Actual state in the diagnosis of fetal lung maturity

Uwe Lorenz, Volker Ragosch, and Hans K. Weitzel

Department of Obstetrics and Gynecology, University Clinic Steglitz, Free University of Berlin, West Germany

1 Introduction

More than one decade has passed since GLUCK et al. [4], based on preceding work of E. SCARPELLI [12, 13] could show the interrelationship between phospholipids, detectable in human amniotic fluid, and the respiratory distress syndome (RDS) of the newborn. The change in concentration of various phospholipids, in particular of lecithin and sphingomyelin, or the ratio of both, takes place according to the progress of gestation with a sharp rise in lecithin concentration at about 35/36 weeks. Simultaneously the incidence of neonatal RDS declines [2]. Nowadays, the problem of lowering neonatal mortality is reduced to the problem of mortality of preterm born infants, whose main cause of death remains up to now the respiratory distress syndrome.

There is still no effective prevention of prematurity by means of tocolysis in sight, so in the science and clinical practice, three main routes of investigation have been followed by obstetricans and neonatologists:

— Evaluation of fetal lung maturity by amniotic fluid analyses;
— Antenatal pharmacological treatment of the fetus in cases with known or supposed lung immaturity: in some way a sort of preventive treatment;
— Postpartum treatment of neonates with obvious lack of sufficient surfactant.

Different ways for the determination of fetal (lung) maturity have been walked on: biophysical, cytological, physiological and biochemical ones. Each of the various methods uses different fetal parameters, which change with advancing intrauterine development and maturation of the unborn child. Biophysical methods measure the surface tension lowering ability of surfactant fragments in amniotic fluid [11]; cytological methods show changes in the composition and properties of amnion epithelial cells; physiological methods deal with changes in the procoagulatory activity under the influence of rising phospholipid levels [7] or with changes in microviscosity [9] and finally the biochemical methods deal with changes in concentration of various phospholipids in amniotic fluid, which originate from the fetal lung and which are essential parts of the fetal lung surfactant.

Our data will show which biochemical methods can be used in daily practice by the obstetrical clinics.

Curriculum vitae

UWE LORENZ, M.D. was born on April 5, 1945 in Marburg/Lahn. From 1963—1969 he studied medicine at the universities of Mainz, Hamburg and Frankfurt and received his doctoral degree in 1969. He spent one year at the Institute of Pharmacology in Munich and was a resident at the department of obstetrics and gynecology in Heidelberg from 1971 to 1978 (Head: Prof. Dr. F. KUBLI). He qualified as a university lecturer in 1980 and has been on the staff of the University Women's Hospital Steglitz in Berlin since 1985 (Head: Prof. Dr. H. WEITZEL). His main scientific interests include perinatology, gynecological oncosurgery and reconstructive breast surgery.

2 Material and methods

One hundred forty eight amniotic fluid samples from 130 patients, obtained either by transabdominal amniocentesis or by vaginal sampling after premature rupture of membranes were analyzed. In about 2/3 of the samples (n = 96) the time interval between amniotic fluid collection and delivery was below 72 hrs. This period of time is important if any correlation between test result and respiratory status of the newborn is looked for.

The following methods of phospholipid analysis in amniotic fluid were used:
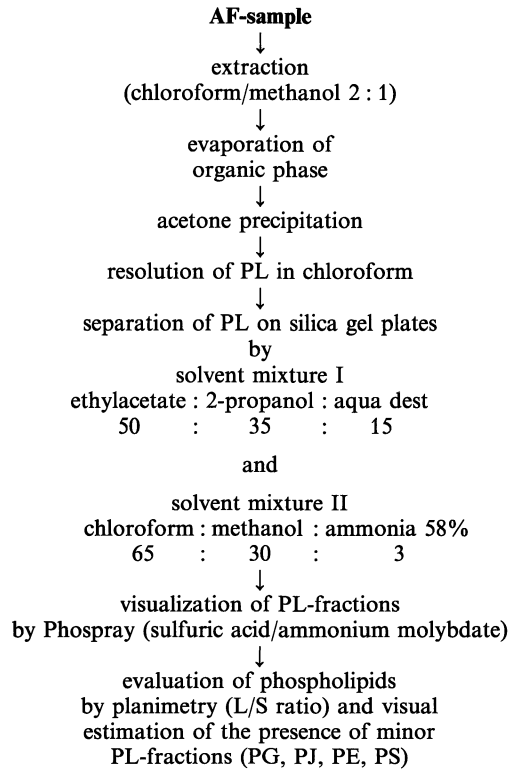
— A miniaturized version of an one-dimensional thin layer chromatographic determination of the lecithin/sphingomyelin ratio [5];
— A one-dimensional thin layer chromatographic separation of all phospholipid components in amniotic fluid [3];
— A completely emzymatic determination of amniotic fluid lecithin concentration [1, 10];
— An immunological method for semiquantitative measurement of phosphatidylglycerol [8].

