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Are Serum Protein Biomarkers Derived from Proteomic Analysis Useful in Screening for Trisomy 21 at 11–13 Weeks?

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Key Words
Trisomy 21 · Proteomics · First-trimester screening · Biochemical markers

Abstract
Objective: The aim of this study is to identify potential biomarkers for fetal trisomy 21 from previous publications using proteomic techniques and examine the potential value of such biomarkers in early screening for this aneuploidy. ***Methods:*** This was a case-control study of 25 pregnancies with fetal trisomy 21 and 50 euploid controls undergoing first-trimester screening for aneuploidies by a combination of maternal age, fetal nuchal translucency (NT) thickness and maternal serum free β-human chorionic gonadotrophin (β-hCG) and pregnancy-associated plasma protein-A (PAPP-A). The maternal serum concentrations of afamin, apolipoprotein E, clusterin, ceruloplasmin, epidermal growth factor, fetuin-A, pigment epithelium-derived factor glycoprotein and transthyretin were determined using an ELISA and compared in the euploid and trisomy 21 groups. ***Results:*** In pregnancies with fetal trisomy 21, the median maternal age, fetal NT thickness and serum free β-hCG were increased, whereas serum PAPP-A was decreased. However, there were no significant differences between cases and controls in any of the biomarkers. ***Conclusion:*** Proteins identified as potential biomarkers for trisomy 21 using proteomic techniques have not been found to be useful in early screening for this aneuploidy.

Introduction

Effective screening for fetal trisomy 21 is provided in the first trimester of pregnancy by a combination of maternal age, fetal nuchal translucency (NT) thickness and biochemical measurement of maternal serum free β-human chorionic gonadotrophin (β-hCG) and pregnancy-associated plasma protein-A (PAPP-A) with a detection rate of about 90% for a false-positive rate of 5% [1–3]. Attempts at improving the performance of screening have mainly focussed on the identification of additional sonographic markers, including absence of the nasal bone, tricuspid regurgitation, reversed a-wave in the fetal ductus venosus and increased velocity in the hepatic artery [3]. Several biochemical markers, including ADAM-12, PLGF and PP-13, have also been investigated, but were not found to be useful.

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Identification of the established useful first- and second-trimester biomarkers of aneuploidy was incidental rather than the result of a systematic prospective investigation based on genetic or pathophysiological principles. The emerging field of proteomics has raised high expectations for the discovery of new biomarkers that would improve the performance of screening for trisomy 21. Essentially, proteomics involves detailed examination of the protein profile in biological tissues and fluids and identification of differences in the profile obtained from a pathological compared to a physiological condition [4, 5].

The aim of this study is to identify potential biomarkers for fetal trisomy 21 from previous publications using proteomic techniques and examine the potential value of such biomarkers in early screening for this aneuploidy.

Methods

Study Population

This was a case-control study drawn from a large prospective study to identify potential biomarkers of pregnancy complications in women during their routine first hospital visit in pregnancy at King's College Hospital, London, UK. In this visit, which is held at 11^{+0} to 13^{+6} weeks of gestation, all women have combined screening for aneuploidies by maternal age, ultrasound measurements of fetal crown-rump length (CRL) and NT thickness and biochemical measurement of maternal serum PAPP-A and free β-hCG [1, 2, 6]. Maternal demographic characteristics, ultrasononographic measurements and biochemical results were recorded in a computer database. Karyotype results and details on pregnancy outcomes were added into the database as soon as they became available. Written informed consent was obtained from the women agreeing to participate in the study, which was approved by the King's College Hospital Ethics Committee. They agreed for aliquots of their serum used for the measurement of free β-hCG and PAPP-A to be stored at –80°C for future studies.

The case-control study population consisted of 25 cases with fetal trisomy 21 and 50 euploid controls. Each case of fetal trisomy 21 was matched with 2 euploid controls with blood collected within 1 week of each other. None of the samples in the case-control study were previously thawed and refrozen.

