

Increased maternal serum activin A but not follistatin levels in pregnant women with hypertensive disorders

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Abstract

Activin A levels are elevated in maternal serum of pregnant women with hypertensive disturbances. Because follistatin is a circulating binding protein for activin A, the present study was designed to evaluate whether serum follistatin and activin A levels also change in patients with hypertensive disorders in the last gestational trimester. The study design was a controlled survey performed in the setting of an academic prenatal care unit. Healthy pregnant women (controls, $n=38$) were compared with patients suffering from pregnancy-induced hypertension (PIH, $n=18$) or pre-eclampsia ($n=16$). In addition, the study included a subset of patients with pre-eclampsia associated with intrauterine growth restriction (IUGR, $n=5$). Maternal blood samples were withdrawn at the time of diagnosis (patients) or in a random prenatal visit (controls), and serum was assayed for follistatin and activin A levels using specific enzyme immunoassays. Hormone concentrations were corrected for gestational age by conversion to multiples of median (MoM) of the healthy controls of the

same gestational age. Follistatin levels were not different between controls and patients, while activin A levels were significantly increased in patients with PIH (1.8 MoM), pre-eclampsia (4.6 MoM), and pre-eclampsia+IUGR (3.2 MoM, $P<0.01$, ANOVA). The ratio between activin A and follistatin was significantly increased in patients with PIH (1.5 MoM) and was further increased in patients with pre-eclampsia (4.5 MoM) and in the group with pre-eclampsia+IUGR (2.6 MoM). Follistatin levels were positively correlated with gestational age in control subjects ($r=0.36$, $P<0.05$) and in patients with PIH ($r=0.46$, $P<0.05$) or pre-eclampsia ($r=0.61$, $P<0.01$), while activin A correlated with gestational age only in the healthy control group ($r=0.69$, $P<0.0001$). The finding of apparently normal follistatin and high activin A levels in patients with PIH and pre-eclampsia suggests that unbound, biologically active, activin A is increased in women with these gestational diseases.

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Introduction

Follistatin is a monomeric protein produced by several tissues including gonads, pituitary gland and placenta, and whose physiological functions are closely related to its high-affinity binding to activins (Ueno *et al.* 1987). Serum follistatin levels are much higher during pregnancy than throughout the menstrual cycle, and increase in healthy pregnant women from the first to the third trimester (Wakatsuki *et al.* 1996, Woodruff *et al.* 1997, O'Connor *et al.* 1999), probably reflecting placental secretion into the maternal compartment (de Kretser *et al.* 1994, Petraglia *et al.* 1994). The role of follistatin in normal pregnancy is not fully understood. The major role of follistatin may be to antagonize the actions of circulating activins by neutralising their biological activity (Mather

et al. 1993, Muttukrishna *et al.* 1996, McConnell *et al.* 1998). Indeed, the molar concentration of both proteins would suggest that most of the circulating activin A in healthy pregnant women is in a follistatin-bound state (Muttukrishna *et al.* 1996). *In vitro* studies have shown that follistatin antagonises activin-induced progesterone and human chorionic gonadotrophin release from human placental cells (Petraglia *et al.* 1994), suggesting that follistatin may regulate some paracrine actions of activins within human placenta.

High activin A levels are present in maternal and fetal serum (Petraglia *et al.* 1993, Muttukrishna *et al.* 1996) and in amniotic fluid (Petraglia *et al.* 1993), and even higher levels are found in association with some gestational diseases. Maternal serum activin A is increased in pre-term labour (Petraglia *et al.* 1995b), gestational diabetes

Table 1 Characteristics of the study groups. Data are means \pm s.d.

	Control	PIH	Pre-eclampsia	Pre-eclampsia + IUGR
Maternal age (years)	27.1 \pm 3.8	26.0 \pm 4.3	29.2 \pm 5.5	28.4 \pm 3.3
Parity	0.9 \pm 0.9	1.0 \pm 0.9	0.6 \pm 1.1	0.8 \pm 0.9
Gestational age at delivery (weeks)	39.4 \pm 1.2	38.7 \pm 1.6	31.8 \pm 4.6*	32.2 \pm 3.3*
Fetal birth weight (g)	3436 \pm 285	3292 \pm 348	1647 \pm 954*	1684 \pm 715*

* $P < 0.05$ vs control (one-way ANOVA).

