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Second-Trimester Maternal Serum Invasive Trophoblast Antigen: A Marker for Down Syndrome Screening, *Raj Pandian,[1*] Laurence A. Cole,[2] and Glenn E. Palomaki,[3]* ([1] Quest Diagnostics Nichols Institute, San Juan Capistrano, CA; [2] University of New Mexico, Albuquerque, NM; [3] Foundation for Blood Research, Scarborough, ME; * address correspondence to this author at: Quest Diagnostics Nichols Institute, 33608 Ortega Hwy., San Juan Capistrano, CA 92690; e-mail Raj.M.Pandian@questdiagnostics.com)

More than 2 million pregnancies in the United States are screened for Down syndrome each year, most often in the second trimester, by a test using three biochemical markers in combination with maternal age *(1)*. The "triple" test includes measurements of serum α-fetoprotein (AFP), unconjugated estriol (uE3), and human chorionic gonadotropin (hCG). In the general pregnancy population, the triple test can identify ~65% of Down syndrome pregnancies at a 5% false-positive rate *(2, 3)*. Recently, a fourth test, dimeric inhibin-A (DIA), was added to form the "quadruple" test, which has a higher sensitivity of up to 75% *(4, 5)*. Combining first-trimester biochemistry (free β-subunit of hCG and pregnancy-associated plasma protein A) and ultrasound markers can yield higher performance *(6–8)*, but the highest performance reported to date is from a combination of both first- and second-trimester markers into the "integrated" test *(9)*. The search continues for even better markers that can be used to both increase the detection rate and reduce false positives. One such marker was originally identified as hyperglycosylated hCG. Cole et al. *(10)* used a specific antibody to measure hyperglycosylated hCG [recently called invasive trophoblast antigen (ITA)] in random urine specimens from women with Down syndrome pregnancies. When these authors used only the creatinine-corrected ITA multiple of the median (MoM), 78% of cases were detected at a false-positive rate of 5% *(10)*. The current practice of Down syndrome screening is based on serum samples. Although collecting random urine is not difficult, the additional sample type may be inconvenient and adds expense beyond the measurements of ITA and creatinine. In addition, obtaining a serum sample for AFP analysis would still be required to screen for open neural tube defects. We therefore investigated the possibility of using maternal serum ITA as a Down syndrome marker. To examine whether serum ITA would improve current screening, we also determined the concentrations of other second-trimester serum markers *(11)*.

Blood samples were collected in plain (red-top) evacuated tubes from pregnant women between 14 and 22 weeks of gestation. Samples from women with a Down syndrome pregnancy (defined by fetal karyotype after amniocentesis) were collected at the time of initial counseling (at Yale University; courtesy of Maurice J. Mahoney, MD). Samples from women undergoing second-trimester serum screening (University of Connecticut; courtesy of Peter Benn, PhD) were collected. All were shown to have singleton pregnancy outcomes. Oral consent and demographic and pregnancy-related information were obtained at the time of blood collection. Serum was separated by centrifugation within 4 h of phlebotomy and remained frozen for 3 years at −80 °C. The samples were quickly thawed at Quest Diagnostics and tested immediately, without access to clinical information. ITA concentrations were determined in California by an automated immunochemiluminometric assay as described previously *(12)*. The detection limit of the ITA assay is 0.2 μg/L with a calibration range up to 300 μg/L. The intra- and interassay imprecisions (CVs), as determined by the use of three controls, were <3.5% and <7.4%, respectively. The serum samples were also assayed on the same day for DIA by an assay from Diagnostic System Laboratories and for AFP, hCG, and uE3 on an automated analyzer (Immulite 2000) from Diagnostics Products Corporation. All assays were performed according to the manufacturer's instructions. The intra- and interassay CVs for all four analytes were <8% and <13%, respectively. Data analysis was performed at the Foundation for Blood Research in Scarborough, ME. Confidence intervals were computed using True Epistat. Estimates of screening performance were modeled using gaussian distributions and the maternal age distributions for the United States in 2000 *(13)*.

