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Invasive Trophoblast Antigen (Hyperglycosylated Human Chorionic Gonadotropin) in Second-Trimester Maternal Urine as a Marker for Down Syndrome: Preliminary Results of an Observational Study on Fresh Samples

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Background: **Down syndrome screening is commonly performed in the US using maternal age and three or four second-trimester maternal serum markers that can identify up to 75% of affected pregnancies by offering diagnostic studies to 5% of women. Invasive trophoblast antigen [ITA; hyperglycosylated human chorionic gonadotropin (hCG)] is a promising marker that can be measured in urine or serum in the first or second trimester. We report preliminary results for urinary ITA in an ongoing observational study.**

Methods: **Women undergoing second-trimester amniocentesis for reasons not associated with biochemical testing provided consent and a urine (and possibly serum) sample that was tested within a few days. Demographic and pregnancy-related information was collected, along with karyotype. Screening performance was modeled for ITA alone and in combination with serum markers**

Results: **Twelve recruitment centers collected urine from 2055 women with singleton pregnancies between 15 and 20 weeks of gestation (2023 unaffected, 28 Down syndrome, and 4 pregnancies with other chromosome abnormalities). After correction for gestational age, urine concentration, and maternal race and weight, the ITA measurements were higher in women with a Down syndrome pregnancy (median ITA, 4.33 multiples of the median). At a 75% detection rate, the false-positive rate could be reduced by substituting ITA for hCG measurements (from 5.6% to 2.6% for the triple test) or by adding ITA measurements to existing combinations (from 3.3% to 2.0% for the quadruple test).**

Conclusions: **Our data provide preliminary confirmation of the potential usefulness of urinary ITA measurements in detecting Down syndrome in a setting that simulates routine usage.**

Down syndrome screening is most often performed in the US using the triple test: a combination of maternal age and second-trimester maternal serum measurements of α-fetoprotein (AFP),[4] unconjugated estriol (uE3), and human chorionic gonadotropin (hCG). When gestational age is estimated by ultrasound examination, the triple test can identify ~70% of Down syndrome pregnancies by selecting 5% of the general pregnancy population for diagnostic testing *(1)*. In an effort to improve screening performance, additional markers are being identified, evaluated, and occasionally, clinically applied. These efforts include adding a fourth second-trimester marker [dimeric inhibin-A (DIA)] to the triple test to form the "quadruple" or "quad" test *(2)* and combining ultrasound measurements of nuchal translucency with biochemical markers in the first trimester *(3)*. Others have suggested incorporating selected first-trimester markers along with second-trimester

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[4] Nonstandard abbreviations: AFP, α-fetoprotein; uE3, unconjugated estriol; hCG, human chorionic gonadotropin; DIA, dimeric inhibin-A; ITA, invasive trophoblast antigen; and MoM, multiple(s) of the median.

biochemical markers into a single integrated interpretation *(4)*. Investigators have also examined maternal urine markers, including β core (the final degradation product of hCG) in the second trimester, but the promising early findings have not been confirmed *(5)*.

Another urine marker that shows promise is invasive trophoblast antigen (ITA). ITA is a unique carbohydrate variant of hCG with more complex N- and O-linked oligosaccharide side chains with additional sialyl-*N*-acetylgalactosamine antennae *(6)*. It is produced by poorly differentiated or invasive trophoblast cells; hence the name *(7, 8)*. The production of ITA is independent of regular hCG synthesis from syncytiotrophoblasts *(9, 10)*. In early pregnancy, the concentration of ITA is very high and accounts for nearly all of the hCG-related molecules. The percentage of ITA rapidly decreases as pregnancy advances and is ultimately a minor component of hCG-related molecules. An automated and specific immunochemiluminometric assay has been developed recently that uses a monoclonal antibody (B152) specific for ITA. This assay has minimal cross-reactivity with hCG and is applicable to many sample types, including urine *(11)*.

