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Proteomic analyses of amniotic fluid : Potential application in health and diseases

THESE

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Pierre-Alain Queloz

Médecin diplômé de la Confédération Suisse
Originaire de Saint-Brais

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Protéomique du liquide amniotique

Résumé :

Le liquide amniotique est essentiel pour le développement du fœtus durant la grossesse. Sa production est directement associée à l'intégrité fonctionnelle des compartiments fœtal, placentaire et amniotique. Le liquide amniotique étant produit et modifié durant la grossesse par différents sites, son profil protéique reflète les changements physiologiques et pathologiques du fœtus et de la mère.

Différentes techniques d'électrophorèse bidimensionnelle, couplées à l'analyse par spectrométrie de masse, on été utilisées pour :
i) déterminer le potentiel analytique de ces méthodes dans l'identification de biomarqueurs. Différentes quantités d'inhibiteur de la trypsine de soja et de myoglobine équine ont été ajoutées au liquide amniotique. Dans ce modèle, une quantité minimale de 5 à 10 ng de ces biomarqueurs artificiels ont été détectés.
ii) évaluer la place de ces méthodes dans la comparaison de liquides amniotiques issus de grossesses normales, obtenus à 17 semaines d'aménorrhée et à terme, et de grossesses où le fœtus présentait une hernie diaphragmatique congénitale. Les protéines exprimées de manière différentielles ont été identifiées.
Finalement, le potentiel de l'électrophorèse bidimensionnelle a été évalué par l'étude de plasma contenant des protéines spécifiques du liquide amniotique dans un modèle de rupture prématurée des membranes. Les résultats obtenus montrent que les technologies utilisant l'électrophorèse bidimensionnelle gardent leur place dans l'analyse de liquides biologiques, tel le liquide amniotique.

Two-Dimensional Gel Electrophoresis Based Technologies for Potential Biomarkers Identification in Amniotic Fluid: A Simple Model

P.-A. Queloz[1], D. Crettaz[1], L. Thadikkaran[1], V. Sapin[2] and J.-D. Tissot[1,*]

[1]*Service régional vaudois de Transfusion Sanguine, Lausanne, Switzerland;* [2]*CHU, Laboratoire de Biochimie Médicale, F-63000, Clermont-Ferrand, France*

Abstract: To assess the efficiency of two-dimensional fluorescence difference gel electrophoresis (2D-DIGE) and of two-dimensional electrophoresis and ammoniacal silver staining (2D-E), different amounts of soybean trypsin inhibitor and horse myoglobin were added to amniotic fluid. In this model, a minimum of 5 to 10 ng of "artificial" biomarkers was detected.

Keywords: Amniotic fluid, Biomarker, CyDye, Proteomics, Two-dimensional electrophoresis.

INTRODUCTION

Amniotic fluid (AF) is essential for fetus development during pregnancy. Its production is directly related to the functional integrity of the fetal, placental and amniotic compartments and varies throughout pregnancy (reviewed in [1]). Since AF is produced and modified throughout pregnancy by different sites, the protein profile of AF would reflect both physiological and pathological changes, which affect the fetus and the mother. Preliminary proteomic works yet published on AF have been limited to identify specific proteins of the amniotic fluid as potential biomarkers with promising results [2-8]. Here, we used 2D fluorescence difference gel electrophoresis (2D-DIGE; Ettan™ DIGE) as well as 2D-E and silver staining to assess the analytical potential of these methods to identify biomarkers in AF.

MATERIAL AND METHODS

Samples Preparation

Pure normal AF samples were obtained from women at term after informed consent as previously described [3]. AF proteins were concentrated to 34 mg/ml using Vivaspin Concentrator (cut-off 5 kDa) from Vivascience (Hannover, Germany). AF protein concentrations were measured by a protein-dye binding coloring method [9], using albumin as standard. Lyophilized soybean trypsin inhibitor (Kunitz-type trypsin inhibitor A) was purchased from Fluka Chemie (Buchs, Switzerland) whereas lyophilized horse myoglobin was obtained from Sigma-Aldrich Chemie (Steinheim, Germany). These two components were used as artificial biomarkers. The lyophilized biomarker proteins were diluted in "buffer A" (urea 8 M, TRIS 40 mM and CHAPS 4%). Various volumes of the latter solution were added to AF in order to obtained AF containing artificial biomarkers (BAF). BAF samples containing from 1 ng to 500 ng of each biomarkers/50 µg of AF proteins were prepared. Control AF samples (CAF) were prepared similarly, but without biomarkers.

2D-E, Silver Staining, Gel Analysis and MALDI-TOF MS

For 2D-E and ammoniacal silver staining, 25 µg of proteins were loaded onto the first dimension isoelectric gels. All methods have been described in detail elsewhere [3,10-13]. Briefly, isoelectric focusing was performed under paraffin oil, using linear immobilized pH gradients (Immobiline Dry-Strip, pH range 3-10, 18 cm) from GE Healthcare (Uppsala, Sweden). The strips were rehydrated in a solution containing 8 M urea, 2% CHAPS, 10 mM DTE, 2% Pharmalyte™ pH 3-10, and traces of bromophenol blue. Samples were then loaded on the cathodic side of the gels. After the migration, strips were placed on the top of 9–16% gradient polyacrylamide gels for the second dimension.

Ammoniacal silver staining was done according to standard protocols [10]. Briefly, at the end of the run, the gels were washed in water, then soaked in ethanol:acetic acid:water (40:10:50) for 1 h and ethanol:acetic acid:water (10:5:85) overnight. After a water wash, the gels were soaked 30 min in glutaraldehyde (1%) buffered with sodium acetate (0.5 M) and then washed with water. The gels were soaked in a 2,7-naphtalenedisulfonic acid fresh solution (0.05%, w/v) for 30 min and rinsed again with water. The gels were stained in a freshly made ammoniacal silver nitrate solution for 30 min . The images were finally developed in a solution containing citric acid (0.01%, w/v) and formaldehyde (0.1%, w/v). Development was stopped with an acetic acid:water (5:95) solution. The silver stained gel images were captured with high resolution (Pixel size 100 micron) densitometry reading with the Personal Laser Densitometer (GE Healthcare). All informatic analyses were performed with the software ImageMaster 2D Platinum (GE Healthcare).

For preparative gels, 125 µg of proteins were loaded onto the gels and Coomassie Blue staining was performed. Briefly, after the run, the gels were washed several times with water and incubated overnight with Colloidal Coomas-

*Address correspondance to this author at the Service régional vaudois de transfusion sanguine, Rue du Bugnon 27, CH-1005 Lausanne, Switzerland; Tel: +41 21 314 65 89; Fax: +41 21 314 65 97;
E-mail: jean-daniel.tissot@chuv.ch

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sie Blue Brilliant. Gels were then rinsed and stained spots were identified by MALDI-TOF, as described elsewhere [3,11,12].

