

# Tissue Phospholipids during Human Pregnancy by $^{31}\text{P}$ NMR: Myometrium, Decidua, Placenta and Fetal Membranes

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The biophysical environment formed by phospholipids, rather than the amount of functional proteins, can be rate limiting for factors controlling myometrial contractility and pregnancy maintenance. We therefore studied myometrial, decidual, placental and fetal membrane phospholipids using the  $^{31}\text{P}$  NMR spectrum. This enabled us to identify bulk phospholipids over 0.05 mmol/kg. The method was checked for reliability for the reproductive tissues studied. The chemical shift of phospholipid standards was slightly different according to whether a single compound or a mixture was analyzed. The bulk phospholipids found were phosphatidylcholine (PC), phosphatidylethanolamine, sphingomyelin (SM) and phosphatidylinositol. The ratio PC/SM decreased during pregnancy in the decidua, placenta and fetal membranes, but not in the myometrium. Pregnancy did not induce significant changes in the total myometrial phospholipids. Their composition was stable even during clinical labor. The fetal tissues, placenta and fetal membranes contained about twice as much phospholipid as the maternal tissues, myometrium and decidua. There was no sign of lysocompounds, cardiolipin or phosphatidic acid. This supports the view that the extraction and analyzing techniques used earlier probably created artefacts. The increased fluidity of the myometrial and placental phospholipids during pregnancy may depend on factors other than the composition of phospholipids.

## INTRODUCTION

The amount of functional protein is not rate limiting during the hydrolysis of mixed micelles of phospholipids (PLs) and detergent by phospholipase A2, a key enzyme in phospholipid metabolism.<sup>1</sup> Rate limiting is the physico-chemical environment, created through PL composition, which also regulates the myometrial receptor ligand binding of the steroids estradiol and progesterone. The pregnancy-specific tissues, the decidua and the fetal membranes, closely connected to the myometrium, have a PL concentration high enough for this function.<sup>2</sup> The structure which has main control over myometrial contractility is the gap junction.<sup>3</sup> The channel formation increases one hundred fold when modified by the PL environment.<sup>4</sup> Gap-junctional communication and dissociation is directly inhibited by lysoPLs, such as lysophosphatidylcholine (LPC), lysophosphatidylinositol (LPI) or simple lysophosphatidic acid (LPA).<sup>4,5</sup> LPC is associated with the electrophysiological derangement of the muscle which is already at a low level,<sup>6</sup> and found earlier in the myometrial tissue.<sup>7,8</sup> When considering the significance of the physico-chemical environment for protein function in plasma and other cellular membranes it is important to know whether the lytic, potent detergent PLs, like PLC, can actually be detected in normal human

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**Abbreviations used:** AUC, area under curve; CL, cardiolipin; LPA, lysophosphatidic acid; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; LPI, lysophosphatidylinositol; PC, plas. phosphatidylcholine plasmalogen; PE, phosphatidylethanolamine; PE, plas. phosphatidylethanolamine plasmalogen; PI, phosphatidylinositol; PG, phosphatidylglycerol; PL, phospholipid; PS, phosphatidylserine; SM, sphingomyelin; TEP, triethyl phosphate.

myometrial tissue.<sup>2</sup>

We studied the myometrial composition of PLs using the  $^{31}\text{P}$  NMR method, which eliminates the artefacts that occur in conventional tissue phospholipid analyses.<sup>9</sup> The methods used were selected and controlled so as to meet this requirement. The shortcomings of earlier methods include the artificial increase of lysocompounds during the analysis.<sup>10-12</sup> The technique used allowed us to determine whether during clinical labor active PL detergents such as LPC, LPA or phosphatidic acid (PA) appear at tissue level (myometrium, decidua, placenta and fetal membranes), or whether there are changes in basic structures, e.g. the ratio of phosphatidylcholine (PC) to sphingomyelin (SM), significant for the concomitant fetal pulmonary maturation.