(Petraglia *et al.* 1995b) and hypertensive complications of pregnancy, particularly pre-eclampsia (Petraglia *et al.* 1995a, Muttukrishna *et al.* 1997). The putative role for activin A in these conditions is still unknown, but the relevance of the changes suggests a possible action of circulating activin A in the adaptive responses to the pathological conditions. To assess whether the elevated levels of total activin A are always associated with a corresponding increase in follistatin, the present study evaluated serum levels of both proteins in women with hypertensive disorders of pregnancy.

Materials and Methods

Subjects

Four groups of pregnant women ($n=77$) were included in the present study: one group of healthy volunteers (control group, $n=38$) was compared with patients carrying a pregnancy complicated either by pregnancy-induced hypertension (PIH, $n=18$) or pre-eclampsia ($n=16$). In addition, patients with pre-eclampsia associated with intrauterine growth restriction (IUGR, $n=5$) were compared with those presenting with pre-eclampsia only. All subjects were studied during the third trimester, between 26 and 39 weeks of gestation at the time of diagnosis of the gestational disease or in a random pre-natal visit (controls). None had additional complications such as diabetes, infectious diseases or preterm labour. Additional characteristics of the study groups are summarised in Table 1. The protocol was approved by the local ethics committee and verbal informed consent was obtained from all subjects.

Pre-eclampsia and PIH were diagnosed according to the criteria proposed by Davey and MacGillivray (1986). Pregnancy-onset high blood pressure was defined as diastolic levels ≥ 90 mmHg after week 20 of gestation in a woman who reported normal blood pressure before pregnancy and presented with blood pressure under 140/90 mmHg at booking in during the first trimester. Pregnancy-onset hypertension along with antenatal proteinuria ≥ 300 mg/24 h not resulting from chronic renal disease was classified as pre-eclampsia, whereas

pregnancy-onset hypertension without associated proteinuria was classified as PIH. The diagnosis of IUGR was made by assessing fetal growth by fetal ultrasound measurement starting from 16 postmenstrual weeks. Standard measurements of biometry included the biparietal diameter, head circumference, femur length, and abdominal circumference. IUGR was defined as the finding of abdominal circumference which was below the mean for gestational age by at least two standard deviations, or abnormally high head circumference/abdominal circumference ratio (Craig 1994).

Blood samples were collected from the antecubital vein after at least 2 h bed rest, and allowed to clot in plastic tubes at room temperature. After centrifugation at 1000 g for 15 min, serum from the samples was separated and stored at -20 °C until assayed for follistatin and activin A.

Follistatin and activin A assays

Serum activin A concentrations were measured using a specific two-site ELISA as described previously (Knight *et al.* 1996) with minor modifications. Briefly, after pre-treatment, the standard/samples (100 μ l) were added to the wells and incubated overnight at room temperature. The plates were then washed and biotinylated monoclonal antibody was added and incubated for 3 h at room temperature. The assay was then continued as described previously. The standard used was recombinant human activin A purified as described previously (Robertson *et al.* 1992). The assay had a detection limit of 50 pg/ml, and the mean intra- and interplate coefficients of variation were 8.2% and 10.0% respectively.

Serum follistatin concentrations were measured using a sensitive and specific ELISA, as previously described (Evans *et al.* 1998). The standard used was recombinant human follistatin-288 (FS288; kindly provided by NIH, Bethesda, MD, USA). The assay had a sensitivity of 19 pg/ml and the mean intra- and interassay coefficients of variation were 9.0% and 11.0% respectively.

All assays were performed blinded as to whether the samples were from a pathological or a normal control pregnancy.