Sample Labeling for 2D-DIGE and Scanning

Each protein sample was labeled with CyDye DIGE Fluor according to the manufacturer instructions (GE Healthcare). BAF and CAF samples (50 µg of proteins) were diluted in "buffer A" to a final volume of 30 µl. Cyanine dyes were reconstituted in 99.8% anhydrous N,N-dimethylformamide from Sigma (Steinheim, Germany), and added to labeling reactions in a ratio of 400 pmol CyDye to 50 µg protein. Protein labeling was achieved by incubation on ice in the dark for 30 min. The reaction was quenched by the addition of 2 µl of 10 mM lysine from Sigma (Steinheim, Germany), followed by incubation on ice for a further 10 min. The samples were separately labeled, BAF with Cy5 and CAF with Cy3. They were then mixed and diluted to a final volume of 100 µl by adding "buffer B" (urea 7 M, thiourea 2 M, CHAPS 4%, DTE 2%, pharmalyte 3-10 2% and pharmalyte 4-7 1%), and loaded onto the first dimension gels (cup loading). CyDye labeled gels were finally scanned using a Typhoon 9400 Variable Mode Imager (Pixel size 100 micron; GE Healthcare).

RESULTS AND DISCUSSION

As shown by (Fig. **1**), soybean trypsin inhibitor (Swissprot P01070; M_r 24275, calculated pI 4.99) were resolved as a double overlapping spot in an area of the gel corresponding to 23 - 26 kDa and pI of 4.5 to 5, whereas horse myoglobin (Swissprot P68082; M_r 16941, calculated pI 7.36) was detected as two separate spots in an area corresponding to 16 - 17 kDa and pI of 7 to 8. No other contaminant spots appeared in the gel, meaning that the purity of the two artificial biomarkers was adequate for the study. Fig. (**2**) shows the detection of the two artificial biomarkers in AF (50 ng of each biomarker). The detection limit was identical for 2D-DIGE and 2D-E with ammoniacal silver staining, and was of 10 ng for trypsin inhibitor and of 5 ng for horse myoglobin (Fig. **3**). In our hands, a final amount of 100 ng of each biomarker was necessary to be detected after staining with colloidal Coomassie Blue Brilliant. When analyzed by MALDI-TOF MS, colloidal Coomassie Blue Brilliant stained spots were correctly identified. The sequence coverage was identical (31% for trypsin inibitor and 80% for horse myoglobin) when either 100 or 500 ng of Coomassie stained proteins were analyzed. In optimized conditions, the 2D-DIGE system was claimed to be able to detect protein amounts down to 15 pg per spot, whereas silver staining technologies appeared to be limited to detect about 1 ng per spot [14,15]. We have to mention here, that the objective of our study was to find the detection limit of artificial biomarkers in an environment rich in proteins (50 µg). In our study, 5 to 10 ng of each biomarkers were clearly identified, when 100 µg (50 µg BAF and 50 µg CAF) were loaded on the gels. The settings of the PMT scanner (600 to 650 volt) were chosen in order to try to detect both high and low abundance proteins. Higher

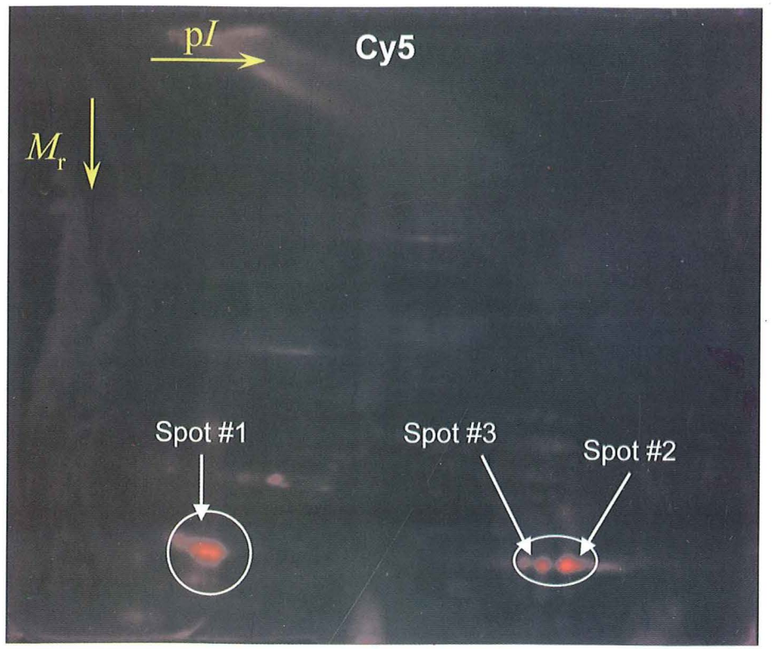

Figure 1: 2D image of a mixture of soybean trypsin inhibitor (40 ng; spot # 1) and of horse myoglobin (40 ng, spots #2 and #3), stained with Cy5.