## MATERIAL AND METHODS

### Tissue sampling

Myometrial tissue samples were collected (with the informed consent of patients and the approval of the Ethical Committee of the University of Turku) from 19 patients. The three nonpregnant patients at midcycle had a clinically normal myometrium when hysterectomy was performed for menorrhagia. The samples from 16 pregnant patients (gravida 2.8 SD 1.8, para 1.1 SD 1.0, 29 SD 5.8 years old) not in clinical labor ( $n=9$ ) or in labor ( $n=7$ ) were taken during cesarean section from the upper medial lip of the uterine incision, with the following indications: fetopelvic disproportion ( $n=6$ ); twins ( $n=3$ ); prolonged labor ( $n=3$ ):

preterm premature rupture of membranes ( $n=2$ ); fetal presentation ( $n=1$ ); or fetal asphyxia ( $n=1$ ). The myometrium was carefully freed from concomitant tissues (decidua, serosa) and washed free of blood with isotonic saline. Small sections of uterine muscle were frozen in liquid nitrogen and stored at  $-70^{\circ}\text{C}$ . The tissue was pulverized with a microdismembrator, using liquid nitrogen for cooling, weighed and used for the extraction of PLs.

Samples of decidua ( $n=11$ ), placenta ( $n=4$ ) and fetal membranes ( $n=6$ ) were obtained from three first-trimester patients admitted for pregnancy termination by suction curettage and from eight late-pregnant patients at cesarean section, as described for the myometrium. The lens tissue was collected from seven patients undergoing extracapsular cataract operation, to confirm the efficacy of the present methods for detecting lysocompounds such as LPC.

### Phospholipids and other chemicals

The chemical shift reference triethyl phosphate (TEP) was purchased from Merck. Table 1 shows the PLs used and their suppliers.

### Extraction of phospholipids

Tissue powder samples, about 1 g each, were suspended in 2 ml of water. The PLs were extracted twice into 4 ml of 1-butanol as described previously.<sup>2</sup> Butanol was removed in a stream of nitrogen and the residue dissolved in 0.5 ml of a chloroform-methanol (2:1) mixture. An aliquot of 0.1 ml was stored at  $-20^{\circ}\text{C}$  for the analyses of total lipid phosphorus as described by Duck-Chong.<sup>13</sup> The remaining 0.4 ml was dried and stored at  $-20^{\circ}\text{C}$  for  $^{31}\text{P}$  NMR analysis within a week. The efficacy of 1-butanol in extracting PLs was tested by adding known amounts of PLs to the myometrial homogenate. The PL recovery was the difference between internal and total (internal plus added) amounts of PLs in the myometrium by  $^{31}\text{P}$  NMR analysis.

The extraction method used by Manimekalai<sup>7</sup> was repeated, using the placental tissue, to see whether there was artificial occurrence of LPC in  $^{31}\text{P}$  NMR.

**Table 1. Phospholipids and their suppliers**

L- $\alpha$ -phosphatidylcholine, from egg yolk (Sigma P 3556)
L- $\alpha$ -phosphatidylcholine, 30% plasmalogen, from bovine heart (Sigma P 9513)
L- $\alpha$ -phosphatidylethanolamine, dipalmitoyl (Koch-Light Lab. 1002)
L- $\alpha$ -phosphatidylethanolamine, 60% plasmalogen, from bovine brain (Sigma P 7523)
L- $\alpha$ -phosphatidylcholine, from egg yolk (Sigma L 4129)
L- $\alpha$ -phosphatidylethanolamine, from egg yolk (Sigma L 4754)
DL- $\alpha$ -phosphatidyl-L-serine, dipalmitoyl (Sigma P 1902)
L- $\alpha$ -phosphatidylinositol, Na-salt, from soybean (Sigma P 0639)
L- $\alpha$ -phosphatidic acid, dipalmitoyl, Na-salt (Sigma P 4013)
L- $\alpha$ -phosphatidyl-DL-glycerol, $\text{NH}_4$ -salt, from egg yolk (Sigma P 0514)
L- $\alpha$ -lysophosphatidic acid, oleoyl, Na-salt (Sigma L 7260)
L- $\alpha$ -lysophosphatidylinositol, Na-salt, from soybean (Sigma L 7635)
Cardiolipin, Na-salt, from bovine heart (Koch-Light Lab. 6066-50)
Sphingomyelin, from bovine brain (Koch-Light Lab. 5067-06)

