Double-monoclonal immunofluorometric assays for pregnancy-associated plasma protein A/proeosinophil major basic protein (PAPP-A/proMBP) complex in first-trimester maternal serum screening for Down syndrome

Qiu-Ping Qin,1* Michael Christiansen,1 Claus Oxvig,2 Kim Pettersson,3 Lars Sottrup-Jensen,2 Claus Koch,4 and Bent Nørgaard-Pedersen1

Four double-monoclonal time-resolved immunofluorometric assays (TrIFMAs) have been developed for the specific determination of pregnancy-associated plasma protein A/proeosinophil major basic protein (PAPP-A/proMBP) complex in first-trimester maternal serum samples. The assays have a functional sensitivity of <4 mIU/L and a working range from 4 to 1000 mIU/L. These 4 assays, together with a polyclonal sandwich TrIFMA, were compared for their ability to discriminate between normal pregnancies (n = 149) and pregnancies carrying a Down syndrome fetus (n = 36) in maternal serum screening samples from gestational weeks 4–13. In 26 Down syndrome pregnancies from gestational weeks 7–12, the median PAPP-A multiples of the median concentration in controls (MoMs) determined by monoclonal antibody combinations 234–3/234–2*, 234–4/234–2*, 234–4/234–5*, and 234–5/234–6* were 0.35, 0.37, 0.42, and 0.44, respectively, whereas the median MoM determined by the polyclonal assay was 0.56. ROC curve analysis also showed that better overall diagnostic accuracy and detection rates were achieved by the monoclonal TrIFMAs than by the polyclonal TrIFMA. This report is the first to describe assays that specifically measure PAPP-A/proMBP complex without possible interference from other proMBP-containing complexes.

Down syndrome (trisomy 21; DS5) is the most common congenital cause of severe mental retardation, with an incidence at birth of ~1.3 in 1000. Prenatal diagnosis of DS is performed by chorionic villus sampling (CVS) or amniocentesis (AC), but these invasive procedures carry a risk of ~0.5–1.5% of inducing spontaneous abortion [1]. CVS is not recommended to be performed before 10–11 gestational weeks because of the risk of causing limb reduction defects in the fetus [2], and AC is not recommended before 14–15 weeks of gestation because of the risk of abortion and a greater failure rate of karyotyping than by usual CVS procedures [3]. However, several authors have reported successful implementation of early AC and early CVS [4–6].

Maternal serum screening is a method for assessing the risk for a DS fetus according to maternal age and serum concentrations of feto-placental proteins or hormones. At present, screening programs that utilize age in combination with the measurements of maternal serum human chorionic gonadotropin (hCG) and α-fetoprotein, with or without unconjugated estriol, in the second trimester of pregnancy are routinely carried out in antenatal care units in many countries [7]. This approach detects ~65–75% of Down-affected pregnancies, with a false-positive rate of ~5% [7, 8]. Serum screening for DS before week 15 of pregnancy is still at the experimental stage. However, several markers such as pregnancy-associated plasma

---

5 Nonstandard abbreviations: DS, Down syndrome; CVS, chorionic villus sampling; AC, amniocentesis; PAPP-A, pregnancy-associated plasma protein A; proMBP, proeosinophil major basic protein; TrIFMA, time-resolved immunofluorometric assay; SPI, pregnancy-specific glycoprotein 1 (Schwangerschaftsprotein 1); hCG, human chorionic gonadotropin; MoMs, multiples of the medians.
protein A (PAPP-A) [8–13], free β-hCG [8–9, 13], dimeric inhibin [13, 14], pregnancy-specific glycoprotein 1 (Schwangerschaftsprotein 1; SPI) [11, 15, 16], and unconjugated estriol [13, 17] have been shown to be useful in maternal serum screening for DS in the first trimester. PAPP-A seems to be one of the most promising markers [13, 18].

PAPP-A is a placenta-derived glycoprotein present in circulation at term as a covalent complex with equimolar amounts of the proform of eosinophil major basic protein (proMBP), which is also synthesized in the placenta with fetal DS [8–13]. Consequently, measurement of serum PAPP-A may be useful in first-trimester maternal serum screening for DS.