The method for L/S ratio determination (miniaturized version) is published elsewhere [9]. By sequential one-dimensional development with two different solvent mixtures Sph, Lec, PI, PE, PS and PG can be separated. Evaluation is made visually or densitometrically by comparison with standards of known L/S ratio (2 : 1, 1.5 : 1, 1 : 1) and by evaluation of presence or absence of phosphatidylglycerol and the other phospholipids (PI, PE, PS) (table I).

In the enzymatic determination of lecithin, after enzymatic cleavage of phosphatidylcholine by phospholipase C and cleavage of phosphorylcholine to choline and phosphate by alkaline phosphatase, choline is used as a substrate of a two step enzymatic cascade with leads to the determination of the consumption of NADH, that can be measured easily at wavelength 365 and 334 nm. The amnount of NADH consumption is equimolar to choline and thus to lecithin (figure 1).

The immunological determination of phosphatidylglycerol is based on the precipitation of PG by PG-antibodies, prepared in rabbits (Reagent B), in presence of an ethanolic solution of egg yolk lecithin (Reagent A). By comparison to simultaneously processed PG-standards of known concentration (threshold value 2 mg/l) the presence or absence of PG in the patients pecimen can be confirmed.

Table I. Chart flow of one-dimensional sequential thin layer chromatographical separation of amniotic fluid phospholipids according to GIPS' procedure [3].

AF-sample
↓
extraction
(chloroform/methanol 2 : 1)
↓
evaporation of
organic phase
↓
acetone precipitation
↓
resolution of PL in chloroform
↓
separation of PL on silica gel plates
by
solvent mixture I
ethylacetate : 2-propanol : aqua dest
50 : 35 : 15

and

solvent mixture II
chloroform : methanol : ammonia 58%
65 : 30 : 3
↓
visualization of PL-fractions
by Phospray (sulfuric acid/ammonium molybdate)
↓
evaluation of phospholipids
by planimetry (L/S ratio) and visual
estimation of the presence of minor
PL-fractions (PG, PJ, PE, PS)

3 Results

As to be expected a strong correlation exists between changes in L/S ratio and advancing gestational age (figures 2, 3 and 4). Phosphatidylglycerol, determined chromatographically or immunologically, correlates well to gestational age (figure 5). Enzymatically determined lecithin concentrations shows the expected correlation of values according to advancing gestational age at both choosen wavelengths for photometric evaluation (λ = 365 or 334 nm) (figures 6 a and 6 b).

For comparison of the validity of the various methods in predicting neonatal RDS, 4-field contingency tables and χ^2 test were used. Significant results in prediction of neonatal RDS we found using one-dimensional separation of PL for the L/S ratio, as well as for the chromatographic or immunologic PG determination (table II).
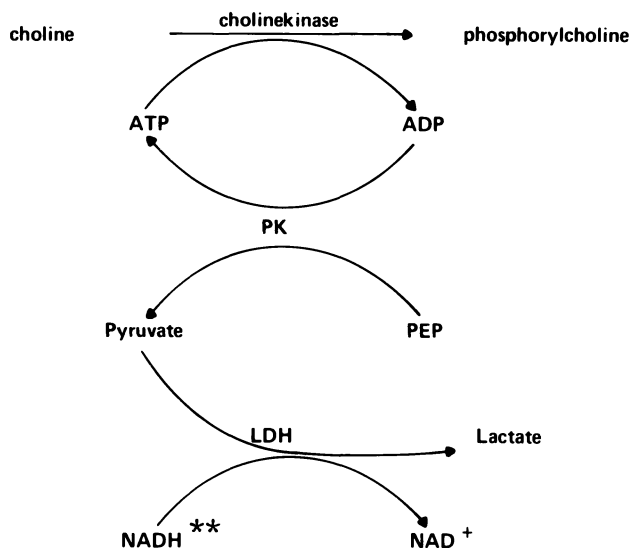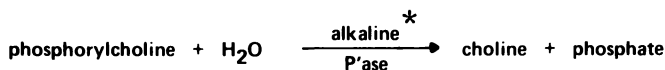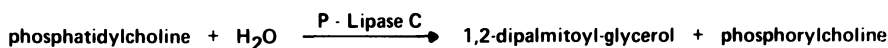
phosphatidylcholine + H_2O $\xrightarrow{\text{P - Lipase C}}$ 1,2-dipalmitoyl-glycerol + phosphorylcholine