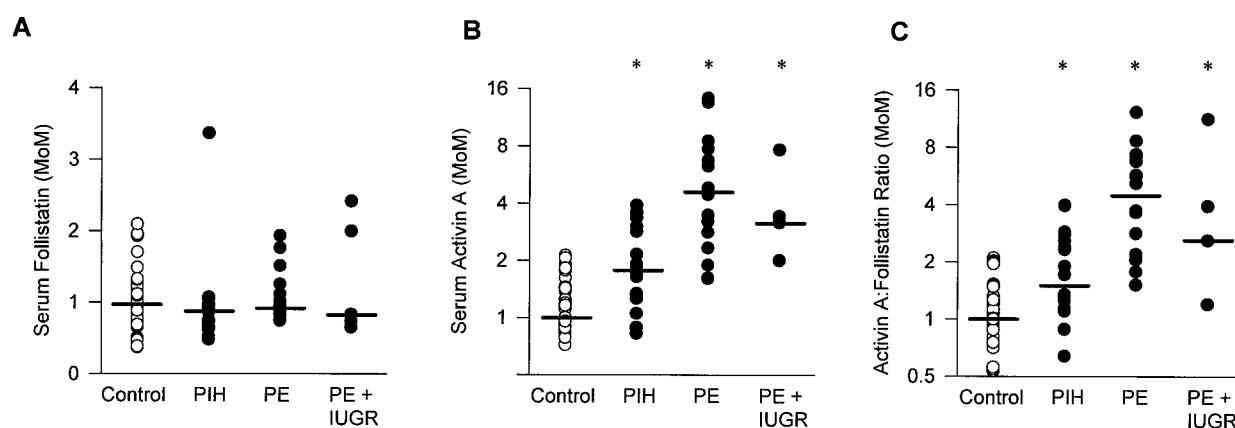


Figure 1 Maternal serum levels of (A) follistatin and (B) activin A, and (C) the ratio between activin A and follistatin in healthy controls (open symbols) and in patients with gestational diseases during the third trimester (closed symbols). PE=pre-eclampsia. Individual values are plotted (expressed as MoM) and horizontal bars represent the group medians. * $P < 0.05$ vs control (Dunn's test).

Data analysis

Serum follistatin and activin A levels were corrected for gestational age by conversion to multiples of median (MoM) of the healthy controls of the same gestational age. The control group (healthy pregnant women) was stratified by gestational age in seven subsets of two weeks each, ranging from 26 to 39 weeks' gestation, and the medians of each stratum were used to convert all values into MoM. Patients were matched with control medians according to gestation length at the time of blood sampling. Follistatin and activin A concentrations (ng/ml) and activin A:follistatin ratios were divided by the median values of control groups pertaining to the same gestation period. This method improves statistical power and permits external validation of results from different populations, besides allowing regression analysis of data in order to reach a day-by-day estimation of the expected normal hormone concentrations (Cuckle *et al.* 1998).

The results are expressed as medians and ranges. Differences between groups were assessed by one-way non-parametric analysis of variance (Kruskal-Wallis ANOVA) and *post-hoc* Dunn's test for multiple comparisons. Correlation between hormone levels and gestational age was assessed by Pearson's linear correlation coefficient (follistatin, normally distributed) or Spearman's rank correlation coefficient (activin A). Between-group differences and correlation coefficients were considered statistically significant whenever $P < 0.05$.

Results

As shown in Fig. 1, serum follistatin levels in control healthy pregnant women (median = 1.0 MoM, range 0.4–2.1 MoM) did not differ significantly compared with PIH (median = 0.9 MoM, range 0.5–3.4 MoM),

pre-eclampsia (median = 1.0 MoM, range 0.7–1.9 MoM), or pre-eclampsia+IUGR (median = 0.8 MoM, range 0.7–2.4 MoM). Conversely, serum activin A levels in patients with pregnancy complicated by PIH (median = 1.8 MoM, range 0.8–3.9 MoM, $P < 0.01$) or pre-eclampsia (median = 4.6 MoM, range 1.6–14.3 MoM, $P < 0.001$) were significantly higher than healthy controls (median = 1.0 MoM, range 0.3–2.1 MoM). In addition, the levels of activin A were equally high in women with pre-eclampsia+IUGR (median = 3.2 MoM, range = 1.5–7.7 MoM; $P < 0.05$ vs healthy controls). The levels of activin A were higher in the group with pre-eclampsia than in the group of patients with PIH ($P < 0.05$). The ratio between activin A and follistatin was significantly increased in patients with PIH (median = 1.5 MoM, range = 0.6–3.9 MoM, $P < 0.05$) and was further increased in patients with pre-eclampsia (median = 4.5 MoM, range = 1.5–12.2 MoM, $P < 0.001$) and in the group with pre-eclampsia+IUGR (median = 2.6 MoM, range = 1.2–16.4 MoM, $P < 0.05$, Fig. 1).

Serum follistatin levels were positively correlated with gestational age in normal third trimester pregnancies ($r = 0.36$, $P < 0.05$, Fig. 2) and also among patients with PIH ($r = 0.46$, $P < 0.05$) and pre-eclampsia ($r = 0.61$, $P < 0.01$). In contrast, serum activin A levels were strongly correlated with gestational age in the control group ($r = 0.69$, $P < 0.0001$) but not in the groups of patients with PIH ($r = 0.39$, $P = 0.10$), or pre-eclampsia ($r = 0.17$, $P = 0.54$, Fig. 3).