Currently, PAPP-A concentrations in sera are measured by isotopic and nonisotopic immunoassays that use polyclonal antibodies, either raised in-house [20, 21] or commercially (A230, from Dako A/S) [8, 11, 12, 22, 23]. Unfortunately, these polyclonal antisera are not specific for PAPP-A. First, the polyclonal anti-PAPP-A antibodies raised thus far are, in fact, anti-PAPP-A/proMBP antibodies, because PAPP-A is isolated from serum as the PAPP-A/proMBP complex. Importantly, proMBP antigen is present in excess of PAPP-A/proMBP complex, thus reflecting the presence of non-PAPP-A-containing proMBP complexes—complexes that do not exist in a constant molar ratio to PAPP-A/proMBP complexes [24]. Second, polyclonal antiserum has also been shown to react with haptoglobin [25] and SPI [22]. Therefore, the concentrations of PAPP-A measured with use of these antibodies may be severely biased by cross-reactivity.

Here we describe four double-monoclonal time-resolved immunofluorometric assays (TrIFMAs) developed for PAPP-A/proMBP complex determination, based on newly produced monoclonal antibodies [26]. We present an evaluation of the performance of these serum assays in first-trimester discrimination between normal pregnancies and DS-affected pregnancies and compare the results with that of a polyclonal version of TrIFMA for PAPP-A [12]. Furthermore, serum concentrations of the complex in nonpregnant women and in men are reported.

**Materials and Methods**

**SERUM SAMPLES**

Serum samples were obtained from a prenatal screening program for severe malformations, infections, and DS at Statens Seruminstitut. The samples had been stored at −20 °C. Both DS samples and apparently normal control samples were matched for gestational age, duration of storage, and times of freezing and thawing. The material included the following: 149 maternal serum samples from normal pregnancies from gestational weeks 4–12 (gestational age was determined from the last menstrual period and in most cases was confirmed by ultrasound examination); 36 maternal serum samples from pregnancies with a DS fetus, diagnosed either by invasive prenatal diagnosis (CVS or AC, n = 30) or at birth (n = 6); 70 serum samples from healthy blood donors, 35 men and 35 women (pregnancy among women was not excluded biochemically, but blood donation is not allowed if the donor is pregnant).

**Calibrators and controls.** Calibrators were made from a 40-week-pregnancy serum pool diluted in dilution buffer [10 mmol/L phosphate buffer, 150 mmol/L NaCl, 2.5 g/L bovine γ-globulin (G 5009; Sigma Chemical Co.), and 10 g/L bovine serum albumin (Sigma A 4503)] and calibrated against WHO IRP 78/610 for pregnancy-associated proteins (WHO International Laboratory for Biological Standards, Statens Seruminstitut, Copenhagen, Denmark). The contents of the ampoule were dissolved in 750 μL of distilled water to give a concentration of 0.1 IU/mL PAPP-A as defined. Three control samples, representing low, medium, and high values of PAPP-A, were prepared from a first-trimester delipidated serum pool (for the low value) and a second-trimester delipidated serum pool (for the medium and high values). Dilution buffer was used as the zero calibrator.

**Eu³⁺-labeled monoclonal antibodies.** Each monoclonal antibody (500 μg) was transferred to labeling buffer (50 mmol/L NaHCO₃, 150 mmol/L NaCl, pH 8.5) by passage through a NAP-5 column (Pharmacia) Then 100 μL of a 2 mg/mL solution of the Eu³⁺-chelate of N₁₁-[(p-isothiocyanatobenzyl)-diethylentriamine-N₁,N₂,N₃,N₄-tetraacetic acid (Wallac Oy) in distilled water was added to the antibody solution and the mixture was left in the dark at room temperature for 24 h. A 100-fold molar excess of Eu³⁺-chelate was used in the labeling reaction. Labeled monoclonal antibody was separated from free Eu³⁺-chelate by gel filtration on a PD-10 column (Pharmacia). The labeled monoclonal antibodies contained 7–15 Eu³⁺ molecules per IgG molecule.

---

Small amounts of monoclonal antibodies may be made available for research purposes upon written request to: Michael Christiansen, Department of Clinical Biochemistry, State Serum Institute, 5 Artillerivej, DK 2300S; fax +45 32 68 38 78; e-mail mchristiansen@cb.diag.ssi.dk.
Biotinylated monoclonal antibodies. Monoclonal antibodies to be biotinylated were dissolved in 50 mmol/L phosphate buffer containing 150 mmol/L NaCl (pH 7.4) and the protein concentration was adjusted to 1 mg/mL. Next, 20 μL of 10 mmol/L biotin isothiocyanate (Wallac Oy) in dimethylformamide and 42 μL of 0.5 mol/L sodium carbonate buffer (pH 9.8) were added to 400 μL of monoclonal antibody solution. The mixture was kept at room temperature for 2 h without shaking. Biotinylated monoclonal antibodies were separated from the chemical reactants by consecutive passage through columns of NAP-5 and NAP-10 (Pharmacia). The concentrations of biotinylated monoclonal antibodies were calculated from the absorbance at 280 nm.