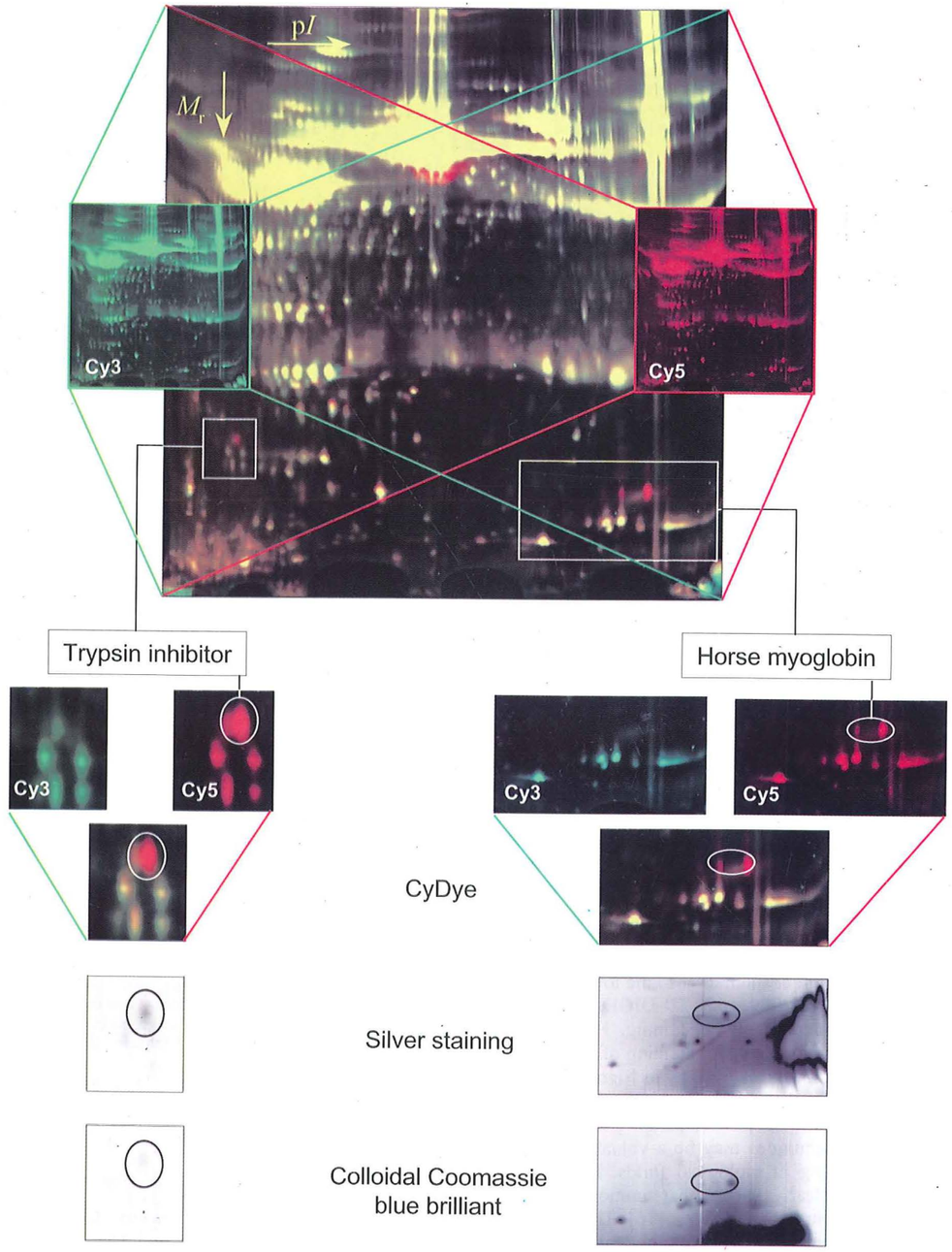

Figure 2: Comparison of the 2D patterns of amniotic fluid proteins without addition of artificial biomarkers (stained with Cy3, inset in green) with that of amniotic fluid proteins with addition of 50 ng of trypsin inhibitor and 50 ng of horse myoglobin (stained with Cy5, inset in red). Fifty µg of proteins of each stained samples were mixed and loaded on immobilized 3 to 10 non linear pH gradient followed by second dimension (9 to 16% polyacrylamide gel electrophoresis. The large 2D gel represents the composite image, and most of the spots, common to the two samples, were detected in yellow. The areas of the gels corresponding either to trypsin inhibitor or to horse myoglobin are highlighted by the frames. The spots corresponding either to trypsin inhibitor or to horse myoglobin are clearly evidenced as additional spots stained in red, in the composite image. These spots were also detected after silver staining (50 ng) and also after colloidal Coomassie Blue Brilliant staining but with higher amount of biomarkers (500 ng).

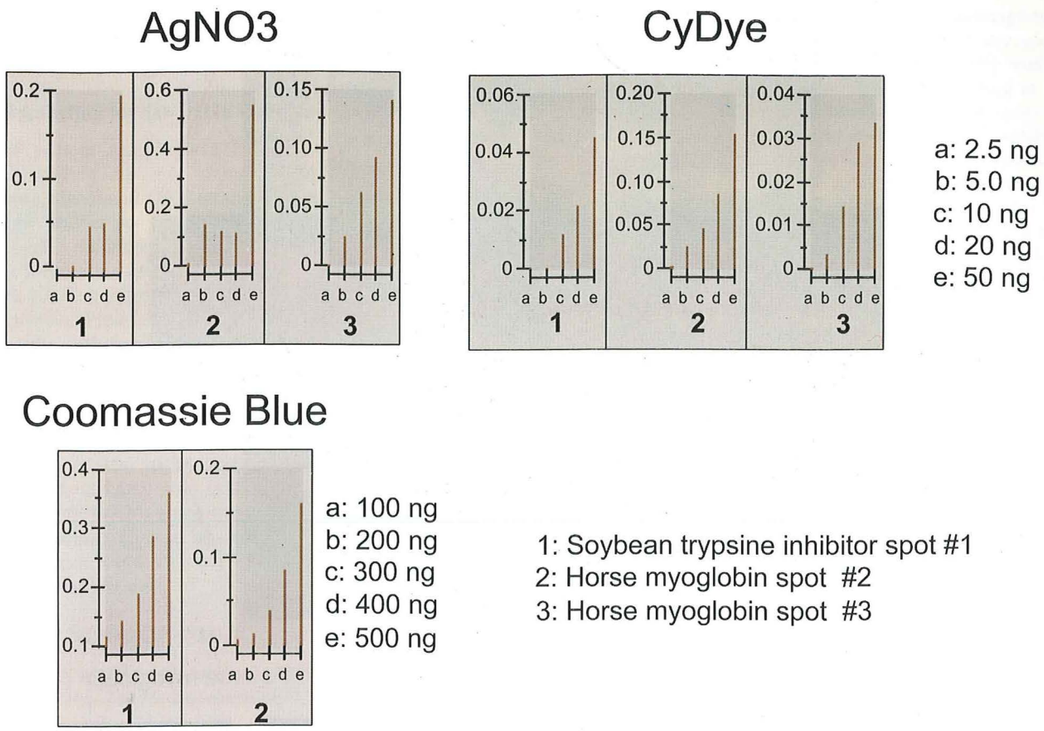

Figure 3: Histograms (%volume) of the spots of soybean trypsin inhibitor (spot #1) and of horse myoglobin (spots #2 and #3), measured according to the different staining procedures, at different concentrations. Note that spot #3 of horse myoglobin was not detected using Coomassie Blue.