Discussion

In the present study we have found normal follistatin levels in patients with PIH or pre-eclampsia in spite of high serum activin A levels, indicating that free activin A levels are likely to be increased in these patients. Since the

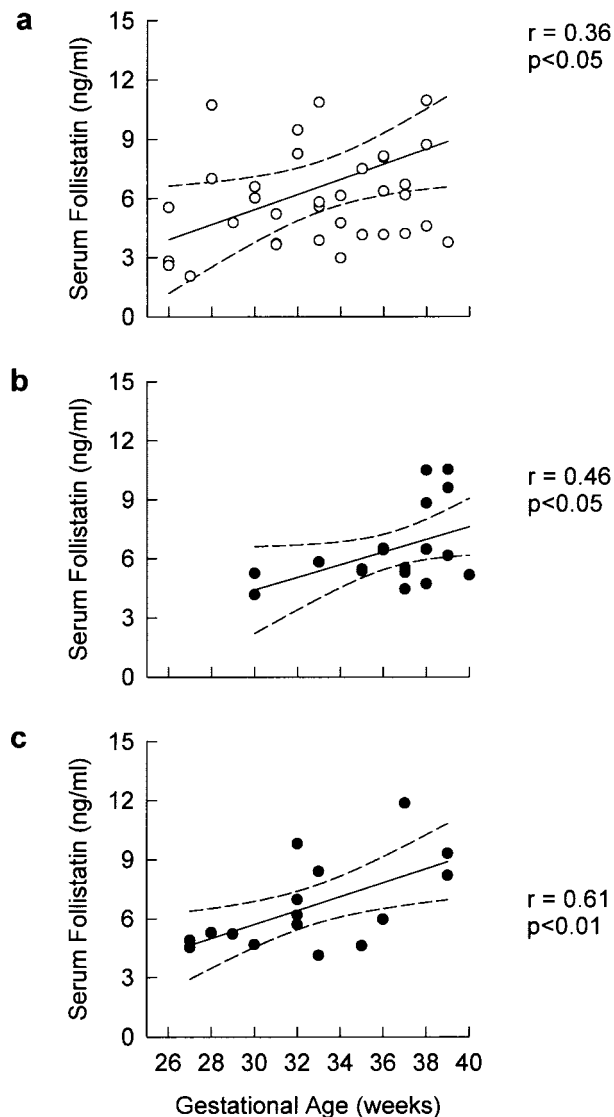


Figure 2 Correlation between gestational age (weeks) and serum follistatin levels in (a) healthy controls, and patients with (b) pregnancy-induced hypertension or (c) pre-eclampsia. Dashed lines represent the 95% confidence intervals for linear regression where Pearson's linear correlation coefficient (r) was statistically significant.

increase of activin A was more pronounced in women with pre-eclampsia than in women with PIH, the rise of activin A seems to reflect the entity of gestational disease rather than a mere placental response to high blood pressure. This specific effect of pre-eclampsia does not preclude the existence of nonspecific mechanisms eliciting activin A secretion in response to fetoplacental disorders, as suggested by the modest but significant increase of activin A in women with PIH. The lack of an additional increase of activin A in women with pre-eclampsia and