PROCEDURES
Monoclonal antibody assays. Maxisorp polystyrene microtiter plates (Nunc) were coated with 1 μg of streptavidin (Zymed Labs.) in 100 μL of 0.1 mol/L citric phosphate buffer (35 mmol/L citric acid, 67 mmol/L disodium phosphate, pH 5.0) per well overnight at room temperature. Plates were washed twice with washing buffer [5 mmol/L Tris-HCl, 150 mmol/L NaCl, 0.05 g/L Tween 20, and 1 g/L Germall II (Wallac Oy), pH 7.5], and blocked with 200 μL of dilution buffer for 2 h at room temperature with slow shaking. After aspiration and washing, the plates were covered with adhesive tape and stored at 4 °C until use. The coated plates were stable at 4 °C for at least 1 month.

To each well of the streptavidin-coated microtiter plates was added 200 ng of biotinylated 234–3 (for TrIFMA 234–3/234–2*) or 400 ng of biotinylated 234–4 (for TrIFMA 234–4/234–2* and TrIFMA 234–4/234–5*) or 400 ng of biotinylated 234–5 (for TrIFMA 234–5/234–6*). All biotinylated monoclonal antibodies were diluted in assay buffer (Wallac Oy). After 1.5 h of incubation at room temperature, the plates were washed with washing buffer. Then 100 μL of either calibrators, ranging from 3.9 to 1000 mIU/L (prepared by serial twofold dilution), or first-trimester serum samples diluted 10-fold with dilution buffer was added to each well in duplicate. Incubations were performed at room temperature for 2 h (for versions of TrIFMA 234–3/234–2* and TrIFMA 234–5/234–6*) or for 3 h (for versions of TrIFMA 234–4/234–2* and TrIFMA 234–4/234–5*). The plates were washed twice and incubated with 100 ng of Eu³⁺-labeled second monoclonal antibody (234–2*, 234–5*, or 234–6*) in 100 μL of enhancement solution (Wallac Oy) and 10 min of slow shaking. After washing twice, 100 ng of Eu³⁺-labeled A230 was dispensed into each well. The plates were incubated at 4 °C overnight, then transferred to room temperature for 30 min and washed 6 times. After addition of 200 μL of enhancement solution (Wallac Oy) and 10 min of slow shaking, the time-resolved fluorescence was measured at 613 nm with the Wallac 1232 Arcus fluorometer; the data were analyzed with a spline algorithm on logarithmically transformed data.

Polyclonal TrIFMA for PAPP-A. This assay was performed as described in detail elsewhere [12]. Briefly, 100 μL of serum samples diluted 10-fold in dilution buffer was added to each well of microtiter plates that had been coated with polyclonal antibody A230 (Dako; lot 025). The calibrators were the same preparations as used in the monoclonal TrIFMAs. The calibrators and samples were incubated at room temperature for 3 h with slow shaking. After washing twice, 100 ng of Eu³⁺-labeled A230 was dispensed into each well. The plates were incubated at 4 °C overnight, then transferred to room temperature for 30 min and washed 6 times. After addition of 200 μL of enhancement solution (Wallac Oy) and 10 min of slow shaking, the time-resolved fluorescence was measured at 613 nm with the Wallac 1232 Arcus fluorometer; the data were analyzed with a spline algorithm on logarithmically transformed data.

Gel filtration. The gel-filtration studies were carried out on a 1.6 cm × 49 cm column of Sephacryl HR-400 (Pharmacia). Elution buffer was 50 mmol/L phosphate buffer, 150 mmol/L NaCl, pH 7.2. The column was operated at room temperature with a flow rate of 1.0 mL/min, and 1.0-mL fractions were collected. A sample of 0.5 mL of a 9th week pregnancy serum pool was loaded on the column. For molecular mass estimation the column was calibrated with catalase (232 kDa), ferritin (440 kDa), and thyroglobulin (669 kDa), all from Pharmacia Biotech. PAPP-A concentrations in the fractions were measured by both the monoclonal TrIFMAs and the polyclonal TrIFMA.