voltage values allowed a lower detection threshold, but was associated with a saturation of the high concentrated proteins present in AF. For 2D-E and silver staining, 5 ng of proteins were identified when a maximum of 25 µg of AF proteins were loaded, because at higher protein content, the silver stained images were of poor quality (data not shown). The ratio between the amount of biomarker to the total amount of proteins loaded were 1/20'000 using 2D-DIGE, and 1/5'000 with 2D-E and ammoniacal silver staining. However, the number of spots detected after silver staining of 25 µg of AF proteins (N=4; 778 ± 65; mean ± SD) was higher (P=0.015, t-test) than those observed after labeling of 50 µg of AF proteins with CyDye (648 ± 67; N=4; mean ± SD). Clearly, combining the two techniques may be a valuable approach for biomarker screening in biological fluids. Interestingly, recent studies have clearly shown that ammoniacal silver staining does not preclude MS analysis [16]. Therefore, image analysis can be combined with protein identification in a single experiment. Furthermore, the Eriochrome black T-silver method, which incorporates a new silver sensitizer, was shown to considerably increase the limit of detection of proteins after staining (up to 0.05 ng) with the advantage to be MS compatible at quantities of 3 to 6 ng of proteins [17]. Our results show that models such as the one described in this study should be used when different techniques are compared. By knowing the limits of detection of 2D based technologies, the best approach can be chosen, taking into account the costs of both equipments and reagents that will be used.

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Proteomic analyses of amniotic fluid: Potential applications in health and diseases

Pierre-Alain Queloz[a], David Crettaz[a], Lynne Thadikkaran[a], Vincent Sapin[b], Denis Gallot[c], Jacques Jani[d], Jan Deprest[d], Didier Lémery[c], Stefano Barelli[a], Jean-Daniel Tissot[a,*]

[a] *Service Régional Vaudois de Transfusion Sanguine, Lausanne, Switzerland*
[b] *CHU, Laboratoire de Biochimie Médicale, F-63000, Clermont-Ferrand, France*
[c] *CHU, Unité de Médecine Materno-Fœtale, Hôtel-Dieu, F-63000, Clermont-Ferrand, France*
[d] *UZ-Gasthuisberg, Leuven, Belgium*

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Abstract

Amniotic fluid (AF) is a potential source of biomarkers for many disorders which may occur during pregnancy. The purpose of this study was to evaluate the place of two-dimensional gel electrophoresis (2-DE) technologies to compare AF in both normal and pathological situations. Two-dimensional fluorescence difference gel electrophoresis (2D-DIGE; Ettan™ DIGE) as well as two-dimensional gel electrophoresis and silver staining followed by image analysis were used. Differentially expressed proteins were identified by mass spectrometry. This approach was used to study electrophoregrams of normal AF obtained at 17 weeks of gestation and at term, as well as AF from fetuses presenting with congenital diaphragmatic hernia. Finally, the potential of two-dimensional electrophoresis was assessed by studying the protein profile of plasma containing AF proteins in a model of premature rupture of the membranes (PROM). Our results clearly show that two-dimensional electrophoresis technologies still have place for analyzing biological fluids such as AF.

Keywords: Amniotic fluid; Congenital diaphragmatic hernia; Proteomics; Two-dimensional electrophoresis

1. Introduction

The biological composition of amniotic fluid (AF) is modified throughout pregnancy. Its protein profile reflects both physiological and pathological changes affecting the fetus and the mother. A number of proteomic studies have been published on AF, and have been recently reviewed [1,2]. Michel et al. identified a series of AF proteins that were absent from plasma, and therefore may be potential markers of premature rupture of membranes (PROM) [3]. In addition, several groups were able to identify proteins that appear useful for the diagnosis of intra-amniotic inflammation [4–8].

Since more than 25 years, two-dimensional gel electrophoresis (2-DE) has been used to identify novel biological markers that can eventually be used for early detection and/or disease diagnosis. Here, we evaluated the role of 2-DE and silver staining as well as of 2D fluorescence difference gel electrophoresis (2-D-DIGE; Ettan™ DIGE) to compare normal proteomic profiles of AF obtained either at 17 or 40 weeks of pregnancy with those of fetuses presenting with congenital diaphragmatic hernia (CDH).

2. Material and methods

2.1. Samples preparation

Pure normal AF samples (30 ml - about 3 mg/ml protein) were obtained after informed consent as previously described [9]. AF were obtained either at 17 ($N=7$) or 40 weeks of pregnancy ($N=7$).

* Corresponding author at. Service Régional Vaudois de Transfusion Sanguine, Rue du Bugnon 27, CH-1005 Lausanne, Switzerland.
Tel.: +41 21 314 65 89; fax: +41 21 314 65 97.
E-mail address: jean-daniel.tissot@chuv.ch (J.-D. Tissot).

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AF and fetal tracheal fluid (TF) were collected from three fetuses requiring a fetoscopic tracheal occlusion procedure (FETO) for severe left-sided CDH (intra-thoracic liver migration and lung-to-head ratio <1). All procedures were performed under the supervision of one of us (JD) after extensive counseling and written informed consent of the mother as described elsewhere [10,11]. Briefly, 5 ml of amniotic and tracheal fluid were taken during the placement (28 weeks of gestation) and removal (34 weeks of gestation) of the tracheal balloon. Samples were immediately centrifuged and frozen. After thawing, protein concentrations were measured by a protein-dye binding coloring method [12], using albumin as standard. The following samples, from three different fetuses, were studied: TF before (TFB) and after plug (TFA), and AF before (AFB) and after plug (AFA).

2.2. 2-DE, silver staining, gel analysis and MS

The methods have been described in detail elsewhere [13]. Briefly, isoelectric focusing (IEF) was performed under paraffin oil, using linear non-immobilized pH gradients (Immobiline Dry-Strip, pH range 4–7, 18 cm from (GE Healthcare, Otelfingen, Switzerland). Samples were loaded on the cathodic side of the gels. Before the second dimension, strips were equilibrated and placed on the top of 9–16% gradient polyacrylamide second dimensional gels. At the end of the run, the gels were stained in a freshly made ammoniacal silver nitrate solution. The silver-stained gel images were captured with high resolution densitometry reading with the Personal Laser Densitometer from GE Healthcare. All informatic analyses were performed using the software ImageMaster 2D Platinium (GE Healthcare). Spots were detected automatically. Manual spot editing (separation of two spots) or deleting (artifacts) was then performed manually. Spots of interest were identified by MALDI-TOF-TOF MS, as described elsewhere [14]. The MASCOT scores, was expressed as $-10 \times \log_{10}(P)$, where P was the absolute probability that the observed match was a random event. So, from the scores, it was possible to calculate the theoretical probability that the found match was a random one, and these were listed in Table 1 as P. Theoretically, it was also possible to calculate the inverse probability (1-P) which was the probability of a match being non random. Each protein score in a peptide mass fingerprint, and each ions score in an MS/MS search, was accompanied by an expectation value. This is the number of matches with equal or better scores that were expected to occur by chance alone. It was directly equivalent to the E-value in a Blast search result and was also given in Table 1. Finally, all our matches have been manually examined and validated on the basis of the mass errors and the series of matched ions.

