Maternal Serum Activin A and Follistatin Levels in Pregnanacies with Down Syndrome

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INTRODUCTION

Seven studies have now shown that maternal serum inhibin A levels are raised on average in second-trimester pregnancies with Down syndrome (Wallace et al., 1996; Wald et al., 1996; Aitken et al., 1996; Cuckle et al., 1996; Lambert-Messerlian et al., 1996a; Spencer et al., 1996; Westrom et al., 1997). Inhibin A is a dimer (α-β2) and the β2 subunit is shared with activin A (βα-βα). A statistically significant increase in maternal serum activin A has been reported in 10 Down syndrome pregnancies compared with 50 controls (Lambert-Messerlian et al., 1996b). Although the magnitude of the increase was not great the confidence limits were wide due to the small number of cases studied. We therefore decided to investigate activin A and its binding protein, follistatin, in a larger series.

MATERIALS AND METHODS

A series of maternal serum samples were selected for activin A and follistatin measurement from a bank of samples which had been stored at −20°C or cooler. A total of 30 singleton Down syndrome pregnancies were studied: 7 at 13 completed weeks’ gestation, 4 at 14 weeks, 11 at 15 weeks and 8 at 16 weeks. For comparison 199 samples from singleton unaffected control pregnancies were also tested at 13–16 weeks’ gestation: 49, 50, 53 and 47 at each completed week. Gestational age was based on ultrasound examination for all cases and controls. Serum samples from cases were taken either routinely as part of Down syndrome screening (23 cases) or specifically for research purposes after an ultrasonic indication (2 cases) or treated control pregnancy (1). The remaining two cases had maternal serum screening; one had a positive result but refused prenatal diagnosis and the second was a false negative.

All tests were carried out without knowledge of which sample was from an affected pregnancy and which a control. Activin A was measured using a previously described enzyme immunoassay (Knight et al., 1996) with minor adjustments. The standard used was a pool of bovine follicular fluid calibrated against recombinant human activin A. Both standards and samples were diluted 1:3 in 5 per cent (w/v) bovine serum albumin in PBS (0.02 M). An equal volume of 10 per cent (w/v) sodium dodecyl sulphate was added to both standards and samples which were then placed in a boiling water bath for 3 minutes to dissociate follistatin–activin complexes. After cooling, hydrogen peroxide was added (Knight and Muttukrishna, 1994) giving a final concentration of ~2 per cent (v/v) and incubated for 30 minutes. The standards and samples were applied to 96-well ELISA plates which had been pre-coated with a mouse monoclonal antibody specific for the βα subunit (E4 (Groome and Lawrence, 1991)). Biotinylated E4 was added and the plates were incubated overnight at room temperature. To detect this complex streptavidin alkaline phosphatase was added after washing the plates, and activity was measured using an ELISA amplification system (Life Technologies, UK). Well absorbances were read at 490 nm using a microplate reader with reference wavelength set at 620 nm and interpreted with Kinecalc software (Bio-Tek Instruments, Winooski, USA).

Follistatin was also measured by enzyme immunoassay (Evans et al., 1998). Briefly, mouse monoclonal antibody 29/9 was coated on to 96-well ELISA plates and treated with a dry coat reagent (Bioonics Ltd, Wyboston, UK) to enable dry storage. The standard

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used was immunopurified follistatin from human follicular fluid which had been calibrated against recombinant human follistatin-288. This had been freeze-dried for storage and, prior to assay, was reconstituted in dissociating solution: 0.02 M PBS containing 84 mM sodium deoxycholate, 3.4 per cent (v/v) Tween 20, 1 per cent (w/v) bovine serum albumin, 5 per cent (v/v) mouse serum. Standards and samples were treated similarly throughout. Both were diluted 1:4 in dissociating solution to dissociate follistatin–activin complexes, and applied to the plates in duplicate. After overnight incubation at room temperature the plates were washed and the Fab fragment of detection antibody 17/2, which was coupled to alkaline phosphatase, was added and incubated at room temperature for two hours. After extensive washes activity was detected using the same system as for activin A.

Results were expressed as multiples of the normal median (MOM) for gestation, to the day. Normal medians were obtained by regression of the observed median in controls for each completed week, weighted for the number tested in the week. The regression equations were \( \log_{10}(\text{activin}) = 3.34246 - 0.001871 \times d \) and \( \log_{10}(\text{follistatin}) = 2.86274 + 0.004165 \times d \), where \( d = \text{gestation in days} \). Maternal weight was allowed for by dividing the MOM by the expected value for the weight based on regression analysis. The equations for activin A and follistatin were, respectively, \( 0.544106 + 27.9509/w \) and \( 0.451521 + 34.8936/w \), where \( w = \text{weight in kg} \).

Each of the 229 samples had been previously tested for \( a \)-fetoprotein (AFP), unconjugated oestriol (uE3), free \( \beta \) human chorionic gonadotrophin (hCG) and inhibin A. The tests on samples taken for screening were done prospectively and the research samples were tested in the next routine analytical batches after collection. Commercial assay kits were used (Amerlex-M, Ortho Diagnostics Ltd, Amersham, UK; DELFIA\textsuperscript{®}, EG&G Wallac Oy, Turku, Finland; Serotec Ltd, Oxford, UK), levels were expressed in MOMs, and adjusted for maternal weight.

