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Development of a Multiplex Selected Reaction Monitoring Assay for Quantification of Biochemical Markers of Down Syndrome in Amniotic Fluid Samples

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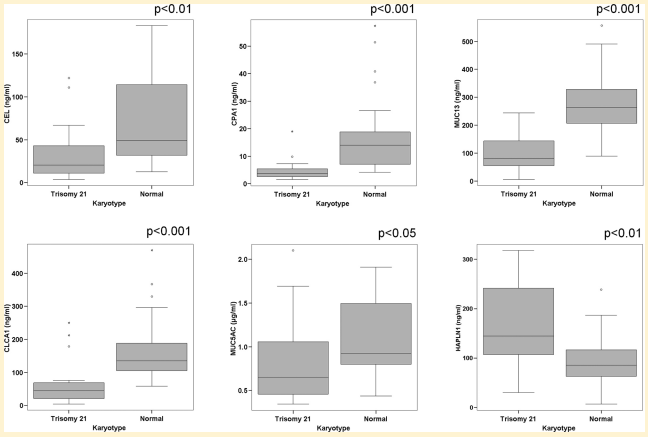
Supporting Information

ABSTRACT: Down syndrome (DS) is one of the most common chromosomal abnormalities affecting about 1 of every 700 fetuses. Current screening strategies have detection rates of 90−95% at a 5% false positive rate. The aim of this study was to discover new biomarkers of DS in amniotic fluid by using a multiplex selected reaction monitoring assay. Nine proteins were analyzed: CEL, CPA1, MUC13, CLCA1, MUC5AC, PLUNC, and HAPLN1, and CGB as positive control and serotransferrin as negative control. One proteotypic peptide for each protein was selected, and internal heavy isotope-labeled peptide standards were spiked into the samples. Fifty-four samples from pregnant women carrying normal (n = 37) or DS-affected (n = 17) fetuses were analyzed. The median protein concentrations for DS and normal samples, respectively, were as follows: 20 and 49 ng/mL (p < 0.01) for CEL; 3.7 and 14 ng/mL (p < 0.001) for CPA1; 80 and 263 ng/mL (p < 0.001) for MUC13; 46 and 135 ng/mL (p < 0.001) for CLCA1; 0.65 and 0.93 μg/mL (p < 0.05) for MUC5AC; 61 and 73 ng/mL (p > 0.05) for PLUNC; 144 and 86 ng/mL (p < 0.01) for HAPLN1; 0.89 and 0.54 μg/mL (p = 0.05) for CGB; 91 and 87 μg/mL (p > 0.05) for serotransferrin. Statistically significant differences were found in six out of the seven candidate proteins analyzed, reflecting a different regulation in DS.



KEYWORDS: *amniotic fluid, biomarker, Down's syndrome, CEL, CLCA1, CPA1, HAPLN1, MUC13, MUC5AC*

INTRODUCTION

Down syndrome (DS) is a genetic condition caused by the presence of all or part of an extra chromosome 21. It is a common birth defect, the most frequent and most recognizable form of mental retardation, occurring in about 1 of every 700 fetuses.[1] Moreover, it is characterized by several dysmorphic features and delayed psychomotor development. Children with DS have a high probability to be born with congenital heart disease, hearing loss, and/or ophthalmological disorders.[2] Respiratory infections and congenital heart defects are the most frequently reported medical disorders on death certificates for individuals with DS. Besides, these individuals have a higher risk of death from other disorders such as dementia, hypothyroidism, seizures, and leukemia.[3]

At present, several screening strategies for the detection of DS are being used. These methods are based on the combination of maternal age with some serum biomarkers, such as fβhCG, PAPP-A, AFP, inhibin A, and uE3, in the first and/or second trimesters of gestation, and the sonographic measurement of fetal nuchal translucency between 11 + 0 and 13 + 6 weeks of pregnancy. The calculation of a patient-specific risk allows detection of 90−95% of DS cases with a 5% false-positive rate (FPR).[4] However, since these screening strategies lack diagnostic power, a sizable number of patients undergo invasive procedures, such as amniocentesis or chorionic-villus sampling, to obtain a diagnostic result. These invasive techniques have a procedure-related miscarriage rate of 0.6−0.7%.[5]

Research efforts now focus on improvement of the sensitivity and specificity of screening, to reduce or eliminate the number of women needing an invasive diagnostic test. Thus, new

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sonographic markers have been added in the first-trimester screening strategies (such as nasal bone, tricuspid flow, and ductus venosus). Also, major efforts are focusing on the detection of free fetal DNA and RNA in maternal blood, and new biochemical markers are being sought.