2.3. Sample labeling for 2D-DIGE and scanning

Prior to CyDye labeling, proteins were concentrated using Vivaspin Concentrator (cut-off 5 kDa) from Vivascience (Hannover, Germany). The labeling was performed according to the manufacturer instructions (GE Healthcare). Fifty μg of proteins were diluted in a buffer containing urea 8 M, TRIS 40 mM and CHAPS 4% to a final volume of 30 μl. Cyanine dyes were reconstituted in 99.8% anhydrous *N*,*N*-dimethylformamide from Sigma (Steinheim, Germany), and added to labeling reactions in a ratio of 400 pmol CyDye to 50 μg protein. Protein labeling was achieved by incubation on ice in the dark for 30 min. The reaction was quenched by addition of 2 μl of 10 mM lysine from Sigma, followed by incubation on ice for a further 10 min. The different samples to be compared were separately "stained" either with Cy5 or Cy3. They were then mixed and diluted to a final volume of 100 μl by adding a buffer containing urea 7 M, thiourea 2 M, CHAPS 4%, DTE 2%, pharmalyte 3–10 (2%) and pharmalyte 4–7 (1%), and loaded onto the first dimension gels (cup loading). CyDye fluor labeled gels were finally scanned using a Typhoon 9400 Variable Mode Imager (GE Healthcare).

3. Results

3.1. Differential protein pattern of AF at term versus AF at 17th week of pregnancy

Fig. 1 shows differential CyDye staining of AF proteins, with proteins particularly more abundant at term being stained in green, whereas proteins more abundant at 17th week of gestation being stained in red in the composite image. Among the differentially expressed protein spots, seven were identified by tandem MS, and corresponded to five different gene products. Some of their characteristics are summarized in Table 1. The SPARC precursor (Swissprot P09486), collagen alpha 1(I) chain (P02452) and collagen alpha 1(III) chain (P02461) were more abundant at 17 weeks of pregnancy, whereas protein Plunc and lipocalin-1 were more expressed at term. In order to confirm our results, an independent 2-DE analysis with silver stained gels was performed. Globally, 1000 ± 141 spots (mean ± SD; $N=7$) for the term samples and 890 ± 187 spots (mean ± SD; $N=7$) for the 17-week samples were detected on the gels. The difference in the number of spots was not statistically significant (t-test, $P=0.238$). However, ascendant heuristic clustering analysis efficiently discriminated the protein pattern of AF at term from that of AF at 17 weeks. When the seven spots showing a clear differential expression between 40 and 17 weeks of pregnancy at 2D-DIGE analysis were expressed as their %volume in our independent 2-DE silver staining analysis, the same differences were observed ($N=7$ in each groups, t-test, all P-values <0.005).

3.2. Acidic shift of tracheal fluid proteins before plug in fetuses presenting with CDH

Two sets of AF (placement and removal of the balloon) from three fetuses presenting with CDH were compared with control AF obtained at 17 weeks of pregnancy and at term. The protein pattern of AF with CDH (1017 spots ± 145) segregated from the one of control AF at 17 weeks, when analyzed by ascendant heuristic clustering of silver stained gels. When a search was performed in order to detect the spots discriminating the two groups, the same set of spots discriminating AF at 17 weeks and at term were identified. Clustering was due to the "age" of the

Table 1
Spots identified by MS/MS using MASCOT

Spot #	SwissProt ID	Abbreviation	Start	End	MASCOT-peptides identified	M_r	p*I*	Mascot score	Coverage (%)	*P*	*E*-value	Comments
1	P09486	SPRC_HUMAN				34′629	4.73	195	35	3.16E-20	1.10E-14	Regulate cell growth, expressed at high levels in tissues undergoing morphogenesis and remodelling
			141	150	K.LHLDYIGPCK.Y (Ions score 49)							
			151	166	K.YIPPCLDSELTEFPLR.M (Ions score 46)							
			173	181	K.NVLVTLYER.D (Ions score 10)							
			206	218	R.LEAGDHPVELLAR.D (Ions score 21)							
2	P09486	SPRC_HUMAN				34′629	4.73	117	31	2E-12	1.40E-07	
			141	150	K.LHLDYIGPCK.Y (Ions score 37)							
			151	166	K.YIPPCLDSELTEFPLR.M (Ions score 5)							
			206	218	R.LEAGDHPVELLAR.D (Ions score 22)							
3	P09486	SPRC_HUMAN	141	150	K.LHLDYIGPCK.Y (Ions score 42)	34′629	4.73	188	33	1.58E-19	2.30E-15	
			151	166	K.YIPPCLDSELTEFPLR.M (Ions score 21)							
			173	181	K.NVLVTLYER.D (Ions score 18)							
			205	218	K.RLEAGDHPVELLAR.D (Ions score 19)							
			206	218	R.LEAGDHPVELLAR.D (Ions score 23)							
4	P02452	CO1A1_HUMAN				138′883	5.66	228	11	1.58E-23	1.10E-18	Member of fibrillar forming collagen, forms the fibrils of tendon, ligaments and bones
			1226	1236	R.DRDLEVDTTLK.S (Ions score 47)							
			1237	1246	K.SLSQQIENIR.S (Ions score 69)							
			1264	1270	K.MCHSDWK.S Oxidation (M) (Ions score 20)							
			1372	1386	K.NSVAYMDQQTGNLKK.A Oxidation (M) (Ions score 27)							
			1387	1399	K.ALLLKGSNEIEIR.A (Ions score 38)							
5	P02461	CO3A1_HUMAN				13′8555	6.18	245	11	3.16E-25	2.30E-20	Occurs along with type I collagen
			1240	1255	K.SVNGQIESLISPDGSR.K (Ions score 61)							
			1267	1273	K.FCHPELK.S (Ions score 18)							
			1274	1286	K.SGEYWVDPNQGCK.L (Ions score 73)							
			1408	1419	K.FTYTVLEDGCTK.H (Ions score 54)							
6	Q9NP55	PLUNC_HUMAN				26′712	5.42	259	34	1.26E-26	8.60E-23	Involved in the airway inflammatory response, may play a role in inate immune responses of the upper airways
			95	109	K.VTSVIPGLNNIIDIK.V (Ions score 61)							
			95	109	K.VTSVIPGLNNIIDIK.V (Ions score 36)							
			110	128	K.VTDPQLLELGLVQSPDGHR.L (Ions score 68)							
			129	138	R.LYVTIPLGIK. (Ions score 39)							
			139	152	K.LQVNTPLVGASLLR.L (Ions score 25)							
			157	167	K.LDITAEILAVR.D (Ions score 41)							
			214	232	K.VLPELVQGNVCPLVNEVLR.G (Ions score 25)							
7	P31025	LCN1_HUMAN				19′250	5.39	32	6	0.000631	0.055	Binding of various ligands, mainly expressed in lachrymal and salivary glands
			140	150	K.NNLEALEDFEK.A (Ions score 32)							

The numbering corresponds to that of Fig. 1.