Four studies have examined the screening performance of ITA in the second trimester of pregnancy. Cole and coworkers *(7, 12)* combined the results from two study sets that measured ITA in urine samples collected at a single center from 1448 control and 39 Down syndrome pregnancies at 14–22 weeks of gestation. ITA was measured by ELISA within a few days. The median concentration of ITA in the Down syndrome specimens was 9.5 multiples of the median (MoM). Seventy-nine percent of cases were above the 95th centile of unaffected pregnancies. A subsequent case–control study of ITA using 45 Down syndrome pregnancies at 10–19 weeks of gestation found a less increased median of 3.63 MoM *(13)*. However, if the analysis was restricted to the 38 cases at 14 weeks and later, the median MoM increased to 4.64, and 45% of cases were above the 95th centile of unaffected pregnancies (5.2 MoM). Several factors may have confounded their findings: (*a*) the mean creatinine concentration in the Down syndrome samples was significantly different from that in the unaffected samples; (*b*) the samples were collected under diverse conditions; and (*c*) the samples underwent multiple freeze–thaw cycles before testing. A fourth study examined several urine markers in ~100 Down syndrome pregnancies *(14)*. Samples were obtained in both the first and second trimesters of pregnancy and were stored for up to 5 years before assay. The Down syndrome pregnancies had a median second-trimester urinary ITA MoM of 3.51, and 40% of these pregnancies had results above the 95th centile of unaffected.

These promising but variable results prompted us to design a prospective, double-blinded study that simulates the collection, handling, and measurement of fresh maternal urinary ITA that would occur as part of routine obstetric practice. Random urine samples were collected from women scheduled to undergo amniocentesis, and ITA was measured within a few days on the fresh, unfrozen samples by an automated assay *(11)*. Preliminary analysis of the findings of this study based on 28 Down syndrome pregnancies is reported here. Our plan is to continue the study until our original target of 50–60 Down syndrome pregnancies have been enrolled. The report was reviewed using the STARD checklist for completeness *(15)*.

Materials and Methods

ELIGIBILITY AND DATA COLLECTION

The study protocols were approved by the State of California Health and Human Services Agency, Committee for the Protection of Human Subjects and, when required, the Institutional Review Board of individual recruitment centers. Study participants were women who were already scheduled for a second-trimester amniocentesis. To be eligible for inclusion, the indication for amniocentesis could not be related to the results of any serum marker study done earlier in pregnancy. In general, the referral indications among eligible women included advanced maternal age, history of Down syndrome, or abnormal ultrasound findings. The women provided consent, which included permission for the karyotype information to be sent to the Data Center at the Foundation for Blood Research in Maine to be linked with the chemistry results. The karyotype was considered to be diagnostic for Down syndrome or any other chromosomal abnormality. All women provided a urine sample, demographic and pregnancy-related information, and selected ultrasound information (e.g., gestational dating by biparietal diameter and number of fetuses). A proportion of women also agreed to provide an optional serum sample. Demographic and ultrasound information was also forwarded to the Data Center along with a unique numeric identifier used for linkage with the pregnancy outcome.

COLLECTION OF URINE AND SERUM SAMPLES

A clean catch of random urine was collected in a sterile container and transferred to a labeled 20-mL sterile conical plastic tube for shipping. If the woman provided a serum sample, whole blood was collected in a red-top tube, allowed to clot for 30 min, and centrifuged at 1000*g* for 30 min at room temperature. The supernatant was then poured into a labeled polypropylene tube for shipment. The urine and serum samples were shipped either the same day or the day after collection by same-day air courier at 4 °C on ice pack to Quest Diagnostics Nichols Institute (San Juan Capistrano, CA). Care was taken that the samples were not subjected to increased temperatures. Samples from one recruitment center (North York General Hospital, Toronto, Canada) were shipped on ice by Federal Express at least once a week. These samples were stored at 4 °C for up to 4 days before shipping, and most were received within 24 h of the shipping date. When received at Quest Diagnostics Nichols Institute, all sam-

ples were aliquoted into 1-mL plastic tubes. One of the urine aliquots (and if present, a serum aliquot) was sent to the laboratory for testing. The remaining aliquots, both serum and urine, were stored at −70 °C. Care was taken that the samples were not subjected to extended periods at room temperature before assay. Most of the samples were assayed within 24 h of receipt in the laboratory, and all were assayed within 48 h.