### Phosphorus NMR spectroscopy and statistics

The PLs were dissolved in 0.6 ml of  $\text{CDCl}_3:\text{CD}_3\text{OD}$  (4:1) v/v in a 5 mm o.d. NMR tube. Phosphorus NMR spectroscopy was performed at  $30^{\circ}\text{C}$  at 202.35 MHz with an NMR spectrometer JEOL JNM A500, equipped with a JEOL NM-50TH5 probe. In order to have a quantitative determination a gated coupling was used. The spectral observation range was 4000 Hz, and 16k data points were used for acquisition. The spectrum was acquired by using a  $60^{\circ}$  pulse with a 12 s pulse interval. Before the actual observation, parallel measurements with different pulse repetition times were performed in order to exclude saturation phenomena. The number of pulses used was 200. Before Fourier transformation, the FID data were multiplied by exponential function by applying 1.5–2.0 Hz line broadening. TEP was used as the chemical shift reference (0 ppm) and as the quantitation standard (2 mmol/l). The peaks were integrated by means of the instrument software.

The Student's *t*-test (two-tailed, unequal variances) or analysis of variance (one-way) with Bonferroni's modified *t*-test were used to compare the PL contents of the different tissues.<sup>14</sup>

## RESULTS

### Methodology

The recoveries yielded by butanol extraction were sufficient for the principal PLs studied (Table 2). In order to check the overall reliability of the  $^{31}\text{P}$  NMR method with the myometrium as a source of PLs, we made repeated measurements of one standard mixture containing PC, LPC, SM and PE. The within-assay variation CV for PC, LPC, SM and PE in six consecutive determinations was 8.1, 6.0, 9.3 and 15.4%, respectively. The corresponding inter-assay CV, when the measurement was repeated four times, was 7.9, 4.9, 7.3 and 20.0%, respectively. One PE determination which gave a 30% lower value than the others resulted in a higher CV of PE.

The PL standards indicated the chemical shifts in  $^{31}\text{P}$  NMR presented in Table 3. There was a slight difference in shifts of single compounds as compared to those in a mixture prepared of the same compounds. This is probably due to the interaction between the standards in the mixture and to the increase of the total concentration of solutes. The compounds studied in the standard mixture of PLs (including the lysocompounds LPC, LPE and LPI) were clearly detectable (Fig. 1). The lowest detectable amount of a given PL was considered to be 0.05 mmol/l, i.e. twice the background noise (except CL with two phosphorus,

**Table 2. Recovery of phospholipids from the human myometrium, when extracted with 1-butanol<sup>a</sup>**

Phospholipid added	Percentage recovery
Phosphatidylcholine, from bovine heart	86.4
Lysophosphatidylcholine, dipalmitoyl	95.9
Sphingomyelin, from bovine brain	71.3
Phosphatidylinositol, from soybean	77.8
Phosphatidylethanolamine, dipalmitoyl	79.8
Phosphatidylglycerol, from egg yolk	76.3

<sup>a</sup> Phospholipids were added to 1 g of myometrium homogenate, concentration 2 mmol/l, and the tissues was extracted twice with 4 ml of 1-butanol.

**Table 3. Chemical shifts of the phospholipid standards in  $^{31}\text{P}$  NMR**

Compound	Symbol	Chemical shift	
		Single	In mixture
Triethylphosphate	TEP	0.00	0.00
Phosphatidylcholine	PC	0.64	0.68
Phosphatidylcholine, plasmalogen	PC plas	n.d.	0.74
Lysophosphatidylcholine	LPC	1.36	1.43
Phosphatidylserine	PS	1.38	1.56
Sphingomyelin	SM	1.69	1.62
Phosphatidylinositol	PI	1.87	1.74
Phosphatidylethanolamine	PE	1.94	1.80
Phosphatidylethanolamine, plasmalogen	PE plas	1.94	1.80
Phosphatidylglycerol	PG	2.17	2.20
Lysophosphatidylinositol	LPI	2.37	2.40
Lysophosphatidylethanolamine	LPE	2.44	2.40
Cardiolipin	CL	2.66	2.50
Phosphatidic acid	PA	2.97	2.97
Lysophosphatidic acid	LPA	5.32	n.d.