phosphorylcholine + H_2O $\xrightarrow[\text{P'ase}]{\text{alkaline}^{*}}$ choline + phosphate

Figure 1. Assay principle of enzymatic determination of amniotic fluid lecithin; * inactivation at 95 – 100 °C, ** photometric measurement of NADH consumption at Hg 365 nm or 334 nm.

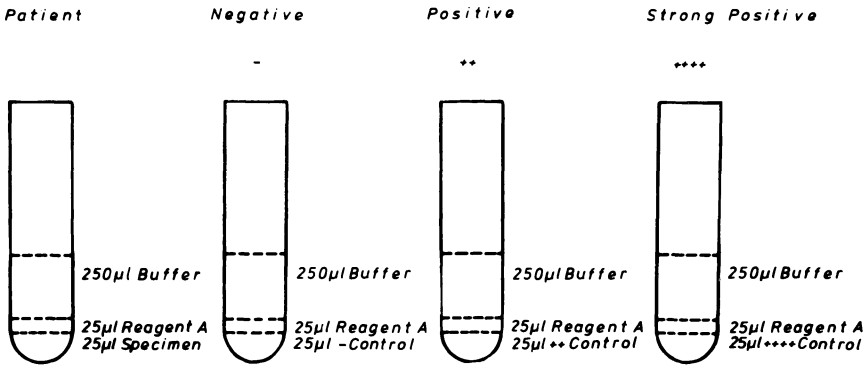**Table II.** Prediction of neonatal RDS by various methods of amniotic fluid phospholipid determination.

Method	Ø RDS	RDS
Miniaturized L/S ratio		
L/S > 2	13	2
L/S ≤ 2	13	6
One-dimensional two step TLC of phospholipids		
L/S > 2	43	0*
L/S ≤ 2	11	4*
Enzymatic lecithin determ. at $\lambda = 365$ nm and $\lambda = 334$ nm ( )		
Lec > 5 mg%	38 (32)	2 (1)
Lec ≤ 5 mg%	16 (38)	2 (7)
Immunologically and TLC ( ) evaluated PG		
PG present	40 (33)	1 (0)*
PG absent	39 (22)	9 (4)*

* indicates significant accordance of AF-PL-result to neonatal RDS

In this study material, for enzymatic lecithin and the miniaturized L/S ratio determination we could not prove statistically significant results due to the small number of RDS cases, but this correlation has been confirmed for both methods in a larger report only recently [10]. For one dimensional thin layer chromatographic determination of L/S ratio as well as for chromatographic or immunologic PG determination a clear correlation to the expected development of RDS in the newborn exists.

The validity of certain test methods has to be described by more than one biostatistical data, namely: sensitivity, predictive correctness of positive or negative result and specificity. The results of these data for the tests evaluated are shown in table III. Throughout all tests we find high values for sensitivity and for the predictive correctness of negative test, that is: good capability of the test to exclude the danger of impending RDS if the threshold value is trespassed. But in all four tests investigated, a low correctness of negative test is observed.