Maternal serum samples were available for 16 Down syndrome and 84 control pregnancies. Fig. 1 shows the ITA results in case and control pregnancies on the logarithmic *y* axis vs the gestational age (based on ultrasound dating) on the *x* axis. The concentration of ITA in control pregnancies vs gestational age fit a log-linear regression well, with a mean decrease of 19% per week. This pattern is distinctly different from that found for hCG during the same period of pregnancy *(14, 15)*. The geometric mean ITA concentration in the Down syndrome pregnancies

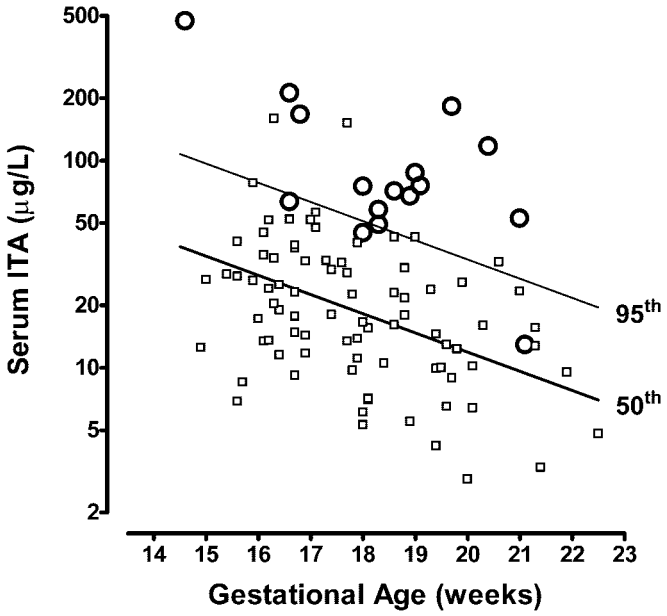

Fig. 1. Scatterplot of second-trimester maternal serum ITA vs gestational age in Down syndrome and unaffected pregnancies.

Serum ITA concentrations are shown on the logarithmic *y axis* for Down syndrome (○) and unaffected pregnancies (□) vs gestational age based on ultrasound measurements (*x axis*). The regressed medians are shown as a *thick solid line*. The 95th centile is shown as a *thin solid line*. Thirteen of the 16 (81%) ITA values in Down syndrome pregnancies are above the 95th centile.

was 5.33 MoM (median, 5.14 MoM), with values ranging from 1.45 to 15.22 MoM. Overall, 13 of the 16 cases (81%) were above the observed 95th centile of 2.9 MoM in unaffected pregnancies. We also determined the univariate performance for the other four serum markers measured in this dataset (AFP, uE3, hCG, and DIA). Table 1 shows the Down syndrome detection rate at a constant false-positive rate of 5% (using the observed 95th or 5th centile as appropriate) along with the median MoM for each analyte. The observed detection rates for AFP, uE3, and DIA measurements were consistent with those expected from one representative study *(16)*, but the observed detection rate of 75% for hCG was considerably higher than the expected 38%. We then calculated the pairwise correlation coefficients between ITA and the other serum markers, which were 0.105, 0.166, and 0.062 for ITA/AFP, ITA/uE3, and ITA/DIA in control pregnancies, respectively. These data indicate that ITA measurements are essentially independent predictors of Down syndrome risk and could be combined with these markers to increase screening performance. However, the correlation coefficient between ITA and hCG was much higher (0.794 in control pregnancies), indicating that the inclusion of both markers in a screening program may not be warranted. The corresponding correlation coefficients in the smaller number of case pregnancies were not significantly different (data not shown), but the confidence intervals were broad.

Table 1. Observed and expected Down syndrome detection rates for ITA and four other second-trimester maternal serum markers.

Maternal serum analyte	Median MoM	Observed DR,[a,b] n (%)	95% CI, %	Expected DR,[c] %
ITA	5.33	13/16 (81)	54–96	
AFP	0.60	4/14 (29)	8–58	25
uE3	0.66	5/14 (36)	13–65	39
hCG	2.63	12/16 (75)	48–93	38
DIA	1.74	5/14 (36)	13–65	45

[a] DR, detection rate; CI, confidence interval.

[b] Sera from all 16 Down syndrome cases were available for ITA and hCG measurements. For 2 samples, AFP, uE3, and DIA measurements were not available.

[c] From Haddow et al. *(4)* and Knight et al. *(16)*, for ultrasound-dated pregnancies.