STATISTICS
Groups were compared by Mann–Whitney U-test or matched signed rank test. The observed (empirical) median values for PAPP-A serum concentrations were determined from the control samples in each of the gestational weeks 4–13. The observed medians, in combination with the number of samples at each gestational week, were subsequently log-linearly regressed to produce the number-weighted, log-linear regressed medians of unaffected samples. Multiples of the medians (MoMs) were calculated by using regressed medians for both unaffected samples and DS-affected samples. Test accuracy was analyzed by receiver-operator characteristics (ROC) curves [27]. Area calculation and comparison of ROC curves was performed by using GraphROC program for Windows (vers. 2.0; Veli Kairisto and Allan Poola, http://WWW.netti.fi/~maxiw).

The version of monoclonal assay is expressed as: Mab1/Mab2*, where Mab1 functions as a capture antibody and Mab2*, labeled with Eu³⁺, serves as a detection antibody in the assay.
Results

Assay Evaluation

Calibration Curve. Calibration curves and intraassay precision profiles of the 4 double-monoclonal TrIFMAs were obtained (Fig. 1). The working range of assays was from 3.9 to 1000 mIU/L for assays 234–4/234–2* and 234–5/234–6*, from 1.95 to 1000 mIU/L for assay 234–3/234–2*, and from 0.95 to 1000 mIU/L for assay 234–4/234–5*—the range being defined as the concentrations where the intraassay CVs were <10%.

Sensitivity (Detection Limit). Sensitivity was estimated by two methods: by calculating the concentration of PAPP-A giving a signal equivalent to that for the 0 calibrator + 2SD (theoretical sensitivity); and by calculating the lowest concentration giving acceptable intraassay variation (CV <10%; functional sensitivity). Table 1 shows these data for the four versions of TrIFMAs.

Reproducibility. Intra- and interassay variation was examined by analyzing 6 serum samples with low, medium, and high concentrations of PAPP-A. Results are shown in Table 2.

Parallelism. Parallelism of the 4 versions of TrIFMAs was examined on 3 serum samples with low, medium, and high PAPP-A content. Agreement between the expected values and the measured values was good (data not shown).

Table 1. Theoretical and functional detection limits (mIU/L).

<table>
<thead>
<tr>
<th>Assay version</th>
<th>Detection limits</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Based on CV &lt;10% in precision profile</td>
</tr>
<tr>
<td>234-3/234-2*</td>
<td>1.95</td>
</tr>
<tr>
<td>234-4/234-2*</td>
<td>3.90</td>
</tr>
<tr>
<td>234-4/234-5*</td>
<td>0.95</td>
</tr>
<tr>
<td>234-5/234-6*</td>
<td>3.90</td>
</tr>
</tbody>
</table>

Recovery. Recovery was examined by analyzing serum samples containing added PAPP-A calibrators. As shown in Table 3 the analytical recoveries of the 4 monoclonal assays were satisfactory.

Comparisons of Five Assays for PAPP-A/promBP Complex

There was an excellent linear correlation between each of monoclonal assays (r >96%) and between the monoclonal and polyclonal assays (r >90%). Difference plots show that PAPP-A/promBP complex concentrations determined by TrIFMA 234–3/234–2* and TrIFMA 234–4/234–2* are very similar to each other, as are those determined by TrIFMA 234–4/234–5* and TrIFMA 234–5/234–6*. However, as shown in Fig. 2, differences were seen, especially in the samples from early pregnancy, between the concentrations obtained by PcAb/PcAb* vs those by TrIFMA 234–3/234–2* and between those by TrIFMA 234–4/234–5* vs TrIFMA 234–4/234–2*.

![Fig. 1. Calibration curves (■) and precision profiles (●) of 4 monoclonal versions of TrIFMAs.](image) Each point was the result of 8 replicates.
GEL FILTRATION
As shown in Fig. 3, PAPP-A/proMBP immunoreactivity eluted as a symmetrical peak, slightly ahead of thyroglobulin. The elution position was that of a globular protein with Mr of ~800,000. The monoclonal combinations 234–3/234–2* and 234–4/234–2* gave concentrations similar to those measured by the polyclonal TrIFMA, whereas the combinations 234–4/234–5* and 234–5/234–6* gave much higher values, even though the peak had the same elution position as that seen with the polyclonal TrIFMA.