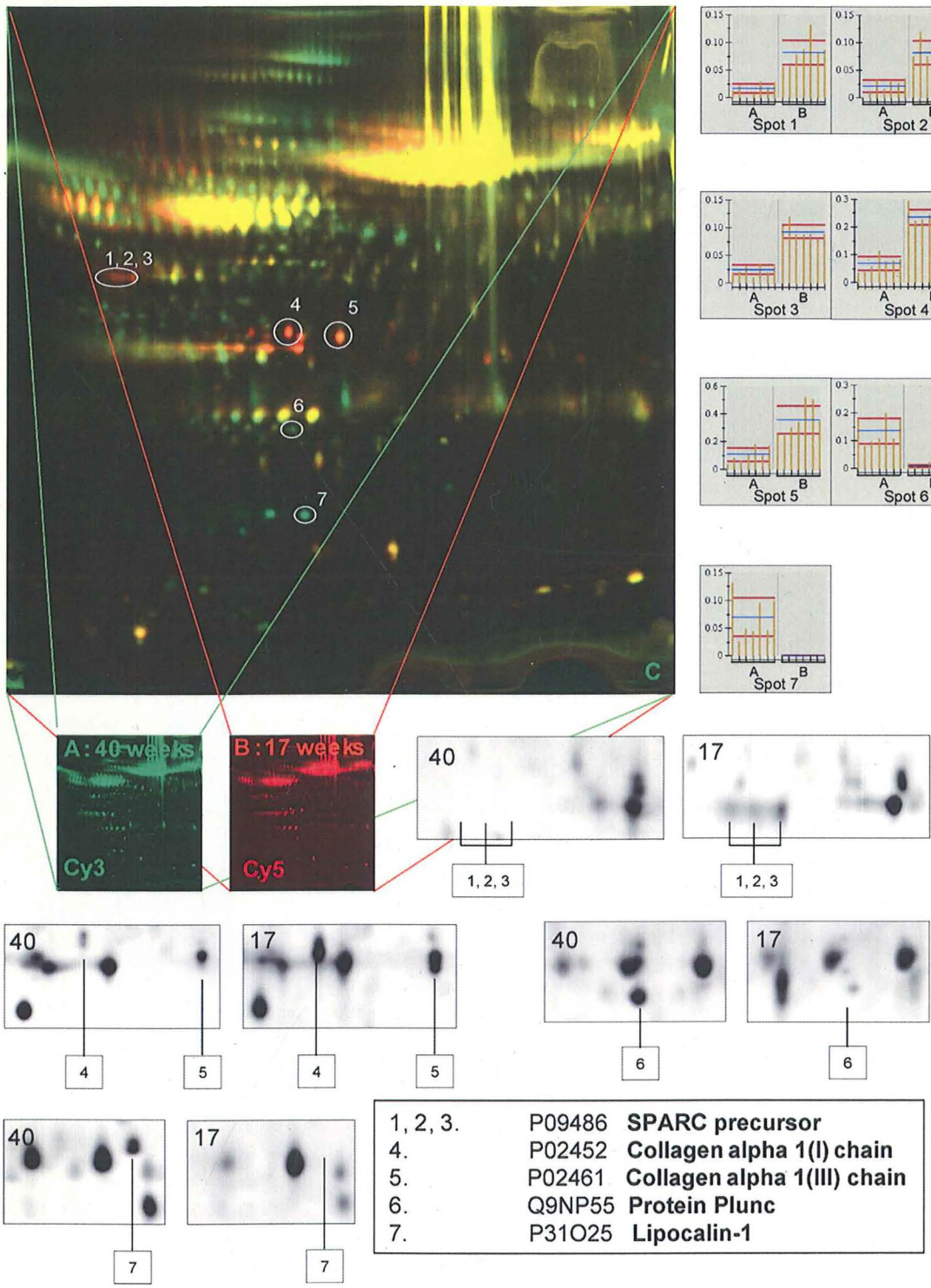

Fig. 1. Two-dimensional electrophoretic technologies evaluating AF samples obtained at term (labeled 40) or at 17 weeks of pregnancy (labeled 17). Color images show comparison of the 2D patterns of AF at term, 50 μg of proteins "stained" using 400 pmol of Cy5 (false color in red, inset labeled A) and of AF at 17 weeks, 50 μg of proteins were "stained" using 400 pmol of Cy3 (false color in green, inset labeled B). Both samples were mixed and loaded on immobilized four to seven non linear pH gradient followed by second dimension (9 to 16% polyacrylamide gel electrophoresis). 2D-DIGE clearly showed seven spots which were differently expressed between AF samples at term or at 17 weeks of pregnancy (panel C). The differential expression of these proteins spots were confirmed by individual spot analysis on different collected samples (%volume; *t*-test, all *P*-values <0.005, histograms on the right of the Figure) after 2-DE and silver staining of samples obtained at term ($N=7$) and samples obtained at 17 weeks of pregnancy ($N=7$). Details of silver stained gels are presented, and the spots numbered according to the 2D fluorescence difference gel electrophoresis color image. Spots were identified by MS analysis, and were named according to the SwissProt database.