Mass spectrometry-based proteomics has become a well-established platform for discovery of novel biomarkers since it allows global analysis of protein expression profiles in any biological sample. Besides, quantitative selected reaction monitoring (SRM) assays performed on a triple quadrupole mass spectrometer has emerged as a robust tool to complement shotgun qualitative studies.[6,7] This technique allows the verification of candidate proteins by quantification of targeted peptides with high selectivity, sensitivity, and a wide dynamic range.[8]

Amniotic fluid (AF) is a watery liquid that surrounds the fetus during pregnancy, protects against mechanical and thermal shock, possesses antimicrobial activity, assists in acid/base balance, and contains nutritional factors.[9] This fluid has great potential to reveal biomarkers that are specific for fetal diseases or complications of pregnancy since it is a reservoir of a large amount of fetal and pregnancy-related proteins. Some of these proteins could help us to better understand the physiology of pregnancy and fetal development.[10]

The purpose of this study was to verify if seven of the proteins identified in our previous studies[11,12] are good AF biomarkers for the prenatal screening of DS. To do so, we developed a mass spectrometry-based SRM assay to quantify these proteins in AF samples from pregnant women with gestational age between 15 and 17 weeks and affected or nonaffected fetuses.

MATERIALS AND METHODS

Materials

RapiGest SF Surfactant was purchased from Waters (Milford, MA, USA). Dithiothreitol, iodoacetamide, and trifluoroacetic acid were from Sigma-Aldrich (St. Louis, MO, USA). Sequencing grade modified trypsin was from Promega (Madison, WI, USA). Ammonium bicarbonate and acetonitrile (grade HPLC) were from Fisher Scientific (New Jersey, USA). OMIX C18 tips were from Varian (Lake Forest, CA, USA). Heavy isotope-labeled peptide standards (Tagged SpikeTides TQL) were from JPT Peptide Technologies (Berlin, Germany). These commercial heavy peptides include a small chemical tag at the C-terminal residue, which has to be cleaved by trypsin digestion.

Amniotic Fluid Samples

AF samples (n = 54) were obtained from pregnant women, with gestational ages ranging from 15 + 0 to 17 + 5 weeks, who underwent amniocentesis and fetal karyotype analysis by the cytogenetics lab at Mount Sinai Hospital (Toronto). Samples were confirmed as being from women carrying chromosomally normal (CN) (n = 37) or DS affected (n = 17) fetuses. These samples were stored at −80 °C until analyzed. The results of alfa-fetoprotein (AFP), in concentration (μg/mL) and multiple of expected median (MoM), measured in these samples for the screening of neural tube defects, with an AutoDelfia analyzer (Perkin-Elmer, Turku, Finland), were collected and used as an external control. Our protocol has been approved by the Institutional Review Board of Mount Sinai Hospital, Toronto, Canada.

Protein Selection

The selection of proteins was based on the score obtained using an in-house selection criteria (Supporting Information, Table S1). It includes three points: relative abundance of proteins in AF, differential expression between DS and CN samples, and tissue-specificity of these proteins. Proteins with high or moderate abundance in AF, with at least 2-fold increase or decrease in expression between DS and CN samples (according to our previous results), and expressed in a restricted number of tissues were preferred. Moreover, we tried to avoid proteins with a moderate or high concentration in the blood of healthy, nonpregnant women.[13] The abundance and differential expression in AF was obtained from our previous studies,[11,12] and the tissue expression specificity was estimated using two databases: BioGPS[14] (biogps.org) and the Human Protein Atlas[15] (proteinatlas.org).

Peptide Selection for SRM Assays

Unique proteotypic peptides for each protein were manually chosen in Scaffold (Proteome Software) using the identification data acquired with an LTQ Orbitrap XL instrument (Thermo Scientific Inc.). Peptides that had clear and intense y-ion fragments, a length of 8−20 amino acids, and without methionine residues were selected. Peptide uniqueness was confirmed by searching against the Basic Local Alignment Search Tool (BLAST).[16] When more than one candidate peptide for a single protein was selected, the peptide with more intense transitions, without interferences, and with no overlap with other peptides in the retention time dimension of the multiplex SRM assay was preferred.