PAPP-A CONCENTRATIONS MEASURED
In healthy blood donors. PAPP-A could be detected by all 4 versions of monoclonal TrIFMAs in serum from nonpregnant women and men. After logarithmic transformation, the distributions of the data in both groups (n = 35 each) were compatible with a gaussian distribution, as judged by Shapiro–Wilks’ test. Table 4 shows geometric means and SD of PAPP-A/proMBP concentrations assayed by 4 versions of TrIFMAs in the serum samples from nonpregnant donors. PAPP-A concentrations in the men’s samples were slightly but not significantly higher (assayed by TrIFMA 234–3/234–2* and TrIFMA 234–4/234–2*) than in the women’s samples, or were clearly and significantly higher (assayed by TrIFMA 234–4/234–5* and TrIFMA 234–5/234–6*; P < 0.05) than in the women’s samples. Results similar to those obtained by TrIFMA 234–4/234–5* and TrIFMA 234–5/234–6* were reported for the PcAb/PcAb* assay in men (n = 78) and women (n = 69) [12]. PAPP-A values determined by TrIFMA 234–5/234–6* differed statistically from those obtained by other assay versions, as did those assayed by TrIFMA 234–4/234–5*. There was no statistically significant difference between the PAPP-A values assayed by TrIFMA 234–3/234–2* and TrIFMA 234–4/234–2*

In unaffected pregnancies. The empirical median values and regressed medians of PAPP-A assayed by each version of TrIFMA in unaffected pregnancies for gestational ages from week 4 to 13 are shown in Table 5. The PAPP-A concentration measured by the polyclonal TrIFMA was higher than that measured by monoclonal TrIFMA 234–3/234–2* and TrIFMA 234–4/234–2* until gestational week 11, and higher than that measured by TrIFMA 234–4/234–5* and TrIFMA 234–5/234–6* until gestational week 8. As pregnancy advanced, the relative differences became smaller. The concentrations measured by TrIFMA 234–3/234–2* were very similar to those obtained by TrIFMA 234–4/234–2*, but different from those seen with TrIFMA 234–4/234–5* and TrIFMA 234–5/234–6*. The PAPP-A value determined by TrIFMA 234–3/234–2* and TrIFMA 234–4/234–2* was lower than that by TrIFMA 234–4/234–5* and TrIFMA 234–5/234–6*, especially in gestational weeks 4–10, where the difference was almost twofold.

In DS pregnancies. Data for DS-affected pregnancies, expressed as MoMs, are given in Table 6. MoM values obtained by use of the monoclonal TrIFMAs were significantly lower than those obtained by the polyclonal TrIFMA (P < 0.01; Mann–Whitney U-test). In the 26 DS pregnancies (gestational weeks 7–12), the median PAPP-A MoM values determined by TrIFMA 234–3/234–2*, 234–4/234–2*, 234–4/234–5*, and 234–5/234–6* were 0.35, 0.37, 0.42, and 0.44, respectively, whereas the median MoM determined by the polyclonal assay was 0.56. No matter which assay version was used, however,
the resulting median MoMs for DS-affected pregnancies were all significantly lower than the median MoM values for the unaffected pregnancies at 7–12 weeks of gestation (\( P < 0.01; \text{Mann–Whitney} \ U\)-test). The distributions of the log MoM values in both the healthy control group and the DS-affected group were compatible with a gaussian distribution as judged from normal plots. Two samples had a very high MoM value in assay combination 234–5/234–6*(4.73 and 7.24), whereas the other combinations gave MoMs <1.0 for the same samples.

**Diagnostic Accuracy**

The diagnostic accuracy of serum PAPP-A determined by 4 monoclonal TrIFMAs in gestational weeks 7–12 for detecting DS-affected pregnancies was evaluated by plotting the ROC curves (see Fig. 4). Areas under the ROC curves and their SEs are given in Table 7. All monoclonal TrIFMAs gave an area significantly (\( P < 0.05 \)) greater than that of the polyclonal TrIFMA. The detection rates for a false-positive rate of 5% varied from 26.9% for the poly-

### Table 4. Geometric means (SD) of PAPP-A (mIU/L) in blood donor samples.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Men</td>
<td>4.72 (2.42)</td>
<td>4.85 (2.38)</td>
<td>3.91 (1.49)</td>
<td>41.12 (4.11)</td>
</tr>
<tr>
<td>Women</td>
<td>3.54 (2.69)</td>
<td>3.74 (2.65)</td>
<td>2.85 (1.65)</td>
<td>21.01 (2.99)</td>
</tr>
</tbody>
</table>

n = 35 for each sex.
clonal TrIFMA to 37.2% for the monoclonal TrIFMA 234–4/234–5*. However, there was no statistically significant difference in the areas under the ROC curves for any of the monoclonal assays ($P > 0.16$).