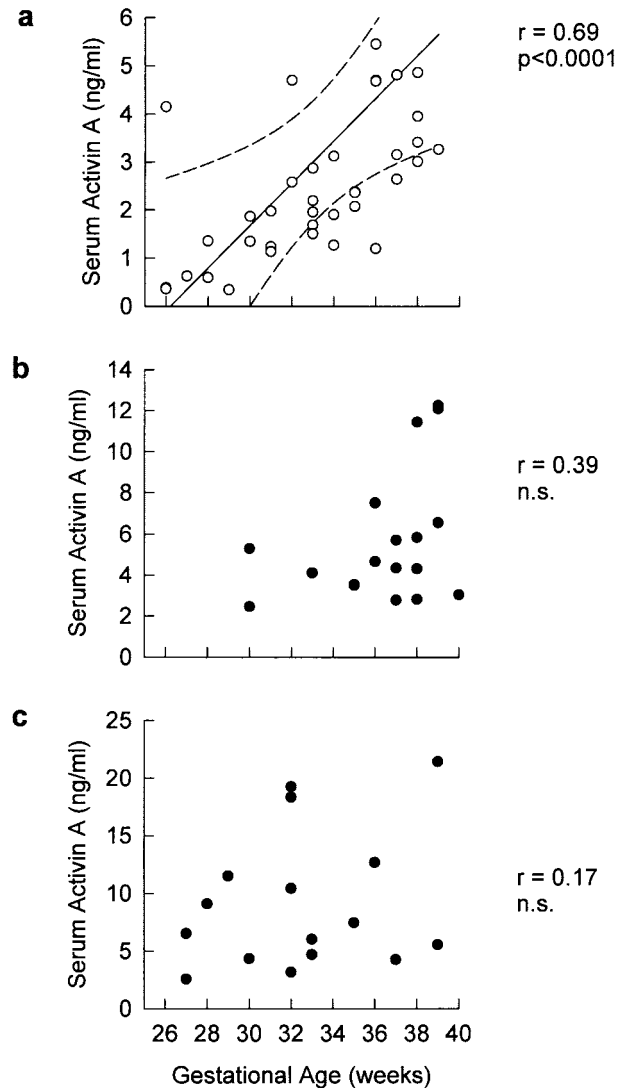


Figure 3 Correlation between gestational age (weeks) and serum activin A levels in (a) healthy controls, and patients with (b) pregnancy-induced hypertension or (c) pre-eclampsia. Dashed lines represent the 95% confidence intervals for linear regression where Spearman's rank correlation coefficient (r) was statistically significant. n.s., not significant.

superimposed IUGR suggests that high maternal serum activin A levels are better associated with the grade of placental rather than fetal compromise.

The role of inhibin-related peptides in gestational diseases is still a matter of speculation. It is recognised that human placenta secretes significant amounts of inhibin A, activin A and follistatin throughout gestation, resulting in a progressive increase of these proteins in maternal circulation up to week 36 (Fowler *et al.* 1998). Abnormally high levels of inhibin A may be detected as early as 22 weeks before the onset of pre-eclampsia (Cuckle *et al.* 1998),

suggesting that inhibin A is a useful marker, and possibly an active component of the placental alterations preceding clinical manifestation of the disease. A role for activin A in the etiopathogenesis of pre-eclampsia may also be postulated, as we have previously shown an increase of activin A secretion preceding the clinical manifestations of superimposed pre-eclampsia in hypertensive patients (Petraglia *et al.* 1995a). The present demonstration that the activin A:follistatin ratio is severely increased in pre-eclampsia adds evidence for a biological effect of activin A, if not in the development of the disease, at least as a fetoplacental adaptive response.

Another critical and intriguing finding of the present study was the absence of a correlation between activin A levels and gestational age in the pathological groups, contrasting with the strong correlation observed in the healthy control group. This difference may be explained because the elevation of activin A probably represents an acute response to the onset of fetoplacental disorders rather than a simple displacement of activin A levels above the physiological curve. In contrast, follistatin levels displayed a weak but significant correlation with the duration of gestation in the third trimester of pregnancy in the healthy control group, as well as in the groups with PIH or pre-eclampsia. This finding suggests that placental follistatin secretion is regulated by mechanisms independent of activin A during the third trimester in pregnancies affected by hypertensive disorders. The physiological increase of follistatin during the third trimester is preserved in these patients regardless of the hypertensive syndrome and in spite of placental distress. Although the rise in serum follistatin levels occurs earlier than activin A in normal pregnancy, and follistatin may be a mechanism to protect the mother from the widespread actions of activin A (O'Connor *et al.* 1999), the present data indicate that this compensatory increase of follistatin does not accompany the abnormal increase of activin A in women with hypertensive disturbances of pregnancy.

The absolute values of follistatin concentration reported here must be interpreted cautiously. Our assay measures predominantly FS288, and recent work suggests that follistatin in human serum may be predominantly FS315 (Schneyer *et al.* 1996). If this is the case, then we would expect our assay to underestimate the true values (Evans *et al.* 1998). A precise quantification of total follistatin concentrations is still unachievable by current immunoassays. Nevertheless, the high starting sensitivity of our method compensates for the reduced cross-reaction with FS315 and permits the detection of minimal differences within and between individuals under several physiological and clinical conditions (Evans *et al.* 1998, Fowler *et al.* 1998, O'Connor *et al.* 1999).

In summary, we have shown that maternal serum follistatin levels during the third trimester of pregnancy are unaffected by PIH, pre-eclampsia or IUGR and remain in the normal range for third trimester gestation. The finding

of apparently normal follistatin and high activin A levels in patients with PIH or pre-eclampsia suggests that unbound, thus biologically active, activin A is increased in women with these types of gestational diseases.

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