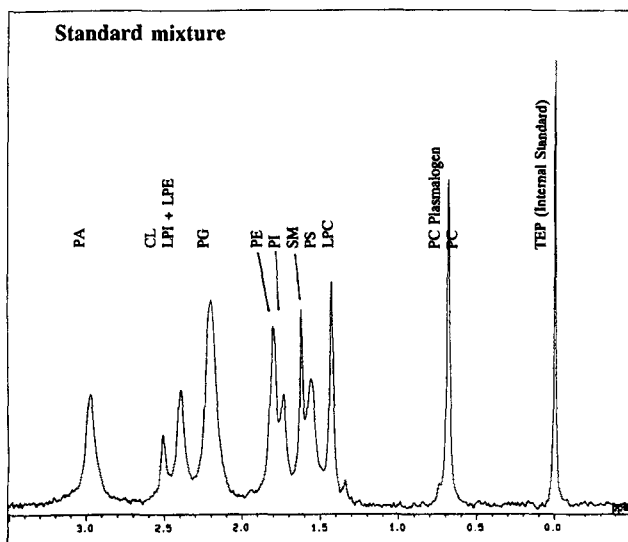
n.d. = not determined.

0.025 mmol/l). LPC was detectable when PLs were extracted from human lens tissue (0.11 mmol/kg), where PE was found to be the major compound (3.39 mmol/kg). When using the extraction method of Manimekalai,<sup>7</sup> placenta tissue appeared to contain LPC (0.31 mmol/kg).

In 37 tissue samples (16 from the myometrium, 11 from the decidua, four from the placenta and six from fetal membranes), total lipid phosphorus was analyzed both chemically<sup>13</sup> and by  $^{31}\text{P}$  NMR, as a sum of AUC (Table 4). The chemical phosphorous content in the myometrium was  $92 \pm 22$  mg/kg, in decidua  $160 \pm 23$  mg/kg, in fetal membranes  $175 \pm 28$  mg/kg and in the placenta  $337 \pm 48$  mg/kg (mean  $\pm$  S.E.). The correlation between the two methods was reasonable ( $r=0.727$ ).

### Composition of the phospholipids

Examples of the PL  $^{31}\text{P}$  NMR spectrum for the different tissues are given in Fig. 2(a)–(d) All the tissue samples analyzed showed PC, SM and PE peaks in  $^{31}\text{P}$  NMR large



**Figure 1.**  $^{31}\text{P}$  NMR spectrum from the standard mixture of phospholipids (4–5 mmol/l). The triethylphosphate served as an internal standard (10 mmol/l).

