are at increased risk of pregnancy loss. Sebire *et al* (Sebire et al., 1997) reported the survival of monochorionic and dichorionic twins from the 10-14 weeks of gestation until pregnancy viability (24 weeks gestation). They reported a fetal loss rate of 1.8% amongst dichorionic twins. In contrast, monochorionic twins, who are always monozygotic, incurred a significantly increased rate of loss rate of 12.2% (Sebire et al., 1997).

Therefore, in consideration of the evidence presented above, it we propose the hypothesis that monochorionic, but not dichorionic twins (which includes all dizygotic twins), are at increased risk of early pregnancy loss and are largely responsible for the 'vanishing twin' phenomenon.

The next step in evaluating this hypothesis would be to examine the rate of losses occurring earlier than 10-14 weeks of gestation in relation to chorionicity. A study to answer this question might be quite readily achieved in IVF cohort examining losses from 6 weeks onwards since an ultrasound is often done around this time to confirm fetal viability and outcomes are usually well documented. In addition, the determination of chorionicity at this gestation is considered to be 100% accurate (Stenhouse et al., 2002).

Part of this hypothesis which will be difficult to investigate is losses occurring among monochorionic twins prior to the 6^{th} week of gestation. Whilst we have been able to

estimate dizygotic twinning survival during this period of 'preclinical' pregnancy (5A), it is difficult to envisage how early monozygotic losses could be explored.

An interesting implication of increased twin losses being largely confined to monochorionicity is how it relates to a recent theory regarding the aetiology of cerebral palsy. It has been proposed by Pharoah and Cooke (Pharoah & Cooke, 1997) that many cases of spastic cerebral palsy arise from monochorionic pregnancies where one of the twins had reduced early in pregnancy. Although our own hypothesis would not directly prove or disprove such an idea, the possibility that increased twin losses might be confined only to monochorionic twinning would lend some support.

4. The pathogenesis of several obstetric diseases may begin very early.

Although a strong association has been established between low early hCG levels and preclinical, or early pregnancy loss (Homan et al., 2000; Macklon et al., 2002), levels at this very early period of pregnancy are not considered to have any significant association with complications later in gestation. We have shown a strong association between low day 15-17 hCG levels and clinical miscarriage (1C), and low birthweight (1D). The biological significance of low early hCG, which may reflect quality of implantation, have been already discussed in the two preceding articles.

Here a model of early origins of miscarriage will be proposed, drawing on the studies relating to pregnancy loss presented in this doctorate. A discussion will then be presented

on how data from this doctorate may be relevant to our understanding on the pathogenesis of low birthweight. Finally, the overall implications of possible very early origins of obstetric diseases will be discussed in terms of biology, and clinical relevance, including potential avenues of therapy and the prevention of disease.

Early origins of miscarriage

The fact that we have observed a strong link between low early hCG and clinical miscarriage (days 15-17, or 4th week of gestation) suggests an early origin of this complication, possibly secondary to a poor quality of implantation, in many cases initiated deliberately by the mother after sensing that the fetus contains a chromosomal error (Fritz et al., 2001). In those who are destined to miscarry, it is possible that a cascade of adverse events starts from the first week of implantation so that by 6-13 weeks of gestation, hCG (2A), MIC-1 (2A) and PAPP-A (2B) have either started to drop, or failed to increase at a sufficient rate to sustain the pregnancy. This lack of a rise may be occurring at both at the maternofetal interface and in the peripheral circulation. Around that same period, the endocrine feedback signal to the ovary from the failing placenta may be weakening, reflected by the fact that the CL is smaller than expected (5A). Thus this model suggests that the 'decision' to miscarry may occur very early in pregnancy and this initiates a pathological cascade which either leads to early pregnancy loss (Macklon et al., 2002; Winter et al., 2002) or clinical miscarriage occurring later.