For statistical purposes standard deviations were estimated indirectly. In unaffected pregnancies the 10–90th centile range divided by 2.563 was used and in Down syndrome, with fewer data, the interquartile range was used instead, dividing it by 1.350. Correlation coefficients were estimated by first excluding outliers exceeding three standard deviations from the median.

**RESULTS**

There was a highly statistically significant elevation in activin A levels among Down syndrome pregnancies (\( p \leq 0.005 \), Wilcoxon rank-sum test, two-tail). Fig. 1 shows the individual levels in cases according to gestation compared with selected centiles in the controls. There were eight cases (27 per cent) with levels exceeding the 90th centile, 1.65 MOM, and only two (6.7 per cent) below the 10th centile, 0.69 MOM. The corresponding proportions for the 95th and 5th centiles were six (20 per cent) and one (3.3 per cent), respectively. The median activin A level for Down syndrome was 1.19 MOM with 95 per cent confidence limits of 0.97–1.46. The standard deviation of \( \log_{10} \) activin A
was 0.20 and 0.15 for the cases and controls, respectively. So the median in Down syndrome was elevated by 0.4 standard deviation (the average for affected and unaffected pregnancies). By comparison in this series of cases the median inhibin A, free β-hCG, AFP and uE₃ levels were 2.25, 2.46, 0.77 and 0.75 MOM, respectively, equivalent to elevations and reductions of 1.7, 1.5, 0.7 and 0.6 standard deviations.

The distribution of follistatin levels in cases was reduced but this did not reach statistical significance (p=0.2, Wilcoxon rank-sum test). The 25th, median and 75th centile levels were 0.71, 0.93 and 1.16 MOM for cases compared with 0.79, 1.02 and 1.67 MOM, respectively, for controls. The 95% confidence limits on the affected median were 0.79–1.09.

For both analytes the median level was higher among the seven cases with samples taken after chorionic villus sampling compared with those taken as part of routine screening. The medians were 1.75 and 1.18 MOM, respectively, for activin A; they were 1.05 and 0.88 MOM for follistatin. These differences were probably due to chance alone but we cannot exclude the possibility of the invasive procedure causing levels to increase. In three of the screening cases post-chorionic villus samples had been stored and so could be used to examine the possibility directly. The activin A levels in the second sample were 87 per cent, 98 per cent and 120 per cent of the first; the follistatin levels were 121 per cent, 127 per cent and 310 per cent.

The results of a correlation analysis between the logarithms of activin A, follistatin and the other marker levels is shown in Table 1. There were highly statistically significant positive associations between activin A and inhibin A among both affected (0.56) and unaffected pregnancies (0.37). The only other marker to be significantly correlated with activin A was free β-hCG but only in controls. The correlation between activin A and follistatin approached statistical significance, but only in cases. Follistatin was itself significantly correlated with inhibin A, but only in cases, and with free β-hCG but only in controls. None of these correlations will have biased the results of the study as the median levels of the established markers in the cases were not atypical.

### DISCUSSION

We found the level of maternal serum activin A to be raised on average in pregnancies with Down syndrome at 13–16 weeks' gestation. Another study examined this analyte in 10 cases at 15–18 weeks (Lambert-Messerlian et al., 1996b) and found a similar median activin A level to the present study (1.25 MOM compared with 1.19 MOM). The individual values were reported so that both series could be pooled. The median value for all 40 cases of Down syndrome combined was 1.22 MOM. A more recent study from the same group examined 20 cases at 15–20 weeks and found a median activin A level 0.82 times that in 100 controls (Lambert-Messerlian et al., 1998). However, these results cannot be directly compared with the other series as the cases and controls were not matched for gestation and values were not expressed in multiples of the gestation-specific median.

The activin A assay used in this study and those of Lambert-Messerlian et al. (1996a) measured both the free and follistatin-bound species. Maternal serum free activin A has also been reported in 20 Down syndrome pregnancies (Lambert-Messerlian et al., 1996a). The median level was 1.16 MOM, although this was not statistically significant.

This is the first study to examine maternal serum follistatin levels in Down syndrome pregnancies. There was a tendency for the distribution of levels to be reduced slightly but this could have been due to chance. The follistatin assay we used measured both free and bound species with a high affinity for follistatin-288. However, the assay has been shown to have considerable cross-reaction with follistatin-315 (Evans et al., 1998). As this is the predominant form in serum (Schneyer et al., 1996) it is possible we have measured only a fraction of the total serum follistatin. This could not be clarified further due to the lack of a specific ELISA for follistatin-315.

Whilst maternal serum activin A levels were increased on average in Down syndrome pregnancies the extent of overlap with unaffected pregnancies was too great for it to be of practical use in screening. Thus,
the inhibin A dimer $\alpha-\beta_A$ is a strong marker of Down syndrome whilst the activin A dimer $\beta_A-\beta_A$ is not.

REFERENCES