MEASUREMENT OF URINARY ITA

The Nichols Institute Diagnostics' Advantage® automated two-site chemiluminometric assay was performed in two steps *(11)*. The anti-hCG monoclonal antibody B207 was labeled with a chemiluminescent acridinium ester and used as the detection antibody. The monoclonal antibody B152, which is specific for ITA, was biotinylated and used as the capture antibody. The Advantage pipetted 15 μL of urine into the cuvette, followed by streptavidin magnetic particles, the capture antibody, and the assay buffer. This mixture was incubated for 30 min at 37 °C, during which time ITA in the sample bound to the biotinylated anti-ITA antibody, which was captured on streptavidin magnetic particles. The magnetic particles were washed three times to remove unbound serum or urine components and biotinylated antibody. The acridinium-ester-labeled anti-hCG antibody and a solution of normal mouse serum were added to the washed magnetic particles. The wells containing washed magnetic particles were then transported to the on-board luminometer, which automatically injected Trigger 1 solution (containing hydrogen peroxide in dilute acid) and Trigger 2 solution (containing diluted sodium hydroxide), initiating the chemiluminescence reaction. The generated "flash" of light was quantified by the luminometer and expressed as relative light units. The amount of bound antibody is directly proportional to the concentration of ITA in the sample. Samples were assayed in singleton. The assay has a calibration range up to 300 μg/L; samples are automatically diluted and reassayed when the ITA concentration exceeds that concentration. The assay has a lower detection limit of 0.2 μg/L. CV were determined by use of three controls with ITA concentrations of 2.0, 17.4, and 181.6 μg/L. The intra- and interassay CV were <3.5% and 7.4%, respectively for all three controls.

OTHER ASSAYS

Creatinine was assayed in a Hitachi automated chemistry analyzer by the Jaffe reaction *(16)*. When available, serum samples were tested for AFP, uE3, and hCG by the Diagnostic Products Immulite 2000 according to the manufacturer's instructions. The serum was also tested for DIA by Diagnostic Systems Laboratories reagents according to the instructions in the package insert.

TEST RESULT SUBMISSION

All urine and serum measurements were sent to the Data Center at the Foundation for Blood Research each week. The results were usually available within 1 week but always before the karyotype information was available. The testing laboratory did not have access to individual karyotypes, thus ensuring the blinded nature of this study.

DATA ANALYSIS

The Data Center combined the information collected at enrollment, the urine and serum measurements, and the karyotype information. The present analysis is restricted to samples from singleton pregnancies collected between 15 and 20 complete weeks of gestation. Urinary ITA results were converted to MoM and corrected for concentration by taking the observed relationship between ITA and creatinine into account *(17)*. The MoM were then corrected for maternal race. Finally, maternal weight was taken into account by use of a published methodology *(18)*. Long-term ITA assay stability was examined by computing the overall median MoM for each calendar quarter. The main analysis focuses on the ability of urinary ITA to differentiate Down syndrome from unaffected pregnancies, but results from pregnancies affected with trisomy 18 and trisomy 13 are also reported. Correlation coefficients between ITA and serum markers in second-trimester unaffected pregnancies were derived after a logarithmic transformation and exclusion of values outside 3 SD.

Correlation coefficients in Down syndrome pregnancies were assumed to be the same as for unaffected pregnancies. Values above (or below) defined truncation limits were set to the upper (or lower) limits for purposes of calculation. This helps ensure reliable risk estimates even when data are in the extreme tails of the distribution. The Down syndrome screening performance was then modeled for selected combinations of urine and serum markers, using the maternal age distribution in the US for 2000 *(19)* and published population distribution parameters for the serum markers *(2, 20)*. Down syndrome risk calculations were based on overlapping gaussian distributions that took into account small pairwise correlation coefficients between the markers to derive a likelihood ratio for each individual's combination of markers *(21–23)*. This likelihood ratio was then multiplied by the a priori Down syndrome risk based on the mother's age to compute the final risk. The maternal age risk was assumed to be independent of the biochemical markers.