Early origins of low birthweight

The weight of recent evidence from animal (Constancia et al., 2000; Constancia et al., 2002) and molecular medicine studies (Abuzzahab et al., 2003; Lawrence et al., 1999) suggest that the IGF/PAPP-A system plays a major role in the pathogenesis of low birthweight. Insulin-like Growth Factor (IGF) I and II have important actions in maintaining a healthy placenta, including steroidogenesis (Nestler, 1990) and nutrient transport (Kniss et al., 1994) and good evidence exists suggesting that deficiencies in this system leads to growth restriction. Mice with an IGF I receptor promoter knock out display a 40% reduction in growth (Constancia et al., 2000) and the reason for the growth defect in this model appears to be a deficiency in the placental transport of nutrients (Constancia et al., 2002). Pregnancy Associated Placental Protein-A (PAPP-A) is an important regulator of the IGF system (Lawrence et al., 1999). Smith *et al* (2002) (Smith et al., 2002a; Smith et al., 2002b) reported a convincing association between depressed maternal serum levels of PAPP-A by 8-12 weeks of gestation and intrauterine growth restriction.

In contrast to the strong association between low days 15-17 hCG and low birthweight (1D), we found that PAPP-A, a regulator of the IGF system, had not begun to rise at this stage. Whilst IGF/PAPP-A system is very likely to play an important role in the pathogenesis of low birthweight, our data give rise to the possibility that a disruption of the IGF/PAPP-A system may not be the primary mechanism responsible for low birthweight, but an important downstream consequence of an earlier pathogenic mechanism occurring during implantation.

Very early origins of obstetric diseases

It may be worthwhile exploring possible early origins of other obstetric complications besides low birthweight and clinical miscarriage. Pre-eclampsia, for instance, has long thought to originate from defective implantation (Khong et al., 1986). We were unable to examine this outcome using our existing IVF database since we could not rule out the possibility that different criteria were used to define pre-eclampsia by different clinicians. Although we failed to find a direct link between preterm delivery under 34 weeks and low early hCG levels (1D), we may have been underpowered to investigate this obstetric complication. It may be worthwhile exploring an association in a study involving larger numbers in light of recent data from animal studies. Bloomfield *et al* (Bloomfield et al., 2003) used an ovine model to show that maternal starvation around the periconception period can induce preterm delivery. In a mouse model, the administration of leukaemic inhibitory factor (LIF) can prolong gestation (Cheung et al., 2003). LIF is known to play a vital role in implantation. Both of these studies point to possibility that the pathogenic mechanism which causes preterm delivery may begin early in some cases.

It is important to note that our studies relating to early hCG and its association with low birthweight and pregnancy loss were done in an IVF cohort. To be accepted as a model relevant to all pregnancies, these studies would ultimately need be replicated in those who spontaneously conceive. This would be an exceedingly costly and difficult study to undertake. In view of this, it would not be unreasonable to firstly conduct further studies in an IVF cohort, such as those relating to the association between early hCG levels and other obstetric diseases.

If a link is confirmed between early hCG levels and a number of obstetric diseases such as pre-eclampsia, preterm delivery, low birthweight, miscarriage and possibly stillbirth, then this information may represent a new area of obstetric research. The exciting implications is that it may lead to therapeutic treatments and advances in preventative medicine. A reduction in the incidence of some obstetric complications may be achieved using therapeutic agents which enhance the quality of implantation. The prospective of treating an embryo at a very early stage in pregnancy around the time of the missed period may in fact be more achievable, realistic and efficacious then developing agents which are administered later when the fetus has become a complex differentiated multi-organ system.

Therapeutic agents which may enhance implantation have already been identified and are under development. The administration of LIF, which is believed to facilitate implantation, has recently been shown not only to enhance the live birthrate in mice, but also increase birthweight by over 10% (Cheung et al., 2003). Multi-centre clinical trials in humans have already commenced. Should it be discovered that LIF does in fact increase the pregnancy rate in humans, it may also be found to decrease the incidence of low birthweight with the knock-on effect of also decreasing the incidence of adult disease. If evidence suggesting that LIF can prolong gestation in murine models (Cheung et al., 2003) is also applicable in humans, it could may be useful in decreasing the incidence of premature delivery. This obstetric complication is currently responsible for a majority of neonatal deaths and nearly one half of all cases of congenital neurologic

disability including cerebral palsy (Goldberg & Rouse, 1998). Another potential therapy which may enhance implantation is inhibin pro αC, already discussed previously.

Regarding preventative medicine, the suggestion that IVF units restrict the number of embryos transferred to lower the incidence of low birthweight (1D) may, in time, represent the first public health measure which aims to prevent adult disease by altering events which occur during the first two weeks of life.

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