**Discussion**

Since Lin et al. [28] reported the crossed immunoelectrophoretic technique for measuring PAPP-A, many different assays have been developed for PAPP-A determination, e.g., rocket immunoelectrophoresis [29], radirocket line immunoelectrophoresis [30], RIAs [31, 32], an IRMA [33], enzyme immunoassays [22, 34–36], and TrIFMAs [11–13]. Until now, the most sensitive RIA for PAPP-A has been the one developed by Sinosich et al. in 1982 [21]. As little as 5 μg/L of PAPP-A can be detected by this RIA (1 μg/L = 5.28 mIU/L). However, it is less sensitive than 2 of our 4 monoclonal TrIFMAs. The most sensitive enzyme immunoassay for PAPP-A is the one established in 1995 by Bersinger et al. [22], which is claimed to measure as little as 40 mIU/L of serum PAPP-A and cover the range of 40–1200 mIU/L. Our monoclonal TrIFMA has a functional sensitivity of <4 mIU/L and a working range of 4–1000 mIU/L for the 10-fold-diluted samples. For the undiluted samples, therefore, the assay should have a sensitivity of <40 mIU/L and a working range of 40–10 000 mIU/L. The monoclonal TrIFMAs have a better than or at least the same sensitivity (as thus defined) as the polyclonal TrIFMA. A low detection limit is necessary for an assay to be useful for determination of PAPP-A in the early first trimester of pregnancy.

PAPP-A exists in pooled pregnancy serum as a heterotetrameric complex of two molecules of PAPP-A and two molecules of proMBP [19]. At least 2 other proMBP complexes are present in term pregnant serum, i.e., complexes with angiotensinogen and complexes with complement 3dg and angiotensinogen [24]. Nothing is currently known about the function of these complexes or their serum concentrations in DS pregnancies.

For analysis of the clinical usefulness of PAPP-A determination in screening, it is important to establish that an immunoassay determines only the concentration of PAPP-A, either free (if that form exists) or complex-bound, and not the concentrations of other serum constituents. Unfortunately, the only commercially available polyclonal antibody against PAPP-A (Dako A230) has been shown to cross-react with proMBP [19, 24], SP1 [22], and haptoglobin [25], all present in pregnancy serum in concentrations greatly exceeding the concentration of PAPP-A. Recently, an absorption procedure to make A230 free of cross-reactivity has been devised that discriminates well between DS and unaffected pregnancies [22]. Absorption procedures have previously been shown to in-

**Table 5. Regressed (and empirical) medians (mIU/L) of unaffected pregnancies determined with different combinations of antibodies.**

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>24.9 (34.5)</td>
<td>4.7 (4.2)</td>
<td>5.2 (3.4)</td>
<td>14.1 (12.1)</td>
<td>17.6 (22.4)</td>
<td>2</td>
</tr>
<tr>
<td>5</td>
<td>41.8 (40.0)</td>
<td>10.2 (5.3)</td>
<td>11.2 (6.9)</td>
<td>27.5 (15.9)</td>
<td>32.3 (26.2)</td>
<td>11</td>
</tr>
<tr>
<td>6</td>
<td>70.4 (60.0)</td>
<td>21.8 (17.6)</td>
<td>23.9 (17.9)</td>
<td>53.6 (43.5)</td>
<td>58.5 (51.8)</td>
<td>19</td>
</tr>
<tr>
<td>7</td>
<td>118.3 (115.5)</td>
<td>46.7 (53.9)</td>
<td>51.1 (66.2)</td>
<td>104.8 (113.7)</td>
<td>109.4 (107.1)</td>
<td>32</td>
</tr>
<tr>
<td>8</td>
<td>198.9 (204.5)</td>
<td>99.9 (126.2)</td>
<td>109.2 (148.7)</td>
<td>204.6 (241.6)</td>
<td>201.4 (244.9)</td>
<td>24</td>
</tr>
<tr>
<td>9</td>
<td>334.5 (416.0)</td>
<td>214.0 (341.4)</td>
<td>233.3 (374.3)</td>
<td>399.6 (689.5)</td>
<td>370.7 (441.4)</td>
<td>27</td>
</tr>
<tr>
<td>10</td>
<td>562.5 (500.0)</td>
<td>458.5 (347.4)</td>
<td>498.4 (382.5)</td>
<td>780.4 (619.4)</td>
<td>682.2 (590.7)</td>
<td>19</td>
</tr>
<tr>
<td>11</td>
<td>945.8 (1291.0)</td>
<td>982.0 (984.4)</td>
<td>1064.9 (1171.1)</td>
<td>1524.1 (1602.4)</td>
<td>1255.5 (1761.4)</td>
<td>7</td>
</tr>
<tr>
<td>12</td>
<td>1590.4 (1414.0)</td>
<td>2103.3 (1359.5)</td>
<td>2275.1 (1237.8)</td>
<td>2976.5 (1592.5)</td>
<td>2310.5 (1765.1)</td>
<td>5</td>
</tr>
<tr>
<td>13</td>
<td>2674.2 (1261.0)</td>
<td>4505.1 (1243.1)</td>
<td>4860.7 (1292.9)</td>
<td>5813.0 (1633.8)</td>
<td>4252.1 (1938.6)</td>
<td>3</td>
</tr>
</tbody>
</table>