Modeling screening performance for a given set of markers entailed a Monte Carlo simulation consisting of randomly generated sets of biochemical measurements drawn separately from selected Down syndrome and unaffected multivariate distributions *(24)*. Each set of measurements was combined as described above with a randomly selected maternal age drawn from the appropriate age distribution to derive a single risk estimate. The final results were a distribution of risks for the two groups. Numbers of case and control samples generated ranged from 25 000 each for univariate analyses to

>2 000 000 each for five-dimensional modeling. Selected risk cutoff values could then be applied to the two groups of risks to determine detection (risks in Down syndrome pregnancies) and false-positive rates (risks in unaffected pregnancies). Continuous variables were compared, after appropriate transformations, by the *t*-test at a two-tailed significance level of 0.05.

FUNDING ORGANIZATION

Quest Diagnostics provided the funding for this project and was involved in the study design. Although the Quest Diagnostics laboratory performed all analytic measurements for this study, they do not have access to individual karyotype information. All statistical analyses were performed by the Data Coordinating Center in Maine.

Results

Between January 2001 and January 2003, 12 recruitment centers participated in the study and collected maternal urine samples from women with 2023 unaffected, 28 Down syndrome, 4 trisomy 18, and 1 trisomy 13 pregnancy. The mean maternal age was 38.9 years, and 91% of the women were age 35 or older at the estimated date of delivery. All women satisfied the inclusion criteria, and their urine samples were subsequently tested for ITA. Median ITA decreased from a high of 17.5 μg/L at 15 complete weeks of gestation to 10.0 μg/L at 18 complete weeks. Observed day-specific median concentrations in unaffected pregnancies fit an exponential model similar to that used for second-trimester serum hCG measurements: median ITA = $26.8483 * e^{[-0.112138*(\text{days} - 98)]} + 8.97$. Using these smoothed medians, we converted ITA measurements from all pregnancies to MoM.

There was no material difference in the mean creatinine concentration between Down syndrome and unaffected pregnancies (0.73 and 0.61 g/L, respectively; $t = -1.20$; $P = 0.23$). The relationship between ITA MoM and creatinine measurements was fitted to a quadratic equation: expected urinary ITA MoM = $-0.08768 * \text{creatinine}^2 + 1.705217 * \text{creatinine} - 0.04976$. This equation was used to calculate a factor to correct each ITA MoM for urine concentration between the truncation limits of 0.1 and 3.0 g/L. The concentration-corrected MoM were then stratified according to four racial/ethnic groups (non-Hispanic Caucasian, Hispanic Caucasian, African American, and Asian). Median ITA values in African Americans (0.76 MoM), Hispanic Caucasians (0.86 MoM), and Asians (1.32 MoM) were significantly different from those in Caucasians ($P < 0.001$), and these differences were taken into account by dividing the MoM by the appropriate factor. The urinary ITA measurements were also corrected for maternal weight *(18)*. The weight adjustment equation for non-Hispanic Caucasians (expected urinary ITA MoM = 95.9435 * 1/weight in pounds + 0.3798) was algebraically modified for the three other racial/ethnic groups by use of the mean maternal weights (155, 157, 178, and 133 pounds in non-Hispanic Caucasians, Hispanic Caucasians, African Americans, and Asians, respectively). Over the eight calendar quarters included in this report, a single set of median values was used. The quarterly median MoM (based on ~200–300 adjusted MoM results) varied randomly between 0.90 and 1.10 MoM with two exceptions: the fourth quarter median adjusted MoM for 2001 was 0.86 ($P = 0.05$), and the fourth quarter median adjusted MoM for 2002 was 1.13 ($P < 0.05$).