Literature Search

We searched Medline and Embase from January 2000 to April 2010 to identify studies which used proteomics techniques to investigate potential biomarkers for fetal trisomy 21 in maternal blood. The search identified four such studies [7–10], but one of these did not report a list of potential biomarkers [7]. One first-trimester study identified seven potential biomarkers [10], but in a subsequent validation study only one of the proteins (epidermal growth factor) was significantly decreased in maternal serum from trisomy 21 pregnancies [11]. One second-trimester study provided a list of nine potential biomarkers [9] and one first-trimester study provided a list of 28 potential biomarkers [8]. There were five proteins with commercially available ELISA kits from the list of the first-trimester study [8] which were also in the list of the second-trimester study [9], and we selected these five proteins for our study (afamin, apolipoprotein E, clusterin, ceruloplasmin and transthyretin). In addition, we selected epidermal growth factor [10, 11] and two additional proteins with large differences between trisomy 21 and euploid fetuses (fetuin-A and pigment epithelium-derived factor glycoprotein) reported in the first-trimester study [8], but not in the second-trimester study [9].

Sample Analysis

None of the samples were previously thawed and refrozen. None of the samples showed any signs of hemolysis or lipemia prior to analysis. The source of the ELISA used, together with intra- and interassay coefficients of variation and minimum detectable limits for each biomarker are summarized in table 1.

Statistical Analysis

The significance of difference between the outcome groups was examined by a χ^2 test or Fisher's exact test for categorical variables and Mann-Whitney U test for continuous variables. The distributions of the biomarkers were made Gaussian after logarithmic transformation and the normality of distributions was examined using histograms and probability plots. In the euploid group, multiple regression analysis was used to determine if any factors amongst the maternal characteristics and gestation were significant predictors of each biomarker. In each euploid and trisomy 21 case, the measured concentration of each biomarker was expressed as a multiple of the median of the euploid group, after appropriate adjustment for those factors found to be significant in the multiple regression analysis in euploid fetuses. The measured PAPP-A and free β-hCG were converted into the multiple of the median after adjustment for gestation, racial origin, weight, parity, cigarette smoking status and method of conception as previously described [12], and the measured NT was expressed as a difference from the expected normal mean for CRL (delta value) [13].

The statistical software package SPSS 16.0 (SPSS Inc., Chicago, Ill., USA) was used for data analyses.

Results

The maternal characteristics and results of the combined test in the cases of trisomy 21 and euploid controls are compared in table 2. In pregnancies with fetal trisomy 21, the median maternal age, fetal NT and serum free β-hCG were increased, whereas serum PAPP-A was decreased.

In the euploid pregnancies, multiple regression analyses demonstrated that the maternal serum concentrations for most biomarkers was not significantly affected by maternal age, weight, racial origin, parity, smoking status, use of ovulation induction drugs or fetal CRL (table 3). The concentrations of apolipoprotein, ceruloplasmin and fetuin-A were affected by maternal weight and apolipoprotein E was also affected by fetal CRL:

Table 1. Source of the ELISA used for the assays, intra- and interassay coefficients of variation, and minimum detectable limits for each biomarker

Biomarker	ELISA	Coefficient of variation, %		Minimum detectable limit
		intra-assay	interassay	
Afamin	EIA-5085; DRG instruments GmbH, Marburg, Germany	4.1–7.2	2.1–8.5	0.01 μg/l
Apolipoprotein E	EA8003-1; Assaypro, St. Charles, Mo., USA	4.6	7.7	10 ng/ml
Clusterin	DCLU00; R&D Systems Europe Ltd., Abingdon, UK	3.4–3.7	6.8–8.4	0.189 ng/ml
Ceruloplasmin[1]	DakoCytomation Ltd.; Cambridgeshire, UK	2.6–3.9	3.9–4.5	0.05 g/l
Transthyretin	EP3010-1; Assaypro, St. Charles, Mo., USA	4.9	7.5	1 ng/ml
Fetuin-A	EG3501-1; Assaypro, St. Charles, Mo., USA	4.8	7.2	5 ng/ml
Pigment epithelium-derived factor glycoprotein	RD191114200R; Biovendor R&D Products, Modrice, Czech Republic	2.9–4.1	5.3–6.6	0.05 ng/ml
Epidermal growth factor	DEG00; R&D Systems Europe Ltd., Abingdon, UK	2.0–4.4	4.3–6.1	0.70 pg/ml

[1] Immunoturbidimetric method rather than ELISA.