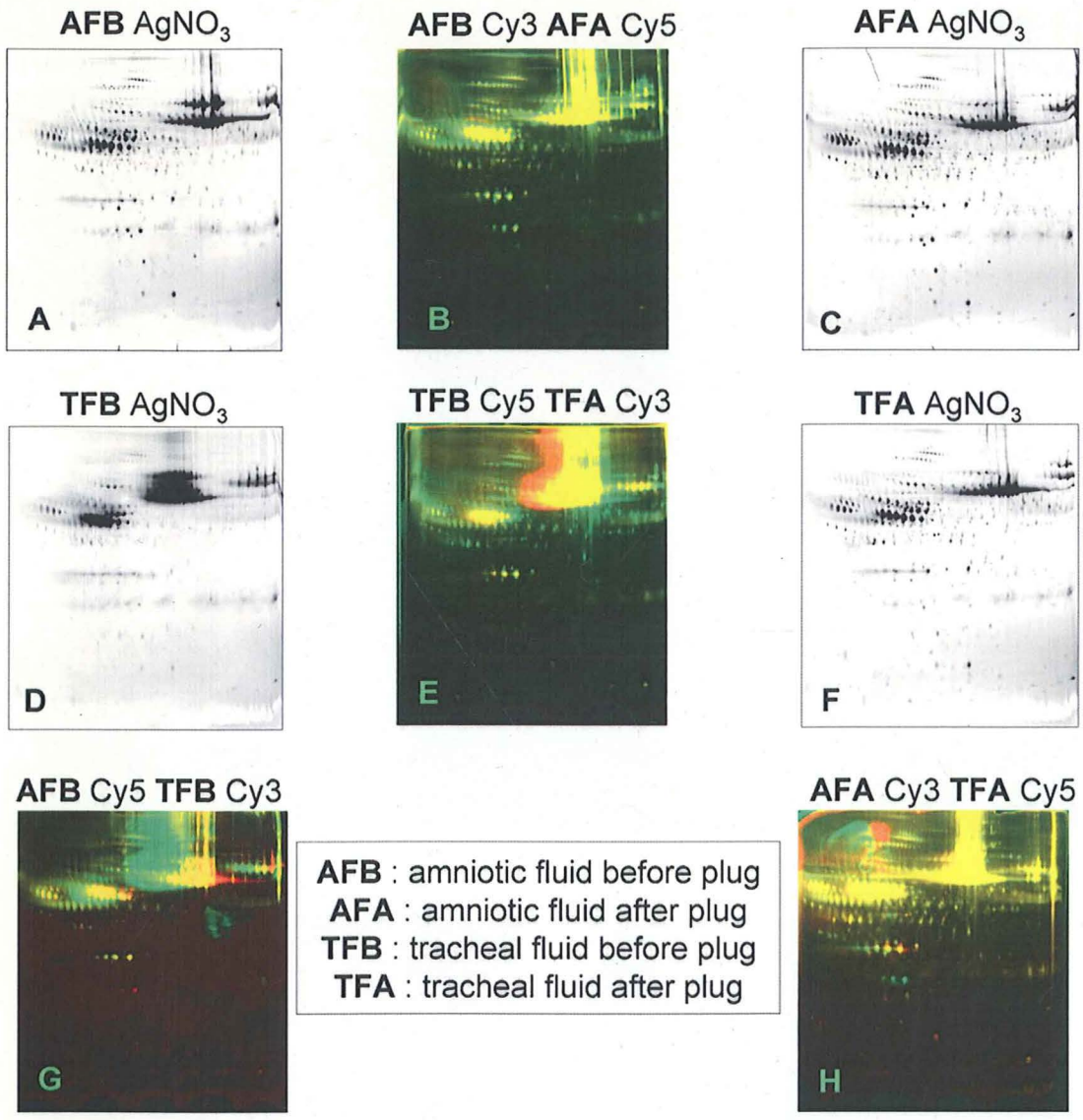

Fig. 2. Comparison of the 2D patterns of AF proteins before and after plug, from patients with congenital diaphragmatic hernia (CDH). Samples were either analyzed using 2D-DIGE and "stained with Cy5 (false color red) or Cy3 (false color green) or with 2-DE and silver staining (labeled AgNO3). The regions that showing the most evident acidic shifts are highlighted by frames, labeled A for the albumin area, and B for that of apolipoprtein-A1. Zooms of these regions are presented in the lower panel. No specific AF or TF protein spot was detected by these 2D technologies. Accumulation of acidic from of most proteins was observed in TF before plug.

samples ($N=3$ at 28 weeks of gestation and $N=3$ at 34 weeks of gestation), but was not related to the disease. When the three groups (AF at term, AF at 17 weeks and AF with CDH) were compared, AF with CDH did not segregate as a single group, indicating the absence of protein spots specific of the diseases on the 2D gels of AF. As shown by Fig. 2A–C, differences were observed between AFB and AFA. When the merged image was evaluated, the left side of many spots stained in green, indicating an acidic shift of the proteins before plug (AFB was stained with the green Cy3). This phenomenon was even more marked when TFB (in this case stained with the red Cy5) was compared with TFA (Fig. 2D–F). Standard MS/MS analyses did not allow to identify particular biochemical modifications that can explain this acidic shift. When silver stained gels were analyzed, no specific spot of CDH were observed either in AF or in TF, before or after plug.

3.3. AF proteins in plasma

As a model of PROM, plasma and AF at term were mixed at different ratio to determine the minimal amount of AF protein detectable in plasma. AF (Fig. 3A, stained with Cy5 in red) and plasma (Fig. 3B, stained with Cy3 in green) have many common proteins which stained in yellow in the merged image (Fig. 3C), with an acidic shift for AF proteins. Many spots were stained in red, representing the AF proteins being largely more abundant compared to plasma, whereas spots in green were spots more abundant in plasma. The two fragments of agrin and perlecan