**Table 4. Phospholipids in human myometrium, decidua, placenta and fetal membranes**

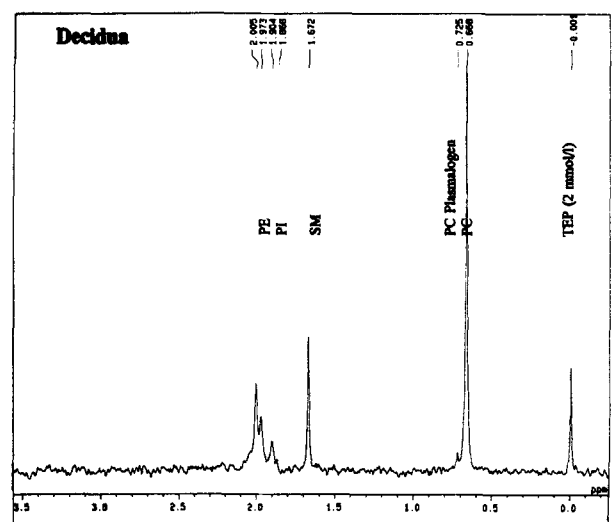
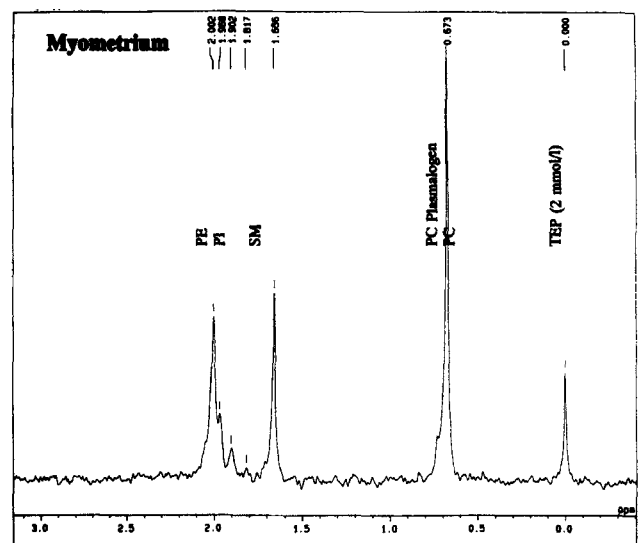
	Myometrium <i>n</i> =19	Decidua <i>n</i> =11	Placenta <i>n</i> =4	Fetal membranes <i>n</i> =6
Total PLs <sup>a</sup>	12.5 $\pm$ 2.3	18.4 $\pm$ 2.8	30.8 $\pm$ 5.1	20.4 $\pm$ 4.3
PC	6.2 $\pm$ 1.2	9.6 $\pm$ 1.8	16.3 $\pm$ 2.8	11.3 $\pm$ 2.4
SM	2.6 $\pm$ 0.4	3.0 $\pm$ 0.5	4.5 $\pm$ 0.5	3.4 $\pm$ 0.8
PI	0.5 $\pm$ 0.1 <sup>c</sup>	1.0 $\pm$ 0.2 <sup>d</sup>	2.4 $\pm$ 0.3	0.4 $\pm$ 0.1
PE	3.5 $\pm$ 0.8	5.4 $\pm$ 0.8	7.6 $\pm$ 1.8	5.2 $\pm$ 1.2
PC/SM <sup>b</sup>	2.2 $\pm$ 0.1	3.4 $\pm$ 0.4	3.6 $\pm$ 0.2	3.5 $\pm$ 0.5

Numbers indicate mmol/kg, mean  $\pm$  SE, <sup>c</sup>*n*=9, <sup>d</sup>*n*=3.

<sup>a</sup> Myometrium and decidua vs placenta and fetal membranes,  $p=0.019$ .

<sup>b</sup> Myometrium vs decidua,  $p=0.01$ ; vs fetal membranes,  $p=0.01$ .

enough to be quantitatively analyzed. A small peak was constantly present at +0.06 ppm from the PC-peak. This was in accord with the PC plas shift standard, but was also similar to what has been described for  $\beta$ -acyl- $\gamma$ -O-alkylphosphatidylcholine.<sup>15</sup> The area of this small peak was included in the PC bulks. The PI was frequently large



**Figure 2.** The  $^{31}\text{P}$  NMR spectra for the phospholipid extracts from myometrium, decidua, placenta and fetal membranes. The highest concentrations of phospholipids were seen in the placenta (compare AUCs to TEP standard, 2 mmol/l). PC, PE, SM and PI (as the smallest bulk compound) can be seen, but not lysocompounds or PA. Limit of detection: 0.05 mmol/kg.