As an example of these calculations, consider a 157-pound Asian woman at 18 weeks, 3 days of gestation with a urinary ITA of 9 μg/L and a creatinine of 0.5 g/L. The expected median ITA at that gestational age is 9.8 μg/L; therefore, her result is 0.92 MoM (9 μg/L/9.8 μg/L). The expected MoM for a creatinine of 0.5 g/L is 0.78, giving a creatinine-adjusted MoM of 1.18 (0.92/0.78). Asian women have, on average, higher ITA concentrations; therefore, the race-adjusted MoM is 0.89 (1.18/1.32). The appropriate weight adjustment equation is found by solving it for the intercept at a MoM of 1.0 and a weight of 133 pounds. This yields an expected MoM for a 157-pound Asian woman of 0.89. Thus, after serially adjusting for gestational age, creatinine, race, and weight, this woman's urinary ITA MoM is 1.00 (0.89/0.89).

The adjusted ITA MoM by gestational age in unaffected and affected pregnancies are shown in Fig. 1. ITA appears to be an equally effective marker for Down syndrome throughout the 15- to 18-week gestational age window in which cases were observed. Overall, 15 of 28

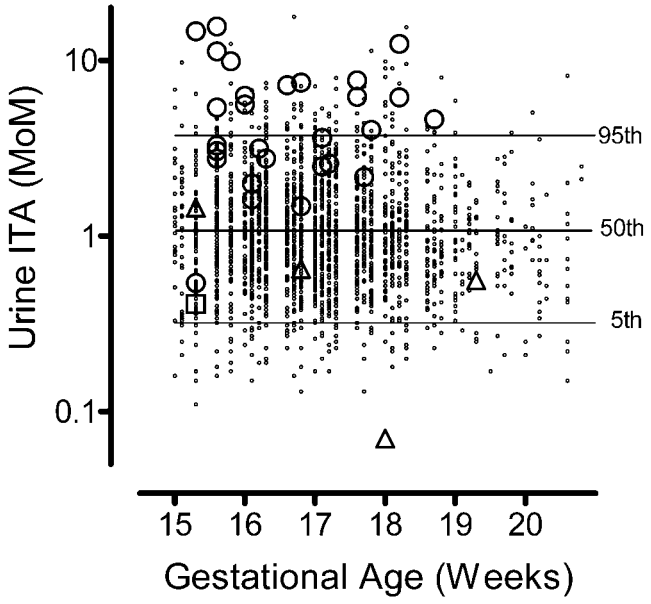

Fig. 1. Second-trimester urinary ITA measurements in Down syndrome and unaffected pregnancies by gestational age.

ITA measurements in concentration-corrected MoM are shown on the logarithmic *vertical axis* for 28 Down syndrome (*large circles*) and 2023 unaffected pregnancies (*small circles*). Four cases of trisomy 18 (*open triangles*) and one case of trisomy 13 (*open square*) are also shown. The *horizontal axis* shows the gestational age (in weeks and days) based on ultrasound measurement of the biparietal diameter. The *horizontal lines* represent the 5th, 50th, and 95th centiles.

Down syndrome pregnancies (54%) were at or above 3.74 MoM, the observed 95th centile of pregnancies with a normal karyotype. The ITA MoM in the one pregnancy affected by trisomy 13 was low, along with three of four cases of trisomy 18. Fig. 2 shows a probability plot for urinary ITA measurements in both Down syndrome and unaffected pregnancies. The data fit a logarithmic gaussian distribution reasonably well in both groups, but to fit the unaffected pregnancies better at higher ITA concentrations, the SD in this group was computed using only the data between the 30th and 99th centiles. The geometric mean ITA concentration in 28 Down syndrome pregnancies was 4.41 MoM [logarithmic mean (SD), 0.6441 (0.3309)]. Among unaffected pregnancies, the median ITA concentration was 1.00 [logarithmic mean (SD), 0.0000 (0.3381)]. The same ITA population characteristics were used in modeling regardless of method of dating. Reasonable truncation limits for urinary ITA are 0.5 and 8.0 MoM (Fig. 2). Table 1 shows the observed and predicted detection rate for Down syndrome and the associated false-positive rates for unaffected pregnancies at selected urinary ITA MoM cutoffs. At a 5% false-positive rate, the modeled univariate detection rate for urinary ITA measurements was 59%. This is in close agreement with the observed rate of 54%.