Table 2. Maternal characteristics and results of the combined test in cases of trisomy 21 and euploid controls

Maternal characteristic	Euploid (n = 50)	Trisomy 21 (n = 25)
Median maternal age, years (IQR)	32.3 (29.0–35.3)	37.1 (30.8–39.3)*
Median maternal weight, kg (IQR)	65.0 (59.0–73.0)	64.0 (59.5–77.5)
Median gestation at sampling, days (IQR)	87.0 (85.0–90.0)	89.0 (86.0–93.0)*
Racial origin, n (%)		
Caucasian	34 (68.0)	21 (84.0)
African	11 (22.0)	2 (8.0)
South Asian	3 (6.0)	0
East Asian	1 (2.0)	1 (2.0)
Mixed	1 (2.0)	1 (2.0)
Nulliparous, n (%)	27 (54.0)	7 (28.0)*
Cigarette smoker, n (%)	2 (4.0)	2 (8.0)
Conception, n (%)		
Spontaneous	47 (94.0)	23 (92.0)
Assisted conception	3 (6.0)	2 (8.0)
Fetal NT thickness		
mm	1.6 (1.4–1.8)	3.8 (3.0–5.0)*
Delta value	0.02 (–0.16 to 0.15)	2.1 (1.1–3.1)*
Serum PAPP-A		
mIU/l	2.73 (1.96–4.28)	1.36 (0.79–2.32)*
MoM	1.00 (0.68–1.39)	0.46 (0.33–0.61)*
Serum free β-human chorionic gonadotrophin		
mIU/l	39.2 (29.3–56.6)	67.0 (43.4–117.5)*
MoM	1.00 (0.74–1.36)	2.12 (1.23–3.10)*

Data presented as either median and interquartile range or as n (%). Comparison with euploid group (χ^2 test or Fisher's exact test for categorical variables and Mann-Whitney U test for continuous variables). * $p < 0.05$. MoM = Multiple of the median.

Table 3. Multiple regression analysis demonstrating the effect of fetal CRL and maternal characteristics on each of the proteomic biomarkers examined in the study

Biomarker	Maternal age, years	Maternal weight, kg	Racial origin	Cigarette smoking	Parity	Method of conception	CRL, mm
Afamin							
Coefficient *(b)*	–0.001	1.2 e^{-04}	–	–0.157	–0.026	0.111	0.002
p value	0.717	0.927	0.749	0.105	0.505	0.125	0.339
Apolipoprotein E							
Coefficient *(b)*	–0.001	0.003	–	–0.012	0.013	–0.086	0.004
p value	0.760	0.006	0.239	0.876	0.695	0.152	0.054
Clusterin							
Coefficient *(b)*	0.002	0.001	–	–0.076	–0.007	0.025	0.001
p value	0.432	0.202	0.356	0.200	0.785	0.571	0.404
Ceruloplasmin							
Coefficient *(b)*	0.001	–0.001	–	0.016	0.013	–0.055	4.1 e^{-04}
p value	0.690	0.048	0.759	0.722	0.470	0.102	0.657
Epidermal growth factor							
Coefficient *(b)*	0.016	0.006	–	–0.223	0.214	0.068	–0.010
p value	0.165	0.161	0.273	0.477	0.103	0.770	0.167
Fetuin-A							
Coefficient *(b)*	4.1 e^{-04}	0.001	–	0.005	–0.003	0.047	0.001
p value	0.722	0.003	0.741	0.874	0.841	0.062	0.137
Pigment epithelium-derived factor glycoprotein							
Coefficient *(b)*	–0.004	0.001	–	–0.004	–0.043	0.113	7.0 e^{-05}
p value	0.244	0.343	0.624	0.967	0.287	0.128	0.974
Transthyretin							
Coefficient *(b)*	3.4 e^{-04}	0.002	–	–0.180	–0.019	0.034	4.8 e^{-04}
p value	0.937	0.361	0.205	0.143	0.702	0.710	0.860

$\log_{10}$ expected apolipoprotein E = 1.199 + 0.003 × maternal weight in kg + 0.004 × fetal CRL in mm; $R^2 = 0.188$, p = 0.003;

$\log_{10}$ expected ceruloplasmin = –0.687 to 0.001 × maternal weight in kg; $R^2 = 0.070$, p = 0.035;

$\log_{10}$ expected fetuin-A = 2.748 + 0.001 × maternal weight in kg; $R^2 = 0.113$, p = 0.010.

In pregnancies with fetal trisomy 21, compared to the euploid group, there was no significant difference in the maternal serum concentration of any of the biomarkers (table 4, fig. 1).

Discussion

This study investigated the value of eight potential biomarkers for trisomy 21 identified from previous studies using proteomic techniques. In the publications there was a 2- to 7-fold difference in the concentration of the selected biomarkers between trisomy 21 and euploid pregnancies. We then used ELISA for the measurement of the maternal serum concentration of these proteins in a group of trisomy 21 pregnancies and euploid controls and found no significant differences between the two.