Sample Preparation

Before analysis, AF samples were thawed at room temperature and centrifuged at 13 500 rpm for 10 min to eliminate cells and other cellular debris. Then, samples were diluted 6-fold with 50 mM ammonium bicarbonate and 9 μL of diluted samples were processed. The proteins were denatured with 0.1% RapiGest for 15 min at 80 °C. Reduction and alkylation were performed using 15 mM dithiothreitol for 15 min at 70 °C and 25 mM iodoacetamide for 40 min at room temperature and in the dark, respectively. Then, a mixture with 65 fmoles of each isotopically labeled peptide was added, and the proteins/heavy peptides were digested with trypsin for 21 h at 37 °C (enzyme/substrate ratio of 1:30). The total protein concentration in each sample was determined using the Coomassie Plus (Bradford) protein assay (Thermo Fisher Scientific). After trypsinization, RapiGest was cleaved with 1% trifluoroacetic acid, and samples were centrifuged at 1500 rpm for 30 min. Peptides were purified and extracted using 10 μL OMIX C18 tips, and then eluted using 5 μL of 65% acetonitrile solution (0.1% formic acid). The final sample was diluted to 130 μL with water (0.1% formic acid) to yield three replicates of 40 μL. All these steps were performed in a 96 well plate.

LC-SRM-MS Conditions

AF samples were loaded onto a 2 cm trap column (C18, 5 μm) with an inner diameter of 150 μm, and the peptides were eluted onto a resolving 5 cm analytical column (C18, 3 μm) with an inner diameter of 75 and 15 μm tip (New Objetive). The LC setup, EASY-nLC (Proxeon A/S), was coupled online to a triple-quadrupole mass spectrometer (TSQ Vantage, Thermo Fisher Scientific Inc.) using a nanoelectrospray ionization source (nano-ESI, Proxeon A/S). A three-step gradient with an injection volume of 40 μL was used. Buffer A contained 0.1%

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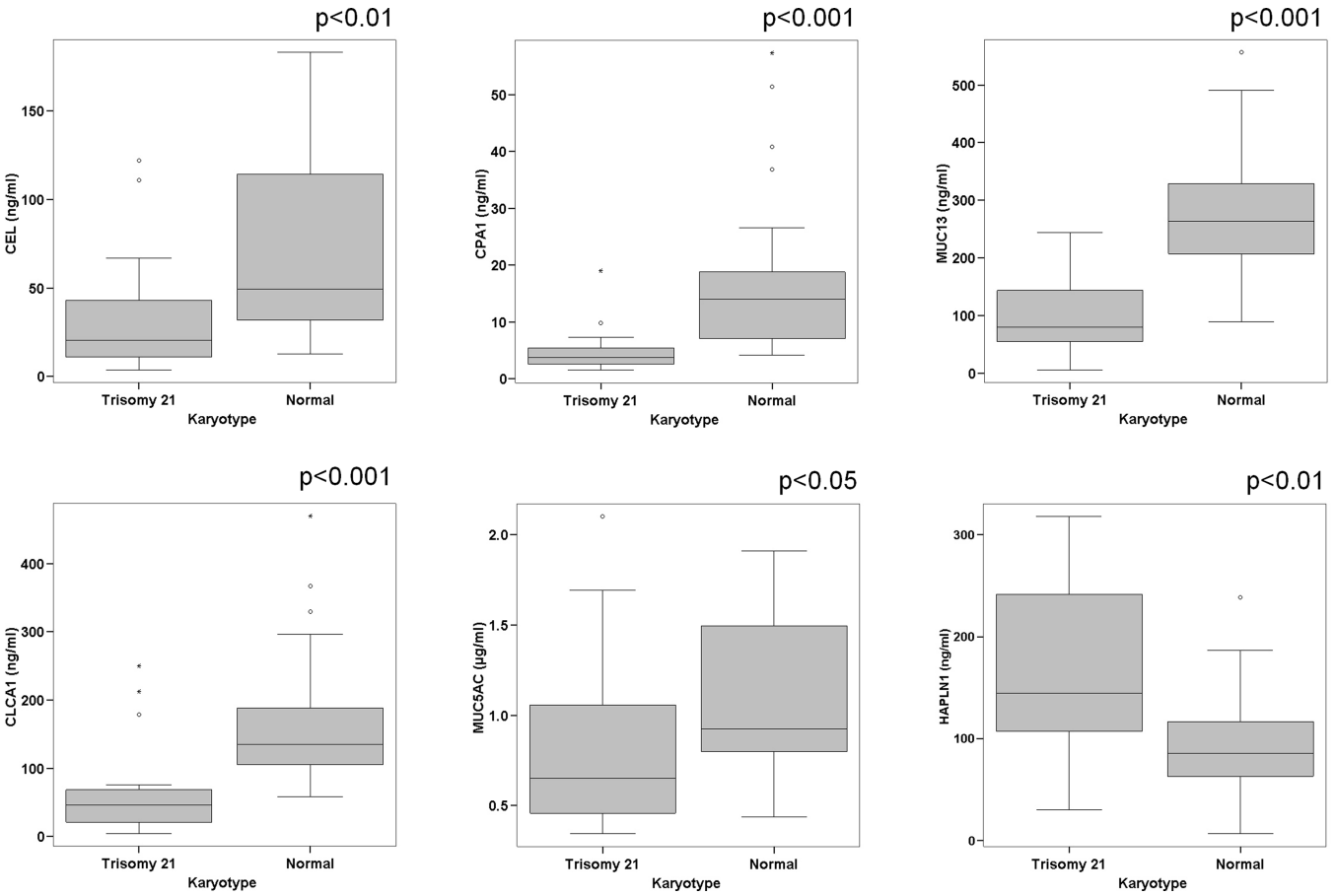