A. Preparation of Lipid Particles (in 12 x 75mm glass tubes)

Patient	Negative	Positive	Strong Positive
	−	++	++++

250 μl Buffer   250 μl Buffer   250 μl Buffer   250 μl Buffer

25 μl Reagent A   25 μl Reagent A   25 μl Reagent A   25 μl Reagent A
25 μl Specimen   25 μl −Control   25 μl ++ Control   25 μl++++Control

B. Preparation of Agglutination Reactions (on agglutination slide)

1. 25 μl Reagent B in each ring
2. 10 μl of each suspension to appropriate ring

Patient   −   ++   ++++
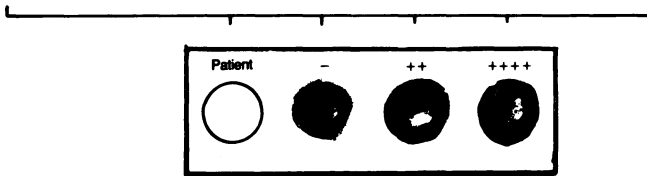
C. Incubation of Reaction Mixtures (Cover and rotate at 60 rpm for 9 min)

D. Examination of Completed Reactions

Figure 2. Immunological determination of amniotic fluid phosphatidylglycerol: assay procedure.

Table III. Predictive values of various amniotic fluid phospholipid determination methods (for abbreviations see table II).

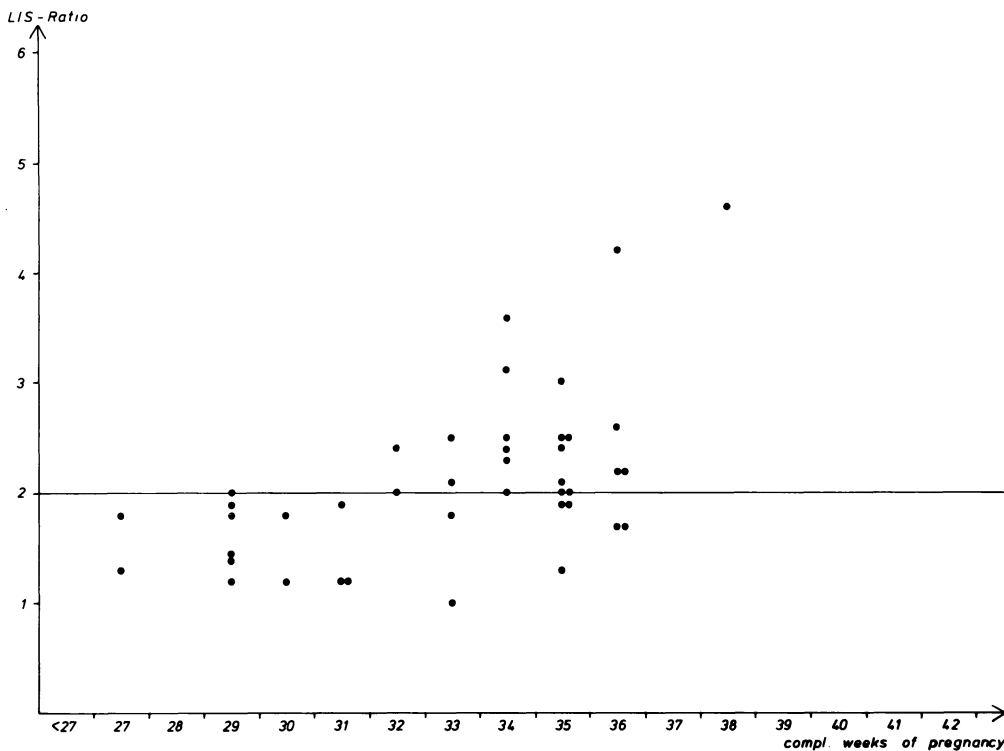|	L/S 1-dim. (miniat.)	L/S 1-dim. 2 step	Lec enzym. $\lambda = 365$ nm	Lec enzym. $\lambda = 334$ nm	PG immun. FLM	PG 1-dim.
Sensitivity	75%	100%	50%	88%	90%	100%
Predictive correctness of positive test	32%	27%	11%	16%	19%	15%
Specificity	50%	80%	70%	46%	51%	60%
Predictive correctness of negative test	87%	100%	95%	97%	98%	100%