The failure to confirm the results of proteomic studies to identify useful circulating maternal biomarkers of fetal trisomy 21 reflects the inherent limitations of proteomic research in identifying biomarkers of disease. Humans produce about 2 million proteins or protein fragments which in turn undergo chemical changes to modify their action and properties resulting in 10–20 million chemically distinct polypeptides [4, 14]. However, more than 95% of these proteins are accounted for by albumin, immunoglobulins and 10 other proteins, which mask less abundant proteins that may be differentially expressed in various disease processes [5, 15]. The first step in proteomic research is depletion of the high-abundant proteins from the sample, but this process inevitably results in a major loss of less abundant markers of disease whose concentration may be as low as nanograms [15–17]. Currently, there is no consensus on how many and which proteins or group of proteins should be

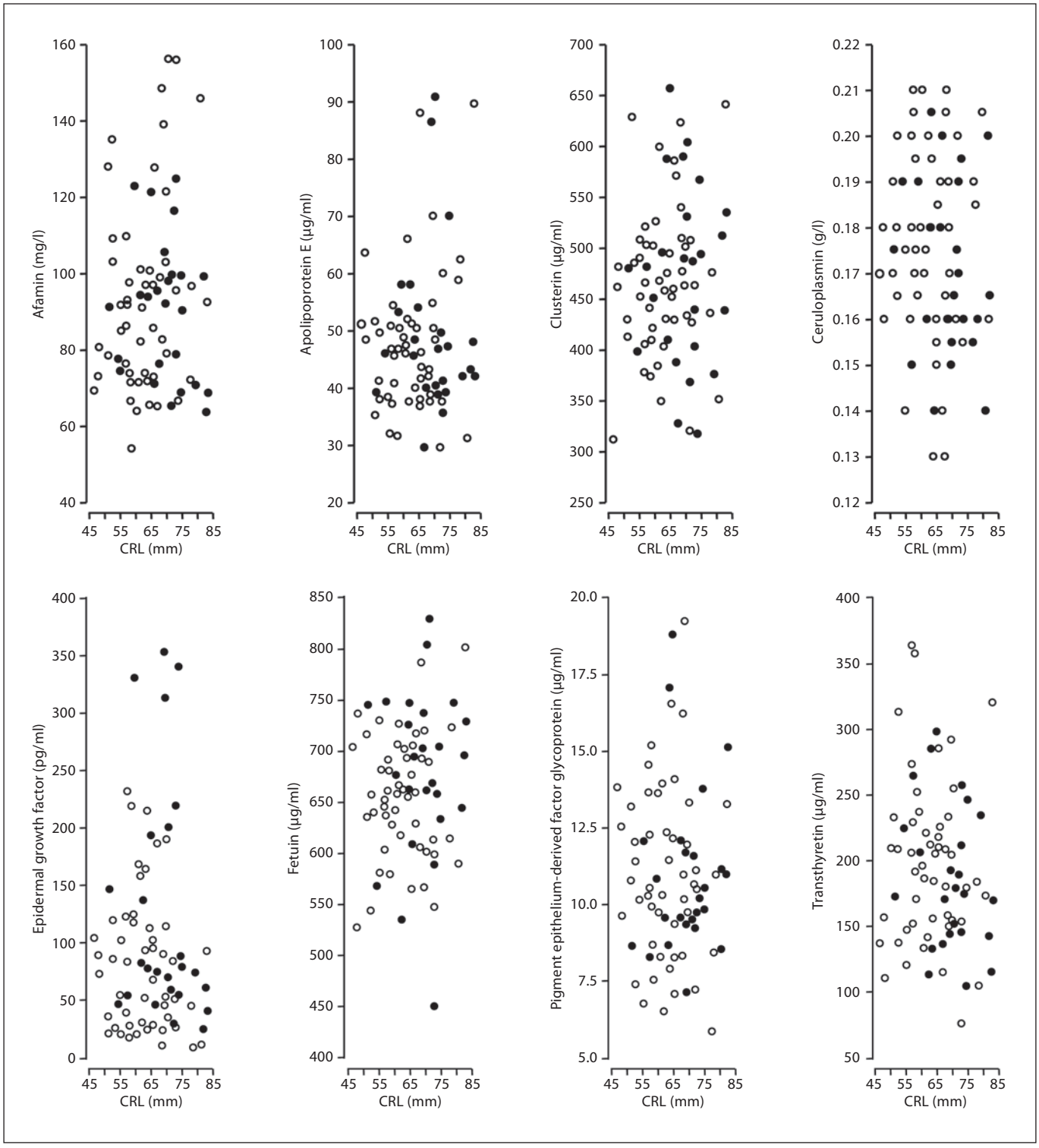

Fig. 1. Scatter plots of maternal serum concentration of afamin, apolipoprotein E, clusterin, ceruloplasmin, epidermal growth factor, fetuin-A, pigment epithelium-derived factor glycoprotein factor and transthyretin in trisomy 21 (open circles) and euploid (closed circles) pregnancies plotted against fetal CRL.

Table 4. Median (IQR) of maternal serum afamin, apolipoprotein E, clusterin, ceruloplasmin, epidermal growth factor, fetuin-A, pigment-derived growth factor and transthyretin in euploid and fetal trisomy 21 pregnancies

Biomarker	Euploid (n = 50)	Trisomy 21 (n = 25)	p value
Afamin			
mg/l	91.3 (73.0–103.0)	92.1 (72.7–99.3)	
MoM	1.00 (0.80–1.13)	1.01 (0.80–1.09)	0.779
Apolipoprotein E			
μg/ml	46.4 (38.0–51.3)	46.0 (40.2–53.6)	
MoM	1.01 (0.84–1.15)	0.88 (0.77–1.12)	0.196
Clusterin			
μg/ml	463.1 (425.3–503.4)	481.2 (400.6–532.6)	
MoM	1.00 (0.92–1.09)	1.04 (0.87–1.15)	0.728
Ceruloplasmin			
g/l	0.18 (0.16–0.19)	0.16 (0.16–0.19)	
MoM	1.02 (0.93–1.09)	0.97 (0.90–1.05)	0.233
Epidermal growth factor			
pg/ml	77.6 (27.7–114.7)	77.0 (53.8–196.6)	
MoM	1.00 (0.36–1.48)	0.99 (0.69–2.53)	0.099
Fetuin-A			
μg/ml	658.5 (611.1–702.3)	694.2 (638.5–741.1)	
MoM	1.01 (0.96–1.06)	1.06 (0.97–1.11)	0.103
Pigment epithelium-derived factor glycoprotein			
μg/ml	10.7 (8.6–13.2)	10.2 (9.3–11.9)	
MoM	1.00 (0.80–1.23)	0.95 (0.87–1.11)	0.686
Transthyretin			