Figure 1. Box and whisker diagrams for CEL, CPA1, MUC13, CLCA1, MUC5AC, and HAPLN1 proteins, in trisomy 21 (n = 17) and chromosomally normal samples (n = 37).

formic acid in water, and buffer B contained 0.1% formic acid in acetonitrile. A 54/59 min method with 30/35 min gradient was used for initial identification of peptides and light/heavy peptide quantification, respectively. Peptides were analyzed by a multiplex SRM method with the following parameters: positive-ion mode, predicted collision energy values, 1.5 s cycle time, 0.2 Da of full width at half-maximum (fwhm) in Q1 and 0.7 Da fwhm in Q3, 1.5 mTorr Q2 pressure, tuned tube lens values, and 1 V declustering voltage. Three transitions for each light/heavy peptides were monitored (Supporting Information, Table S2). In silico digestion and fragmentation, prediction of collision energy, and analysis of results were performed using Pinpoint 1.0 software (Thermo Scientific Inc.). Skyline software[17] was used to predict the retention time of peptides for the initial identification.

Statistical Analysis

Statistical analyses were performed with SPSS 15.0 software package (SPSS Inc., Chicago, IL, USA). A p-value <0.05 was considered statistically significant. Normal distribution was evaluated using Shapiro–Wilk test and by inspection of Q–Q plots. Student's t- or Mann–Whitney U-tests were performed for comparison between independent samples. Receiver-operating characteristic (ROC) curves were plotted and areas under the curve (AUC), with a 95% confidence interval (CI), were calculated.

RESULTS

Selection of Proteins and Proteotypic Peptides

After analyzing 542 proteins with our candidate selection criteria and exclude proteins with high to moderate abundance in blood, a total of 7 candidate proteins were selected: four new candidates, chloride channel accessory 1 (CLCA1), hyaluronan and proteoglycan link protein 1 (HAPLN1), mucin 5AC (MUC5AC), and PLUNC (palate, lung and nasal epithelium associated); and three candidates from our previous study,[11] bile salt-activated lipase (CEL), carboxypeptidase A1 (CPA1), and mucin 13 (MUC13). These candidate proteins showed moderate or high tissue specificity according to the databases analyzed (Supporting Information, Figures S1–S7). Moreover, two more proteins (chorionic gonadotropin, beta polypeptide (CGB) and serotransferrin (TF)) used as internal controls were analyzed. The CGB was selected as positive control, since it is a known biomarker of DS, and TF was selected as negative control, since it is one of the most abundant proteins in AF and its levels were previously demonstrated not to be significantly different between AF samples.[11] Between 1 and 3 proteotypic peptides for each protein were analyzed to finally select the peptide with the best analytical features.

Confirmation of Peptide Identification

The identity of peptides was confirmed by four different ways. First, by prediction of retention times using SRRCalc 3.0 from Skyline software formula to predict the retention time according to the hydrophobicity of peptide (Supporting