**Table 6. Median MoMs of PAPP-A in Down syndrome pregnancies.**

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>1.43</td>
<td>0.44</td>
<td>0.54</td>
<td>0.35</td>
<td>0.35</td>
<td>4</td>
</tr>
<tr>
<td>6</td>
<td>0.69</td>
<td>0.22</td>
<td>0.23</td>
<td>0.18</td>
<td>0.18</td>
<td>6</td>
</tr>
<tr>
<td>7</td>
<td>0.43</td>
<td>0.22</td>
<td>0.22</td>
<td>0.22</td>
<td>0.22</td>
<td>4</td>
</tr>
<tr>
<td>8</td>
<td>0.77</td>
<td>0.58</td>
<td>0.53</td>
<td>0.55</td>
<td>0.55</td>
<td>5</td>
</tr>
<tr>
<td>9</td>
<td>0.45</td>
<td>0.33</td>
<td>0.37</td>
<td>0.35</td>
<td>0.35</td>
<td>6</td>
</tr>
<tr>
<td>10</td>
<td>0.67</td>
<td>0.43</td>
<td>0.42</td>
<td>0.40</td>
<td>0.40</td>
<td>6</td>
</tr>
<tr>
<td>11</td>
<td>0.88</td>
<td>0.46</td>
<td>0.48</td>
<td>0.51</td>
<td>0.51</td>
<td>1</td>
</tr>
<tr>
<td>12</td>
<td>0.75</td>
<td>0.33</td>
<td>0.35</td>
<td>0.38</td>
<td>0.38</td>
<td>4</td>
</tr>
<tr>
<td>5–12</td>
<td>0.69</td>
<td>0.34</td>
<td>0.36</td>
<td>0.35</td>
<td>0.45</td>
<td>36</td>
</tr>
<tr>
<td>6–12</td>
<td>0.57</td>
<td>0.34</td>
<td>0.35</td>
<td>0.35</td>
<td>0.45</td>
<td>32</td>
</tr>
<tr>
<td>7–12</td>
<td>0.56</td>
<td>0.35</td>
<td>0.37</td>
<td>0.42</td>
<td>0.44</td>
<td>26</td>
</tr>
</tbody>
</table>
crease the specificity of polyclonal anti-PAPP-A antibodies [37], but such procedures are difficult to standardize and very time-consuming to perform. Thus, the double-monoclonal assay format is preferred.

In healthy blood donors, a low concentration of PAPP-A was detected by all 4 monoclonal versions of TrIFMA—in agreement with findings with the polyclonal TrIFMA [12]. The source of this immunoreactivity is likely to be seminal fluid, Graffian follicles, corpus luteum, and testes [38]. Furthermore, recent results suggest that PAPP-A mRNA is also transcribed in the brain, but whether PAPP-A synthesized there reaches the circulation is not known [39]. In some samples, the 234–5/

234–6* assay version found much higher concentrations of PAPP-A than did the other assays. This unusual, high value of PAPP-A is thought to be caused by cross-reactants in the sera.