μg/ml	193.4 (152.8–229.7)	174.3 (142.9–229.3)	
MoM	1.00 (0.79–1.19)	0.90 (0.74–1.19)	0.406

Comparison between groups by Mann-Whitney U test.
MoM = Multiple of the median.

removed from the samples of interest prior to proteomic profiling [5, 18].

There are also challenges in the second and third steps of the proteomic analysis, which involve protein separation, by 2D gel electrophoresis or 2D liquid chromatography, and protein identification using mass spectrometry-based methods. The limitations of these methods include, poor reproducibility, low accuracy and inability to identify low molecular weight proteins [4, 19–21]. In addition to such technical problems, there is another inherent limitation in proteomic analysis because the pattern of proteins expressed by a given genome is influenced not only by the disease under investigation, but also by such factors as age, sex, physical activity, time of sampling and tissue of origin [20].

Our results are consistent with the findings of two meta-analyses which essentially highlighted the inability of current proteomic technologies to identify potentially useful biomarkers in a wide variety of diseases in both humans and animals [21, 22]. The studies reported that some proteins or protein families are commonly reported as being differentially expressed regardless of species, in vivo or in vitro conditions, tissues and organs, and experimental objective. Interestingly, some of the proteins which we examined in this study such as afamin, ceruloplasmin and transferrin have also been reported by proteomic studies to be differentially expressed in patients with squamous cell carcinoma of the cervix, ovarian cancer, nasopharyngeal carcinoma and in those with central nervous system damage [23–26].

Comparison of the proteomic profile in patients with a variety of pathological conditions with healthy individuals could potentially improve our understanding of disease and lead to improved methods of screening, diagnosis and monitoring of the effects of therapeutic interventions. However, this high expectation has been met with

limited success [4, 5, 19, 27, 28]. Our findings illustrate the limitations of the proteomic technologies and demonstrate that biomarkers identified from current proteomics studies are not useful in early screening for trisomy 21.

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