Figure 3. L/S ratio values in amniotic fluid according to gestational age. Determination by a miniaturized one dimensional TLC-method [2]. Positive correlation of L/S ratio values vs. gestational age (29 – 38 weeks): y = 0.174 − 3.632; r = 0.566.
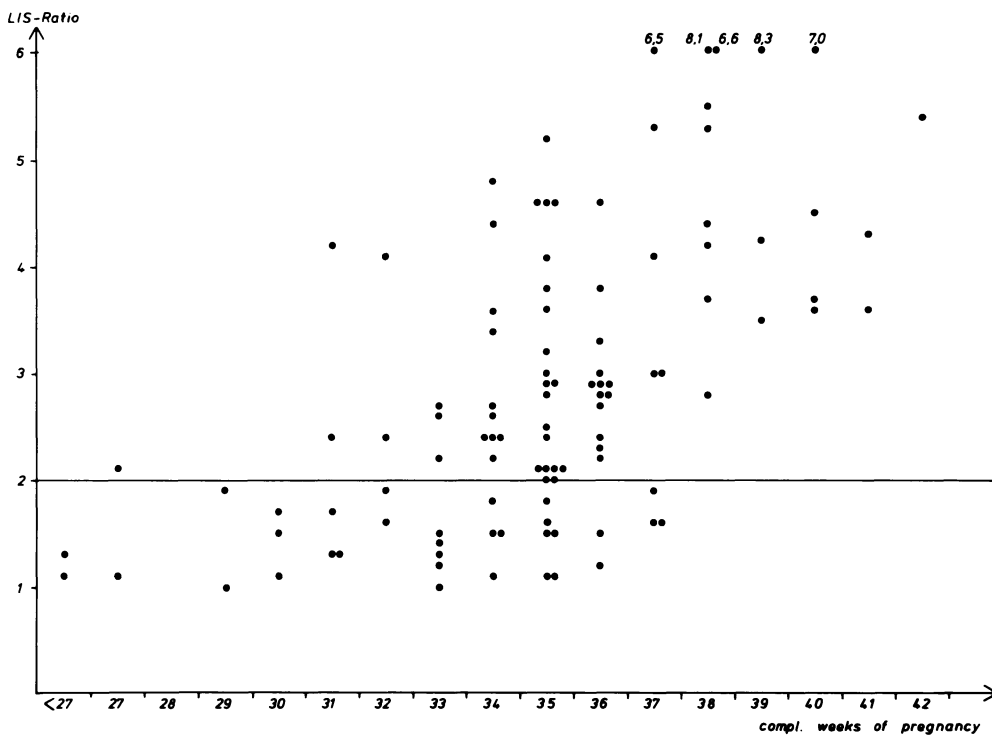