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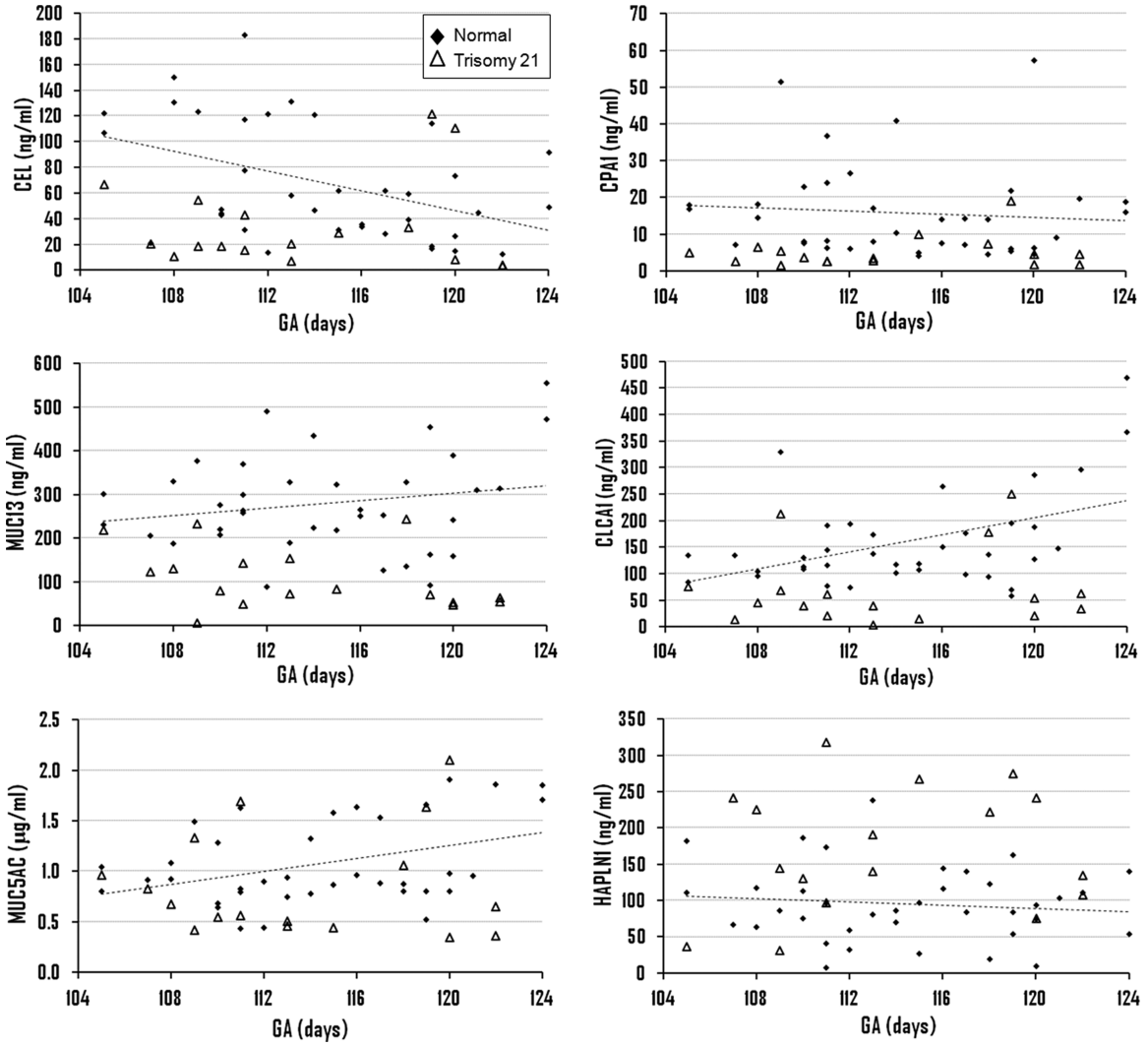

Figure 2. Concentrations of CEL, CPA1, MUC13, CLCA1, MUC5AC, and HAPLN1, according to the gestational age (from 105 to 124 days). The filled diamonds and open triangles represent the normal and trisomy 21 samples, respectively. The dashed line represents the estimation of median values in chromosomally normal pregnancies.

Information, Table S3). Second, by analysis of correlation between retention times in the discovery stage with an LTQ Orbitrap XL (60 min gradient) and verification stage with a TSQ Vantage (30 min gradient) (Supporting Information, Figure S8). Third, observing the coelution of at least 6 transitions for each peptide analyzed. Fourth, comparing the fragmentation pattern (similar intensity of transitions) in the LTQ Orbitrap XL and TSQ Vantage (Supporting Information, Figure S9).

Analysis of Individual Amniotic Fluid Samples

AF samples were analyzed sequentially and in a stochastic order. The peak area for each endogenous peptide was integrated, and the concentration was calculated by extrapolation with the area of internal standard (Supporting Information, Figures S10 and S11). The protein concentration was calculated assuming a ratio peptide/protein of 1:1 and using the molecular weight corresponding to the complete sequence of each protein. Three injections (SRM runs) per sample were performed to estimate the reproducibility of analysis. The average coefficient of variation (CV) for the nine peptides was from 3.3 to 12.4%, with an overall CV lower than 6%. Additionally, the concentration of AFP in these AF samples was used as an external control for subsequent analyses.