Of the 2023 women whose pregnancies had a normal karyotype, 533 also provided a serum sample. In these samples, the pairwise correlation coefficient between urinary ITA and serum hCG was relatively high ($r = 0.692$) compared with the corresponding urine/serum correlation coefficients of −0.056, −0.168, and 0.402 for ITA/AFP, ITA/uE3, and ITA/DIA, respectively. Only four women whose pregnancies were affected with Down syndrome provided a serum sample, too few to derive meaningful performance characteristics or correlation coefficients.

To perform the modeling, the correlation coefficients in Down syndrome pregnancies were set equal to those found in unaffected pregnancies. Table 2 shows Down syndrome screening performance for various combinations of maternal age, urinary ITA, and other serum markers (excluding serum ITA) at fixed false-positive rates. For laboratories currently using the triple test, the

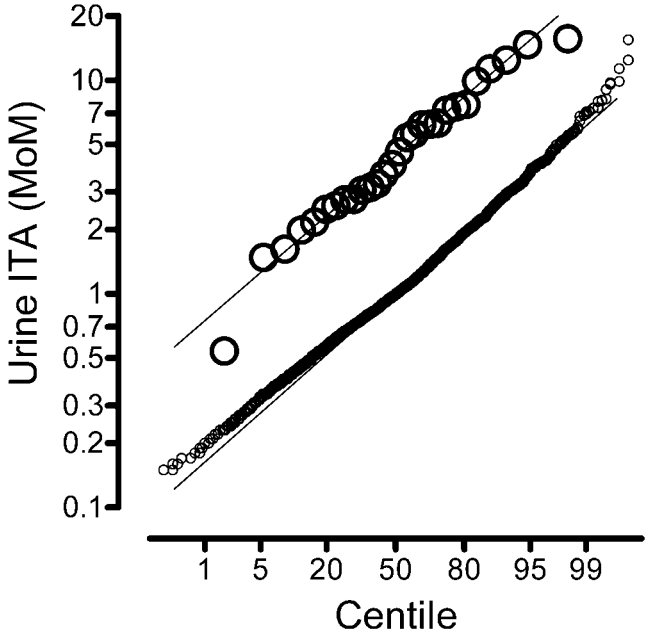

Fig. 2. Probability plot of second-trimester urinary ITA measurements in Down syndrome and unaffected pregnancies.

The ITA measurements in MoM are shown on the logarithmic *vertical axis* for 28 Down syndrome (*large circles*) and 2023 unaffected pregnancies (*small circles*). The MoM values have been corrected for both urine concentration and maternal race and weight. The *horizontal axis* represents the rank of the observations in its respective group. A *straight line* indicates that the data fit a logarithmic gaussian distribution well. The *two straight lines* represent the logarithmic means and SD for Down syndrome and unaffected pregnancies reported in the text.

Table 1. Observed and predicted Down syndrome detection and false-positive rates for second-trimester measurements of urinary ITA.

	Detection rate, %		False-positive rate, %	
ITA[a] MoM	Observed	Predicted[b]	Observed	Predicted[b]
≥1.00	96	97	50	50
≥2.00	86	85	19	19
≥3.00	68	69	8.4	7.9
≥3.74	54	59	5.0	4.5
≥4.00	54	55	4.3	3.7
≥5.00	46	43	2.4	1.9
≥6.00	39	34	1.3	1.1
≥7.00	29	27	1.0	0.6

[a] After adjusting for urine concentration, maternal race, and maternal weight.
[b] Using the logarithmic means and SD reported in the text.

Table 2. Second-trimester Down syndrome detection rates expected at four fixed false-positive rates using maternal age in combination with urinary ITA and other serum markers.