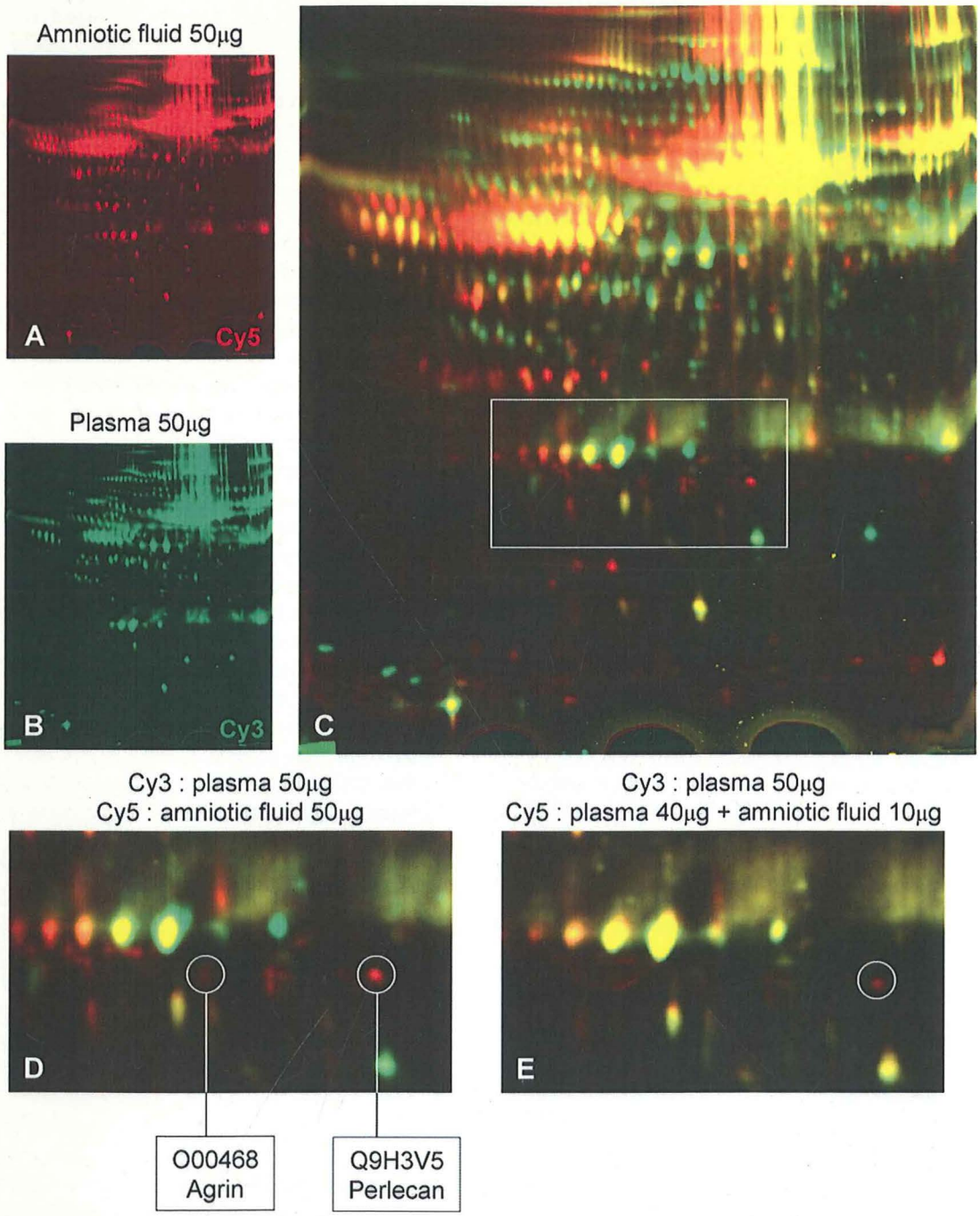

Fig. 3. Comparative analysis of plasma and amniotic fluid proteins. Color images show comparison of the 2D patterns of AF at term, 50 µg of proteins "stained" using 400 pmol of Cy5 (false color in red, inset A) and of the 50 µg of proteins of the corresponding maternal plasma, "stained" using 400 pmol of Cy3 (false color in green, inset B). Both sample were mixed and loaded on immobilized four to seven non linear pH gradient followed by second dimension (9 to 16% polyacrylamide gel electrophoresis). The composite image (inset C) reveals proteins spots that are more abundant in AF in red, whereas those being more abundant in plasma are in green. Common proteins stain in yellow. Note, that for most AF protein present in plasma, an acidic shift is observed. When AF proteins were added to plasma (inset D), they were only detected when at least 10% of the total amount loaded were from AF (inset E).

(Fig. 3D), previously identified as AF "specific" proteins [9] were unambiguously observed when AF proteins represented at least 10% of the total amount of proteins loaded (Fig. 3E).

4. Discussion

Our results confirm that the protein profile of AF is dynamic and that changes occur during development. Some proteins are more abundant early in pregnancy, other being more expressed at term. These observations are particularly important when biomarkers of a specific condition are identified, because adequate age-matched controls must be used. The normal protein profile of AF cannot be established without having normal samples from different fetuses at different stages of gestation. This critical point was confirmed when AF at 17 weeks of gestation was compared with AF of fetuses presenting with CDH. The

group of control was inadequate, and the spots discriminating the two groups by heuristic classification were those discriminating the "age" of AF.

The 2-DE analyses of AF and TF of patients with CDH did not reveal specific protein spots of the disease. However, important changes of the protein patterns were clearly evidenced by 2D-DIGE, and an acidic shift was detected according to the particular differences highlighted. The etiology of CDH remains largely unknown, but various genetic abnormalities have been recently identified [15,16]. It affects 1 out of 2000 to 5000 live births. CDH can now be accurately diagnosed by mid-gestation, and the outcome in individual cases can be predicted on the basis of sonographic measurements of the lung-to-head ratio and the presence or absence of liver herniation in the thorax [17,18]. The aim of the proteomic studies was to determine if specific pulmonary proteins, secreted by respiratory epithelium, could be isolated and later used as a tool to evaluate the pulmonary response to the tracheal occlusion. The samples were collected during fetoscopic tracheal occlusion, which is nowadays considered as a minimally invasive fetal therapy, improving outcome in highly selected cases [19,20]. No specific protein spot was identified in AF or TF before or after the plug. However, an important acidic shift of many proteins was observed in AF, and was particularly marked in TF samples before plug. The origin of the acidic shift is presently not known, but most likely reflects the presence of "aged" oxidized proteins, which accumulate in TF, because of the absence of respiratory movements due to the hernia in the thorax. Most likely, the acidic shift of the TF proteins is a consequence rather as being the cause of CDH. However, using our MS technologies, we were unable to characterize the biochemical origin of the acidic shits observed.

The characterization of the AF proteome is in progress [3,9,21]. However, when 2-DE based technologies are used for comparative studies, an important limitation is the amount of proteins which are present in both plasma and AF. Here, in order to imitate the mixed AF and blood leakage occurring in PROM, AF proteins were added to plasma. When AF proteins represented less than 10% of the total amount of proteins loaded, it was not more possible to identify AF specific proteins using 2D-based technologies. These results indicate that 2-DE is all but optimal for proteomic studies of cervical fluid, at least for PROM biomarker identification. Other approaches, such as surface-enhanced laser desorption ionization time-of-flight mass spectrometry, appear to be more suitable for identifying markers of intra-amniotic inflammation in cervical fluid [22]. These methods could be a potential approach to diagnosis amniotic fluid embolism. This pathology is a catastrophic syndrome occurring during pregnancy or in the immediate post-partum, linked with an abnormal presence of AF in blood [23]. Up to date, the diagnosis of the amniotic fluid embolism is difficult, due to absence of specific and sensitive biomarkers. The optimization of AF proteins detection in maternal blood will strongly increase the clinical management of such pathology.

Taken together, our results show the difficulties of studying AF using 2D-based technologies, and that combination of different approaches will be necessary to better understand the physio(patho)logy of AF in health and diseases. Nevertheless, our results clearly show that two-dimensional electrophoresis technologies still have place for analyzing biological fluids such as AF.

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