Figure 4. L/S ratio values in amniotic fluid according to gestational age. Determination of L/S ratio by one dimensional sequential two step TLC. Positive correlation of L/S ratio values vs. gestational age (33 – 39 weeks): y = 9.536 − 16.01; r = 0.549.
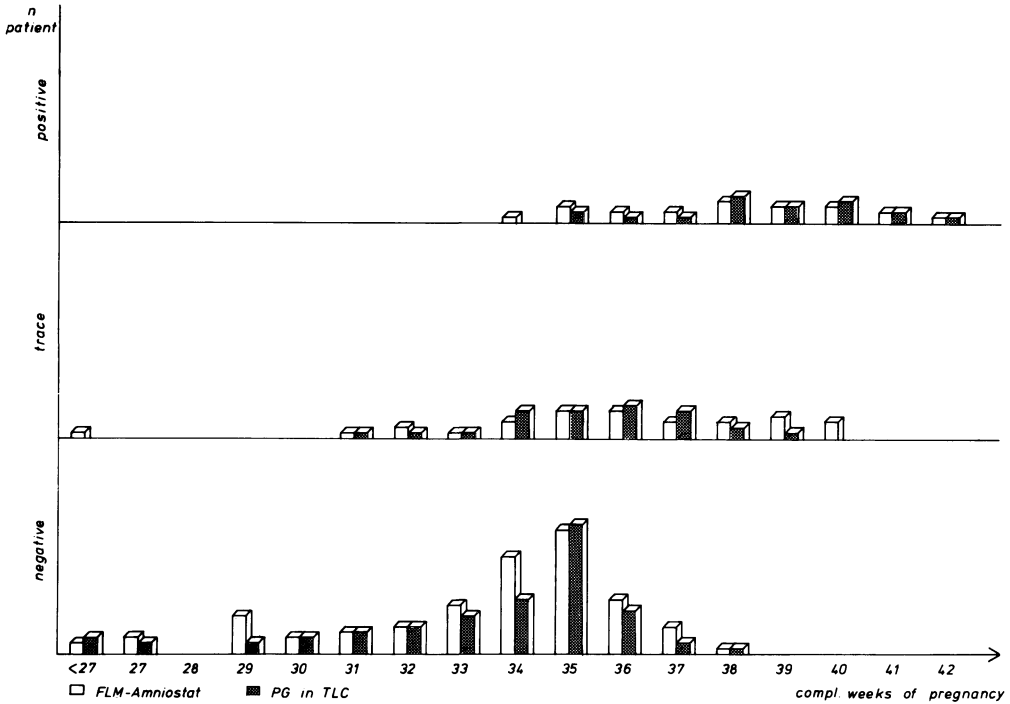
Figure 5. Semiquantitative evaluation of phosphatidylglycerol (PG) in amniotic fluid corresponding to gestational age; Determination of PG by immunological (□) or 1-dimensional sequential two step TLC (■).
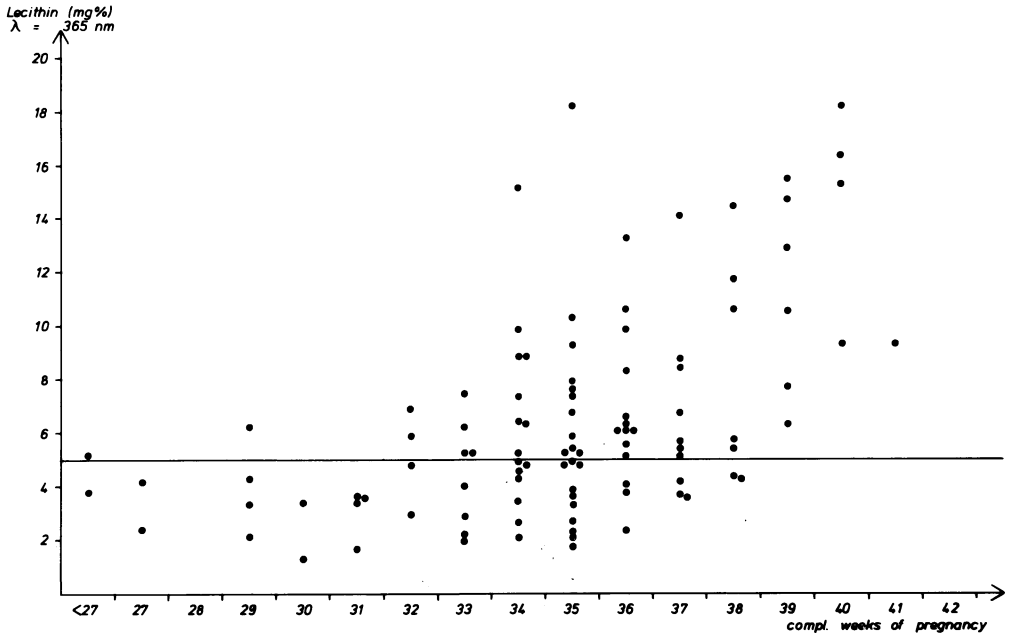