	Down syndrome detection rate, (%) at a FPR[b] of							
	1%		3%		5%		7%	
Maternal age[a] and	LMP[c]	US[c]	LMP	US	LMP	US	LMP	US
AFP and hCG	42	44	58	60	66	68	70	73
AFP and ITA	52	54	69	69	76	76	81	80
AFP, uE3, and hCG	46	51	61	66	69	73	74	77
AFP, uE3, and ITA	55	64	71	77	78	82	83	86
AFP, hCG, and ITA	54	54	70	70	77	78	82	83
AFP, DIA, and ITA	61	63	76	76	82	82	85	86
AFP, uE3, hCG, and DIA	58	60	71	74	78	80	82	84
AFP, uE3, hCG, and ITA	58	60	74	74	81	81	85	85
AFP, uE3, DIA, and ITA	64	66	78	79	84	85	87	88
AFP, uE3, hCG, DIA, and ITA	65	66	80	80	85	85	89	89

[a] Using the maternal age distribution in the US for 2000.
[b] FPR, false-positive rate.
[c] Dating by last menstrual period (LMP) or by ultrasound measurements (US).

addition of DIA or urinary ITA measurements would give essentially equivalent screening performance. However, other four-marker urine/serum combinations may be better than the four-marker serum test. For example, at a 5% false-positive rate, current quadruple marker screening (maternal age in combination with serum AFP, uE3, hCG, and DIA measurements) based on last menstrual period dating is expected to have a detection rate of 78%. However, the urine/serum quadruple marker test (replacing serum hCG with urinary ITA) would increase detection to 84%. Because of the relatively high correlation coefficients between urinary ITA and serum hCG, adding urinary ITA to the serum quadruple test would increase detection only to 85%. Table 3 shows the results of the same modeling, but at fixed detection rates. The potential usefulness of substituting or adding urinary ITA measurements to existing combinations is more apparent when viewed in this way. The number of Down syndrome samples on which the analysis relied was relatively small; therefore, those values (e.g., logarithmic means and SD) may change as more cases are collected. However, given the parameters used to model results for Tables 2 and 3, the precision of the estimated screening performance is high given the large number of case and control observations used in the simulation *(24)*.

To examine the effect of a longer time interval between sample collection and laboratory receipt on ITA measurements, the results for the 13 Down syndrome samples collected from one recruitment center (Toronto) were compared with the results for the 15 samples collected from the remaining recruitment centers. On average, the samples from Toronto were stored for several extra days at 4 °C before express shipping to the laboratory. There was no difference in ITA measurements from unaffected pregnancies (median MoM of 1.05 and 0.98 from the Toronto and other laboratories, respectively; not significant). The median urinary ITA measurement was lower in the Down syndrome samples collected from Toronto (3.05 vs 5.40 MoM), but this difference was not statistically significant ($t = -1.04$; $P = 0.33$). At the 95th centile of ITA measurements (3.73 MoM), the detection rate at Toronto was lower [5 of 13 (38%) compared with 10 of 15 (67%)], but at the 90th centile (2.76 MoM), the detection rates were more similar [9 of 13 (69%) at Toronto and 12 of 15 (80%) from the remaining recruitment centers]. Although these differences were not statistically significant, the more rapid shipping protocol that simulates real practice is now being consistently used at all recruitment sites for the remainder of the study.

Table 3. Second-trimester Down syndrome false-positive rates expected at four fixed detection rates using maternal age in combination with urinary ITA and other serum markers.

	Down syndrome false-positive rate (%) at detection rate of							
	55%		65%		75%		85%	
Maternal age[a] and	LMP[b]	US[b]	LMP	US	LMP	US	LMP	US
AFP and hCG	2.7	2.2	5.3	4.3	10.1	8.6	19.8	17.0
AFP and ITA	1.2	1.1	2.3	2.3	4.5	4.7	9.7	10.0
AFP, uE3, and hCG	1.8	1.2	3.9	2.7	7.7	5.6	16.5	12.2
AFP, uE3, and ITA	1.0	0.5	1.9	1.1	3.9	2.6	8.3	6.4
AFP, hCG, and ITA	1.1	1.1	2.2	2.0	4.2	4.2	8.7	8.3
AFP, DIA, and ITA	0.6	0.6	1.3	1.2	2.9	2.8	6.8	6.4
AFP, uE3, hCG, and DIA	0.8	0.7	1.8	1.5	4.0	3.3	9.4	7.8
AFP, uE3, hCG, and ITA	0.8	0.7	1.6	1.5	3.3	3.1	7.1	6.9
AFP, uE3, DIA, and ITA	0.5	0.4	1.1	0.9	2.4	2.1	5.6	5.0
AFP, uE3, hCG, DIA, and ITA	0.5	0.4	1.0	0.9	2.1	2.0	4.9	4.8

[a] Using the maternal age distribution in the US for 2000.
[b] Dating by last menstrual period (LMP) or by ultrasound measurements (US).