In summary, the current study found second-trimester maternal serum ITA measurement to be a good marker for fetal Down syndrome and with the highest MoM for Down syndrome (5.33) among the tests studied (Table 1). At a 5% false-positive rate, ITA measurements alone were able to detect 81% of cases when gestational dating was based on ultrasound measurements. In a previous study of ITA in 10 other serum samples, 60% were at or above both the 95th and 98.5th centiles *(17)*. We found the observed performance of hCG measurements (a marker that is highly correlated with ITA) to significantly exceed published expectations (positive in 12 of 16 cases). Whether this is attributable to chance or a systematic bias is unknown. When we reviewed the screening history of the Down syndrome patients for referral bias, we found that 8 of 16 were from women who were 35 years of age or older. There was no evidence that any of the 16 women were screen-positive by biochemical marker testing before amniocentesis. However, the possibility that the performance of ITA may be overestimated in this dataset, with its high detection rate for hCG, cannot be excluded. Because of the small sample size, the confidence interval on this detection rate is wide for ITA, ranging from 54% to 96%.

One strength of the current study is that the blood samples were collected and processed within 4 h before being frozen at −80 °C, limiting the possibility of degradation of markers. In addition, ITA was assayed by a sensitive, reproducible, and automated assay suitable for large-scale applications *(12)*. However, there are also additional important considerations: (*a*) a relatively small number of Down syndrome pregnancies were included in this study; (*b*) the case and control samples were collected at separate sites, and although efforts were made to standardize the collection and storage protocols, it is possible that the case and control samples were not handled identically; and (*c*) the case samples were collected after amniocentesis. The retrospective nature of identifying cases also explains why the gestational ages of the cases are, on average, later than the gestational ages in the controls. Currently, as Down syndrome screening programs use multiple serum markers, serum ITA could be combined more readily than urine ITA testing with

existing panels (or replace current markers) as a way to increase the detection rate and/or decrease the false-positive rate. However, before ITA is included in such a panel, these findings must be confirmed in a larger study.

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Direct Detection of Exon Deletions/Duplications in Female Carriers of and Male Patients with Duchenne/Becker Muscular Dystrophy, *Giulia Frisso, Antonella Carsana, Nadia Tinto, Giuseppe Calcagno, Francesco Salvatore, and Lucia Sacchetti** (Dipartimento di Biochimica e Biotecnologie Mediche and CEINGE Biotecnologie Avanzate, Università Federico II, Naples, Italy; * address correspondence to this author at: Dipartimento di Biochimica e Biotecnologie Mediche, Università di Napoli Federico II, and CEINGE Biotecnologie Avanzate, Via S Pansini 5, 80131 Napoli, Italy; fax 39-81-7462404, e-mail sacchetti@dbbm.unina.it)

Duchenne and Becker muscular dystrophies (DMD/BMD) are X-linked allelic neuromuscular disorders that have a prevalence of 1 in 3500 live-born males. The genetic defect is the result of mutations of the dystrophin gene, which encodes a 427-kDa rod-shaped cytoskeletal protein *(1)*. The locus is very unstable: one-third of all DMD/BMD cases represent new mutations without a family history of the disease *(1)*. Approximately 50–70% of DMD/BMD cases are the result of macrodeletions, and partial gene duplications have been reported in ~6% of patients *(2)*. Both macrodeletions and macroduplications are preferentially clustered in two areas, the amino-terminal (exons 3–7) and the central (exons 44–55) regions *(1)*. The remaining cases are presumably attributable to point mutations or small insertions/deletions *(2)* scattered along the entire gene. PCR detection of macrodeletions is very useful in the analysis of affected males *(3, 4)*, but it provides no information about the carrier status of at-risk women. Carrier status within families is usually assessed by haplotype analysis *(5)*, fluorescence in situ hybridization *(6)*, amplification of ectopic transcripts *(7, 8)*, dosage analysis on Southern blots *(9)*, or separation of quantitative PCR products by gel electrophoresis *(10)*. Semiquantitative methods, based on the separation of fluorescently labeled amplified exons by gel or capillary electrophoresis, are also available *(11–15)*.

Here we report a quantitative PCR method, followed by separation by capillary gel electrophoresis of the fluorescently labeled amplified exons of hot spot regions of the dystrophin gene, which allowed us to detect ~99% patients (affected males and female carriers) with macrodeletions and 89% with macroduplications, and to identify small insertions or deletions in those regions. This method is rapid, and unlike other procedures, it includes an internal standard to normalize amplification efficiency and to calculate a diagnostic index. We validated the method by screening 135 DMD/BMD patients previously