Figure 6 a. Lecithin concentration (mg %) in amniotic fluid according to advancing gestational age. Enzymatic determination of lecithin. Photometric evaluation at λ = 365 nm (Hg). Positive correlation of values vs. gestational age (33 – 39 weeks): y = 0.792 – 21.544; r = 0.375.
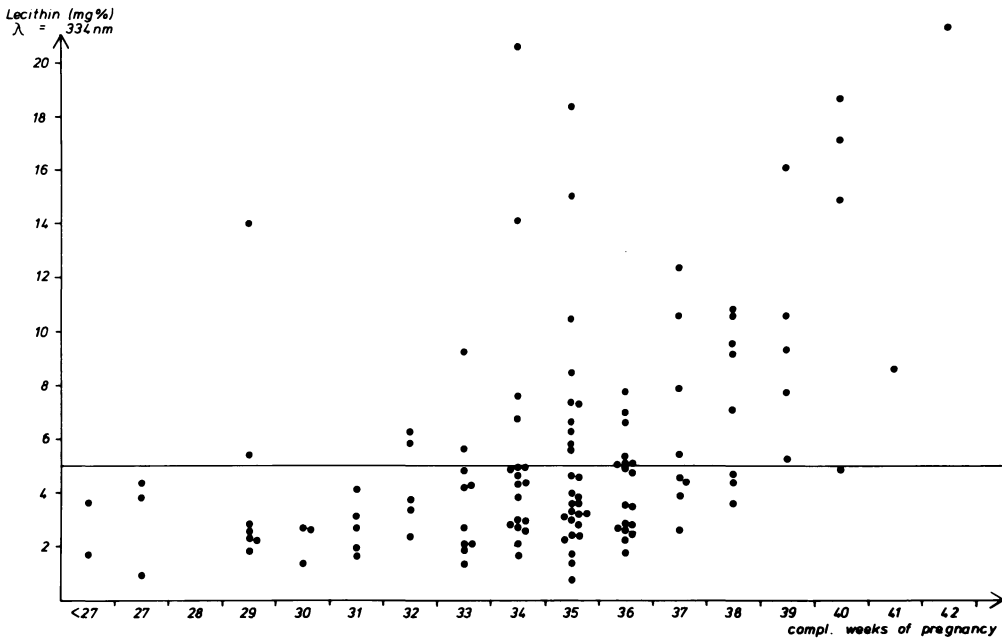
Figure 6b. Lecithin concentration (mg %) in amniotic fluid according to advancing gestational age. Enzymatic determination of lecithin. Photometric evaluation at $\lambda = 334$ nm (Hg). Positive correlation of values vs. gestational age (33–39 weeks): $y = 0.75 - 21.248$; $r = 0.353$.

4 Comment

Insofar we can conclude that under the actual methods of amniotic fluid PL evaluation the fairly simply performable immunological PG determination as well as the one dimensional separation of AF phospholipids, which is easier to run than the original "lung profile" determination of KULO-VICH and GLUCK [6] lead to a good information about exclusion of RDS. On the other hand below the threshold value, be it an L/S ratio below 2 or an undetectable PG value, a discriminating parameter is lacking, which may be able to indicate the real danger of the fetus to suffer from RDS if born.

Summary

Four methods of phospholipid analysis in amniotic fluid were compared:
— miniaturized version of a one-dimensional thin-layer chromatographic determination of the lecithin/sphingomyelin ratio [5];
— A one-dimensional thin-layer chromatographic separation of all phospholipid components in amniotic fluid [3];
— A completely enzymatic determination of amniotic fluid lecithin concentration [1, 10];
— An immunological method for semiquantitative measurement of phosphatidylglycerol [8].

The various phospholipid parameters (L/S ratio, lecithin concentration, phosphatidylglycerol detection either by thin-layer chromatography or immunological methods) show a strong correlation to advancing gestational age.

While we were not able to prove a statistically significant correlation between enzymatic lecithin values or L/S ratio values and the occurrence of neonatal RDS cases in this study due to the relatively small number of RDS cases, the L/S ratio values obtained by one-dimensional thin-layer chromatography and the phosphatidylglycerol values, determined either chromatographically or immunologically, showed a clear correlation to the expected development of RDS in the newborn.

Thus we can conclude that under the actual methods of amniotic fluid phospholipid evaluation immunological phosphatidylglycerol determination as well as the one-dimensional separation of amniotic fluid phospholipids, which is easier to run than the "lung profile" determination of KULOVICH and GLUCK [6] provide good information about the exclusion of RDS.

Zusammenfassung

Aktueller Stand in der Diagnostik der fetalen Lungenreife
Es wurden vier Phospholipidbestimmungsmethoden im
Fruchtwasser miteinander verglichen:
— eine miniaturisierte Version der eindimensionalen
  dünnschicht-chromatographischen Bestimmung des
  Lezithin-Sphingomyelin-Onotientan [5];
— eine eindimensionale dünnschichtchromatographi-
  sche Trennung aller Fruchtwasser-Lipidkomponen-
  ten [3];
— eine vollständige enzymatische Bestimmung der Lezi-
  thinkonzentration im Fruchtwasser [1, 10];
— eine immunologische Methode zur semiquantitativen
  Messung von Phosphatidylglycerol [8].
Die verschiedenen Phospolipidparameter (L/S-Quotient,
Lezithinkonzentration,   Phosphatidylglycerolnachweis
durch Dünnschichtchromatographie bzw. immunolo-
gisch) zeigen eine enge Korrelation zum Gestationsalter.
Aufgrund der relativ kleinen Anzahl von Fällen mit
Atemnotsyndrom waren wir nicht in der Lage, eine
statistisch signifikante Korrelation zwischen enzyma-