The median protein concentrations for DS and CN samples were 20 and 49 ng/mL ($p < 0.01$) for CEL; 3.7 and 14 ng/mL ($p < 0.001$) for CPA1; 80 and 263 ng/mL ($p < 0.001$) for MUC13; 46 and 135 ng/mL ($p < 0.001$) for CLCA1; 0.65 and 0.93 µg/mL ($p < 0.05$) for MUC5AC; 144 and 86 ng/mL ($p < 0.01$) for HAPLN1 (Figure 1); 61 and 73 ng/mL ($p > 0.05$) for PLUNC, 91 and 87 µg/mL ($p > 0.05$) for TF; 0.89 and 0.54 µg/mL ($p = 0.05$) for CGB; 9.3 and 13.9 µg/mL ($p < 0.001$) for AFP (Supporting Information, Figure S12). The mean gestational age was 114 days in both groups of samples.

Moreover, the concentrations were plotted according to gestational age (Figure 2 and Supporting Information, Figure S13), and the results from CN samples were used to estimate the median protein concentration, by calculation of linear regression. Then, the concentrations were transformed to MoM, and the ratio DS/CN of median MoM was calculated (Table 1), resulting in CEL (0.23), CPA1 (0.26), MUC13 (0.30), CLCA1 (0.31), MUC5AC (0.65), HAPLN1 (1.68), PLUNC (0.80), TF (1.00), CGB (1.81), and AFP (0.59). The MoMs obtained for AFP with this linear regression were

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Table 1. Calculation of the Median MoM of Each Protein, in the Cases of Trisomy 21 and Normal Karyotype

	median MoM		ratio
protein	trisomy 21	normal karyotype	trisomy 21/normal
CEL	0.22	0.94	0.23
CPA1	0.22	0.84	0.26
MUC13	0.30	0.98	0.30
CLCA1	0.28	0.92	0.31
MUC5AC	0.59	0.90	0.65
PLUNC	0.62	0.78	0.80
HAPLN1	1.56	0.93	1.68
TF	1.00	1.00	1.00
CGB	1.56	0.86	1.81
AFP	0.59	1.00	0.59

compared with the MoMs used in the prenatal screening program of neural tube defects (Mount Sinai Hospital) and calculated with a large number of AF samples, resulting in a difference of MoMs from −6.3 to +10.6%.

Evaluation of Potential Value of Biomarkers Analyzed

The MoMs obtained for all patients (n = 54) were used to calculate the ROC curve and the corresponding AUC for proteins with significant differences in concentration (MUC13, CPA1, CLCA1, CEL, HAPLN1, and MUC5AC) and positive controls (CGB and AFP) (Table 2 and Supporting Information, Figure S14). MUC13 showed the greatest AUC (0.900; 95% CI: 0.815−0.985), followed by AFP (0.895; 95% CI: 0.778−1.012), CPA1 (0.882; 95% CI: 0.778−0.986), and CLCA1 (0.825; 95% CI: 0.681−0.969). Therefore, MUC13, CPA1, and CLCA1 seem to be the most interesting candidates. Finally, the comparison of MoMs obtained in the cases of DS (Supporting Information, Table S4), shows that candidate proteins may generate different and complementary information to that provided by known biomarkers, as in cases 7, 11, and 13 where the results of CGB and AFP weakly (or not) increase the risk of DS and the results of some of the candidate proteins are very abnormal.

Table 2. Area under the Curve (AUC) and Confidence Interval at 95% (95% CI), for Each Candidate Protein (CEL, CPA1, MUC13, CLCA1, MUC5AC, and HAPLN1) and Positive Controls (CGB and AFP)

protein	AUC	95% CI
MUC13	0.900	0.815−0.985
AFP	0.895	0.778−1.012
CPA1	0.882	0.778−0.986
CLCA1	0.825	0.681−0.969
CEL	0.774	0.617−0.932
HAPLN1	0.762	0.611−0.912
CGB	0.684	0.517−0.850
MUC5AC	0.636	0.451−0.821