Discussion

Our results indicate that second-trimester maternal urinary ITA measurements may be better at identifying Down syndrome pregnancies than any of the currently available second-trimester serum markers. At a 5% false-positive rate, the predicted univariate Down syndrome detection rate for urinary ITA is 59% (approximate 95% confidence interval, 39–78%) compared with estimates from the literature of 25%, 38%, 39%, and 46% for AFP, hCG, uE3, and DIA, respectively *(2, 20)*. This rate is lower than the 79% reported in an earlier study *(7)*, but higher than the 40–45% reported in others *(13, 14)*.

Enthusiasm for urine markers in Down syndrome screening has waxed and waned because of the variability of results from different studies. The earliest report of a urine marker was the β-core fragment of hCG. Two initial studies *(25, 26)* estimated detection rates of 60–70% (at a 5% false-positive rate). However, subsequent studies failed to confirm these initial findings *(27, 28)*. The cause of this variability may be related to the assays, sample collection methods, or storage conditions. One study showed that freezing–thawing causes polymerization of the measured β-core fragment, possibly explaining the poor results obtained with frozen stored samples *(29)*. In support of this hypothesis, a prospective study using fresh urine specimens assayed within 1 week of collection showed a 62% detection rate, which is similar to rates obtained in the earlier studies *(5)*. Regardless of the reason, the β-core fragment of hCG has not received wide application. Other urine markers have also been investigated, including hCG, free β-hCG, and total estriol *(28, 30)*, but the results for these analytes were less promising, and they have not been incorporated into screening protocols.

Stability studies of urinary ITA have also found that, as with β-core fragment of hCG, losses in ITA concentrations might occur after multiple freeze–thaw cycles *(31)*. The current study was designed to avoid the possible confounding effect of freezing–thawing or long-term storage by use of prompt shipment of fresh specimens directly to the laboratory for immediate assay. Remaining samples

were frozen for future evaluation. When the enrollment phase of the study is complete, a nested matched case–control study can be performed to compare screening performance of urinary ITA on fresh vs frozen specimens. There is some indication that the longer time between sample collection and testing at the Toronto center (responsible for nearly one-half of the Down syndrome samples) contributed to poorer screening performance. Although the differences were not statistically significant, future adherence to a single collection protocol may show that these preliminary results underestimate the true effectiveness of ITA as a marker for Down syndrome.

Because only a limited number of women in our study provided a serum sample, the multivariate screening performance of urinary ITA was modeled using published population parameters for the second-trimester serum markers *(2, 20)*. When urinary ITA measurements were added to the triple test (AFP, hCG, and uE3), the Down syndrome detection rate increased from 69% to 81% at a 5% false-positive rate. Alternatively, if the detection rate were held at 75%, adding urinary ITA to the triple test would reduce the false-positive rate from 7.7% to 3.3%. Smaller improvements in performance were obtained when ITA was added to the quadruple test (the triple test plus DIA measurements). The extent of improvement was consistent with that reported in a recent report exploring serum, urine, and ultrasound markers for Down syndrome *(14)*, although the current study found ITA to be a somewhat better marker. This is probably attributable to the mitigating effect of the other markers on overall screening performance estimates. The addition of ITA measurements to a second-trimester maternal serum screening test would require collection of a random urine. This would not be difficult because urine is routinely collected throughout pregnancy, transportation costs would increase only slightly, and the necessary creatinine measurement is relatively simple and inexpensive. Furthermore, the assay for ITA is highly reliable and automated *(11)*. If these findings for urinary ITA in the second trimester are confirmed in a larger number of affected pregnancies, Down syndrome screening programs might then consider adding urinary ITA measurements as one way to improve screening performance.

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