In pregnant women, PAPP-A serum concentrations are found to increase with gestational age. However, absolute concentrations seem to differ widely between investigators [9–13], probably because of differences in the calibrators or labeled tracers and antibodies used. Compared with the findings of other groups, the medians obtained here are lower. This has no consequences for maternal screening, however, because the MoM, not the absolute concentration, is used to calculate the individual risk that a pregnancy is affected by DS. When the unaffected controls and affected samples are determined with the same assay, the MoM value obtained for each sample should be the same as that obtained by the other assays.

PAPP-A has been described to exhibit immunological heterogeneity, and several plasma proteins have been suggested as having epitopes that are cross-reactive with PAPP-A [37, 40]. We think it surprising that some monoclonal assay combinations, i.e., 234/234–5* and 234–5/234–6*, give much higher PAPP-A concentrations than do the polyclonal TrIFMA and the two other monoclonal combinations, given that the immunoreactivities determined by all of the assays elute as one predominant peak

**Table 7. ROC curve analysis.**

<table>
<thead>
<tr>
<th>Version</th>
<th>Area under the ROC curve</th>
<th>Detection rate, %, at a 5% false-positive rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>PcAb/PcAb*</td>
<td>0.734 ± 0.052</td>
<td>26.90</td>
</tr>
<tr>
<td>234-3/234-2*</td>
<td>0.830 ± 0.039</td>
<td>27.10</td>
</tr>
<tr>
<td>234-4/234-2*</td>
<td>0.799 ± 0.044</td>
<td>30.80</td>
</tr>
<tr>
<td>234-4/234-5*</td>
<td>0.822 ± 0.041</td>
<td>37.20</td>
</tr>
<tr>
<td>234-5/234-6*</td>
<td>0.806 ± 0.043</td>
<td>30.80</td>
</tr>
</tbody>
</table>

*All monoclonal TrIFMAs gave an area significantly (P < 0.05) greater than that of the polyclonal TrIFMA.

Detection rates were derived from ROC curves.
of $M_r \sim 820,000$. The differences in the relative size of the peak could be caused by epitope sharing with other high-$M_r$ substances or perhaps more likely by differential reactivity of monoclonal antibodies to different glycoforms of the PAPP-A/proMBP complex (carbohydrate content 17.4% [19]). It could also be attributable to variations in attached ligands or perhaps preanalytical modifications. Whether the cause is cross-reactivity or variations in the detection of isoforms of PAPP-A/proMBP, we find it noteworthy that the discrimination between DS pregnancies and unaffected pregnancies obtained with the double monoclonal assays is very similar to each other and better than that with the polyclonal TrIFMA. The fact that the difference in concentrations determined by different assays for PAPP-A/proMBP complex depends on gestational age suggests that different isoforms of PAPP-A/proMBP complex or that other complexes containing either or both of the two proteins PAPP-A and proMBP may exist in different stages of pregnancy. This possibility is currently under investigation.

Our finding of an approximate size of 800 kDa for the PAPP-A/proMBP complex is in agreement with the findings of others [28, 40]. However, this value is much higher than the mass determined from the molecular structure of PAPP-A/proMBP, 474 kDa. The reason for this discrepancy is not clear; maybe the PAPP-A/proMBP complex has an asymmetrical structure. The proMBP part of the complex is glycosylated and carries a linear glycosaminoglycan [41].

In terms of diagnostic accuracy, at a 5% false-positive rate, the rate of DS detection was 27.1–37.2% as found by the monoclonal assays and 26.9% by the polyclonal assay, both lower than that (42%) reported by Wald et al. [13], but close to (by PcAb/PcAb* and TrIFMA 234–3/234–2*) or higher than (by TrIFMA 234–4/234–2*, 234–4/234–5*, and 234–5/234–6*) that reported by Spencer et al. [8]: 28%. The differences in detection rates from these two published studies are most likely the result of using different antibodies. The detection rate obtained in our study, using the same polyclonal antibodies, is 26.9%, which is in close agreement with the result of Spencer's study.

Irrespective of whether polyclonal or monoclonal TrIFMAs are used, maternal serum PAPP-A concentrations are lower in DS-affected pregnancies. This finding is in accordance with previous studies [8–12]. Compared with polyclonal TrIFMA, monoclonal TrIFMAs seem to better discriminate between unaffected pregnancies and DS pregnancies. However, some of the monoclonal combinations are unsuitable because of high amounts of PAPP-A immunoreactivity in nonpregnant serum.

We thank Mona-Maria Ilola for excellent technical assistance. L.S.-J. was supported by the Danish Biomembrane Center and by a basic research grant from the University of Arhus.

References