DISCUSSION

Current screening strategies of DS allow detection of approximately 95% of cases at 5% FPR.[4] This imperfect diagnostic power leads a considerable number of invasive procedures (amniocentesis; chorionic-villus sampling), increasing the risk of pregnancy complications and the costs of maternal care. At present, many studies showing progress in the noninvasive diagnosis of DS have been published.[18] These studies are based on the analysis of fetal DNA or RNA in maternal blood by using different techniques. The massively parallel shotgun sequencing (MPSS) is one of the techniques that have shown the most promising results. Thus, Palomaki et al.[19] reported a DS detection rate of 98.6% at 0.20% FPR. However, this technology is not diagnostic yet, and since the test is complex, and the resources needed are considerable, it cannot be offered as a screening test to all pregnant women, but appears to be an adequate second line test. Therefore, the improvement of current screening strategies may be very useful in identifying pregnant women with highest risk of DS for further analysis with MPSS or similar techniques. The inclusion of new serum markers of DS in the current screening programs is an easy and cheap way to improve the performance of these maternal care strategies. These new biomarkers must meet two basic characteristics to have an impact on screening. First, they have to have a serum concentration significantly different between DS and normal karyotype, and second, they need to show complementarity with known biomarkers.

In recent years, several proteomic studies focused on the search for new biomarkers of DS, in both amniotic fluid and maternal blood, have been published. Some of these studies use different approaches for the quantification of candidate proteins, such as SRM[20,21] and iTRAQ[22,23] technologies, among others. However, the results are not always consistent. Thus, for instance, Heywood et al.[20] have recently reported the up-regulation of serum amyloid P protein in the maternal blood of pregnant women carrying fetuses affected by DS, both in first and second trimesters, while Lopez et al.[21] previously reported the down-regulation of this protein in the first trimester, despite the fact that the same proteotypic peptide was used in both studies for the quantification of serum amyloid P protein with a SRM method.

The most important limitation of using maternal blood for the discovery of DS biomarkers is that because of the complexity of this fluid, only highly abundant proteins can be detected and studied. Besides, the direct association of these proteins with the fetal development is unlikely. On the other hand, the AF has great potential to reveal biomarkers that are specific for fetal diseases or complications of pregnancy. However, the major limitation of using AF samples is that it is not possible to predict whether these proteins will cross the placental barrier until the maternal blood circulation and, therefore, if the differences in concentration between AF and maternal serum samples are similar or not.

In our previous studies,[11,12] we have used the AF as source of potential biomarkers of DS and the mass spectrometry as a tool of protein identification and preliminary verification of some candidate proteins. In this study, we present the quantification, in an independent set of AF samples, of seven potential biomarkers of DS by using the SRM technology. Here, one heavy isotope-labeled peptide for each candidate protein was used as internal standard. Positive and negative controls were included in the multiplex SRM assay, and the results of normal pregnancies were used to calculate the MoMs and establish ROC curves.

The AF samples analyzed were from pregnant women with gestational ages ranging from 15 + 0 to 17 + 5 weeks. The purpose was to study the candidate proteins in a narrow gestational interval, to reduce the variability of protein concentration due to the evolution of pregnancy, and at the same time, maximize the likelihood of finding a useful

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biomarker in first and/or second trimesters of pregnancy since these gestational weeks are the closest to first trimester, in which the amniocentesis can be performed.

The selection of studied proteins was based on three major criteria with the aim to have good candidates for further future analysis in maternal blood. Thus, proteins with moderate or high abundance in AF were preferred, in order to avoid an excessive dilution in maternal blood. Priority was given to the proteins with at least 2-fold increase or decrease expression between DS and CN samples (according to our previous results) and with strong expression in relevant tissues, such as placenta (e.g., HAPLN1) or tissues where it has been described that children with DS show issues,[24] such as gastrointestinal tract (e.g., MUC13) and respiratory system (e.g., MUC5AC). If these proteins are expressed and involved on the development of these fetal tissues, then they can be good biomarkers for the prenatal screening of DS. On the other hand, the blood concentration of proteins studied was estimated using different ways, previous proteomic studies[13] and bioinformatic tools,[14,15] avoiding those proteins with high concentration in maternal blood of nonpregnant women, since the serum protein concentration of maternal origin may mask the differences observed in the AF. These requirements are fulfilled by the known biomarkers AFP and CGB.