tisch bestimmten Lezithinkonzentrationen bzw. L/S-
Quotienten und dem Auftreten eines neonatalen Atem-
notsyndroms in dieser Studie nachzuweisen. Dagegen
zeigten die L/S-Ratiowerte, die auf dem Wege der eindi-
mensionalen Dünnschichtchromatographie bestimmt
wurden, und die Phosphatidylglycerolwerte, entweder
dünnschichtchromatographisch oder immunologisch be-
stimmt, eine eindeutige Korrelation zu der zu erwarten-
den Entwicklung eines Atemnotsyndroms des Neugebo-
renen.
Wir können feststellen, daß unter den aktuellen Metho-
den der Phospholipidbestimmungen aus dem Frucht-
wasser sowohl die immunologische Bestimmung von
Phosphatidylglycerol als auch die eindimensionale Tren-
nung der Fruchtwasserphospholipide, die einfacher
durchzuführen ist als die Bestimmung des „lung profile"
nach KULOVICH und GLUCK [6], eine gute Information
hinsichtlich des Ausschlusses eine drohenden Atemnot-
syndroms des Neugeborenen geben.

Schlüsselwörter: Atemnotsyndrom des Neugeborenen, Chromatographie der Phospholipide im Fruchtwasser, im-
munologische Bestimmung des Phosphatidylglycerol, Lezithin-Sphingomyelin-Ratio, Phospholi-
pidbestimmungsmethoden im Fruchtwasser.

Résumé

État actuel du diagnostic de maturité pulmonaire foetale
On compare quatre méthodes d'analyse des phospholipi-
des dans le liquide amniotique:
— Version miniaturisée de la détermination du rapport
  Lécithine/sphingomyéline par chromatographie uni-
  dimensionelle en couche mince [5];
— Séparation par chromatographie uni-dimensionelle
  en couche mince de tous les phospholipides du li-
  quide amniotique [3];
— Dosage de la lécithine dans le liquide amniotique par
  méthode enzymatique totale [1, 10];
— Détermination semi-quantitative du phosphatidyl-
  glycérol par méthode immunologique [8].
Les différents paramètres phospholipidiques (rapport L/
S, taux de lécithine, déterminations du phosphadidylgly-
cérol que ce soit par chromatographie en couche mince
ou par immunologie) sont fortement corrélés avec l'aug-
mentation de l'âge gestationnel. Bien que nous ne soyons
pas capables de prouver une corrélation statistiquement
significative entre les valeurs de la lécithine par méthode
enzymatique ou les valeurs du rapport L/S et la survenue

du SDR néonatal dans cette étude du fait du nombre
relativement faible de SDR, toutefois les valeurs du
rapport L/S obtenues par du nombre relativement faible
de SDR, toutefois les valeurs du rapport L/S obtenues
par du nombre relativement faible de SDR, toutefois les
valeurs du rapport L/S obtenues par chromatographie
uni-dimensionelle en couche mince et les taux de phos-
phatidylglycérol, trouvés soit par chromatographie soit
par méthode immunologique, montrent trouvés soit par
chromatographie soit par méthode immunologique,
montrent une corrélation nette avec le risque de dévelop-
pement d'un SDR pour le nouveau-né.

Aussi, pouvons-nous conclure que sous couvert des
méthodes actuelles d'étude des phospholipides du liquide
amniotique qu'il s'agisse de la détermination immunolo-
gique du phosphatidylglycérol ou de l'étude des phos-
pholipides du liquide amniotique par chromatographie
uni-dimensionelle qui est plus aisée à réaliser que la
détermination du « profil pulmonaire » de KULOVICH et
GLUCK [6], nous pouvons abtenir de bonnes informa-
tions concernant l'élimination du risque de SDR.

Mots-clés: Analyse des phospholipides du liquide amniotique, chromatographie des phospholipides dans le liquide
amniotique, détermination immunologique du phosphatidylglycérol, rapport lécithine/sphingomyéline,
syndrome de détresse respiratoire néonatale.

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Prof. Dr. Uwe Lorenz
Universitätsklinikum Steglitz der FU Berlin
Frauenklinik und Poliklinik
Hindenburgdamm 30
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