The results obtained in the 54 samples assayed indicate that six out of the seven proteins studied are potential candidates for the detection of DS. Five of the proteins (CEL, CPA1, MUC13, CLCA1, and MUC5AC) showed a significant down-regulation in DS cases, and only one protein (HAPLN1) showed up-regulation. Moreover, three of these proteins (CPA1, CEL, and MUC13) were analyzed in our previous study[11] but in a smaller set of samples, showing similar results. The inclusion in the analysis of positive (CGB and AFP) and negative (TF) controls allowed for increased confidence in the obtained results. Thus, the comparison of concentrations (in CN and DS samples) for TF (negative control) did not show significant differences and for CGB (positive control) were within the limit of significance (p = 0.05). The subsequent estimation of medians and calculation of MoMs seems to be reasonable since for AFP, the differences found between the estimated MoMs and the MoMs used in the screening program of neural tube defects were ±10%. The median MoM obtained for DS cases, after correction with the value for CN cases, was 1.00 MoM (for TF), 1.81 MoM (for CGB), and 0.59 MoM (for AFP). It is important to point out that because of the technology used (SRM), the results for CGB correspond to total hCG. Similar results were described by Spencer et al.[25] for CGB (1.84 MoM) and AFP (0.56 MoM), using immunoassays and AF samples from pregnant women carrying DS (n = 91) or CN (n = 240) fetuses, at 15−21 gestational weeks. Therefore, the negative control did not show any difference between groups, and the positive controls showed very similar differences to those described previously.

With respect to the candidates, HAPLN1 is a protein that stabilizes the interaction between hyaluronan and several proteoglycans, such as versican and agrecan, in cartilage formation.[26] This protein is highly expressed in decidual cells of placenta as well as ovary, heart, and brain.[15] Several studies have suggested that HAPLN1 may have important roles in heart development[26,27] and as a survival antiapoptotic factor in luteinizing granulosa cells. Moreover, the expression of HAPLN1 in ovary and granulose cells seems to be stimulated by hCG.[28] The overexpression of HAPLN1 in DS-AF samples may be related with the overexpression of hCG in these pregnancies and/or with the development of heart malformations in DS fetuses.

MUC5AC is a member of the gel-forming mucin subfamily of secreted vertebrate mucins. It is secreted by epithelial goblet and the glandular mucous cells, protecting the mucosa from infection and chemical damage.[29] In the respiratory tract, mucus has a protective function by trapping inhaled foreign debris and bacteria and clearing them from the airways.[30] The down-regulation of MUC5AC may be related to poor protection of airways and frequent respiratory infections, reported in individuals with DS.[3]

CLCA1 protein is normally expressed in the goblet cells of intestinal and respiratory tracts, playing an important role in the pathogenesis of respiratory diseases.[31] Kim et al.[32] described that histamine induces the expression of MUC5AC via the upregulation of CLCA1. Moreover, it has been reported that there is a deficit of histamine in DS brains,[33] which may induce the down-regulation of CLCA1 and MUC5AC observed in AF samples.

MUC13 is a transmembrane glycoprotein normally localized to the apical surface of epithelial cells in the gastrointestinal system, playing a role in protection and lubrication of the mucosal surface.[34] Its role in DS may be similar to that of the MUC5AC protein; however, the regulation of MUC13 expression has not been studied yet.

CEL and CPA1 are two proteins highly expressed in the pancreas. CEL is synthesized primarily in the acinar cells of the pancreas, and it is secreted into the lumen of the intestine. This protein catalyzes the hydrolysis of cholesteryl esters to nonesterified cholesterol and fatty acids,[35] but its role in hepatic and plasma cholesterol metabolism remain unclear. Interestingly, DS appears to be a protective factor regarding the development of atherosclerosis, and higher levels of cholesterol in blood samples of fetuses with DS have been reported.[36] Finally, CPA1 is a monomeric pancreatic exopeptidase involved in zymogen inhibition but with unknown biological function.

■ CONCLUSION

Statistically significant differences were found in six out of the seven proteins analyzed, reflecting a different regulation in DS. These proteins may be useful biomarkers in the screening of this pathology in the first and/or second trimesters of gestation. Further analyses with maternal serum samples must be performed to elucidate the real potential of these candidates in DS prenatal diagnosis.

■ ASSOCIATED CONTENT

Supporting Information

Tables S1−S4 and Figures S1−S14. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS

AF, amniotic fluid; AFP, alfa-fetoprotein; AUC, area under the curve; CEL, bile salt-activated lipase; CGB, chorionic gonadotropin, beta polypeptide; CI, confidence interval; CLCA1, chloride channel accessory 1; CN, chromosomally normal; CPA1, carboxypeptidase A1; CV, coefficient of variation; DS, Down syndrome; FPR, false-positive rate; fwhm, full width at half-maximum; HAPLN1, hyaluronan and proteoglycan link protein 1; MoM, multiple of expected median; MPSS, massively parallel shotgun sequencing; MUC13, mucin 13; MUC5AC, mucin 5AC; PLUNC, palate, lung and nasal epithelium associated; ROC, receiver-operating characteristic; SRM, selected reaction monitoring; TF, serotransferrin

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