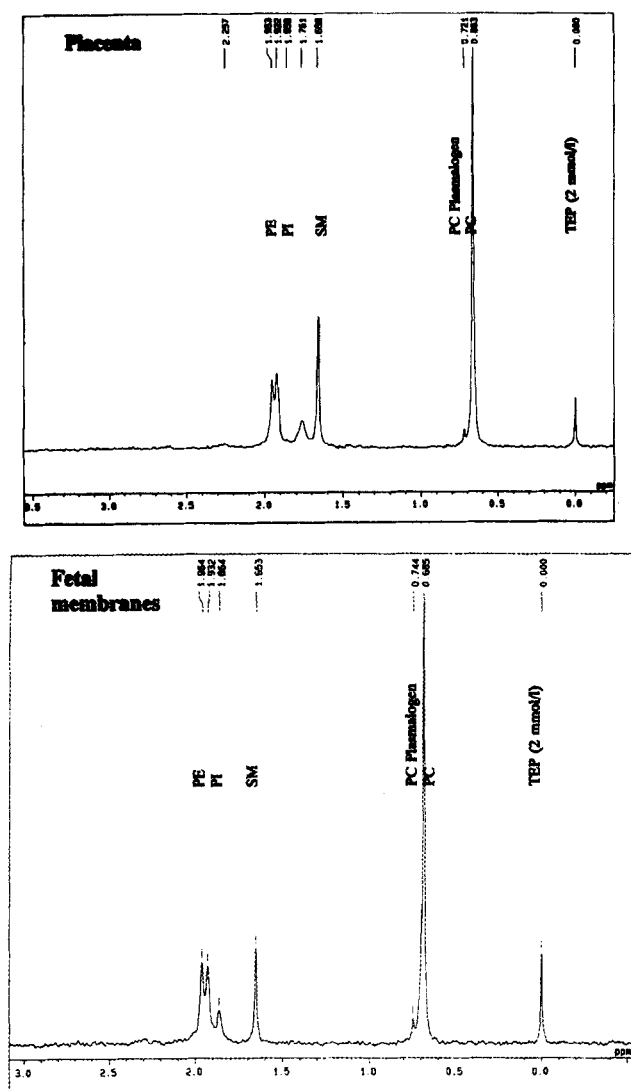


Figure 2. continued

enough to be analyzed, but was also overrun by noise: analyses of PI were possible in 8/19 myometrial samples, in 3/11 decidual samples, and detectable in all samples of fetal origin (placenta, fetal membranes). PE regularly contained two separate peaks. The PE plus shift could not be distinguished from total PE. Instead of the usual two peaks, total PE contained in a few instances three separate peaks. Because the separate PE-peaks were not identified, PE was analyzed as a single pool. In one case, the decidua contained a detectable amount of PG (0.87 mmol/kg). In no experiment were the lysocompounds (LPC, LPE, LPI, LPA), CL or PA found, nor suspected to be present.

### Comparison of phospholipid contents

The contents of total PLs, PC, SM, PI and PE are given in Table 4. The fetal tissues, placenta and fetal membranes had about twice the amount of PLs as the maternal tissues, the myometrium and the decidua ( $p=0.019$ ). In spite of the wide variation in the myometrial concentrations of PC and SM during pregnancy, their ratio remained constant: the range for PC was 0.7–19.6 mmol/kg and for SM 0.3–4.9 mmol/kg, but for PC/SM only 1.5–4.0. In the nonpregnant state, all the corresponding values were stable: for PC 2.9–4.0; for SM 1.6–1.9; and for PC/SM 1.9–2.2.

Table 5. Phospholipids in the myometrium according to the condition of the patient

	Nonpregnant <i>n</i> =3	Pregnant, no labor <i>n</i> =9	Pregnant, labor <i>n</i> =7
Total PLs	9.6±0.7	11.7±3.9	14.9±5.6
PC	4.4±0.4	5.7±1.2	7.6±3.0
SM	2.1±0.1	2.7±0.6	2.8±0.7
PI	0.7±0.2	0.1±0.0 <sup>a</sup>	0.5±0.2 <sup>b</sup>
PE	2.4±0.3	3.3±1.1	4.3±1.8
PC/SM	2.1±0.1	2.1±0.1	2.4±0.3

Numbers indicate mmol/kg, mean±SE, <sup>a</sup> *n*=2, <sup>b</sup> *n*=4.

The PC/SM ratio was higher in the pregnancy-specific tissues than in the myometrium (Table 4).

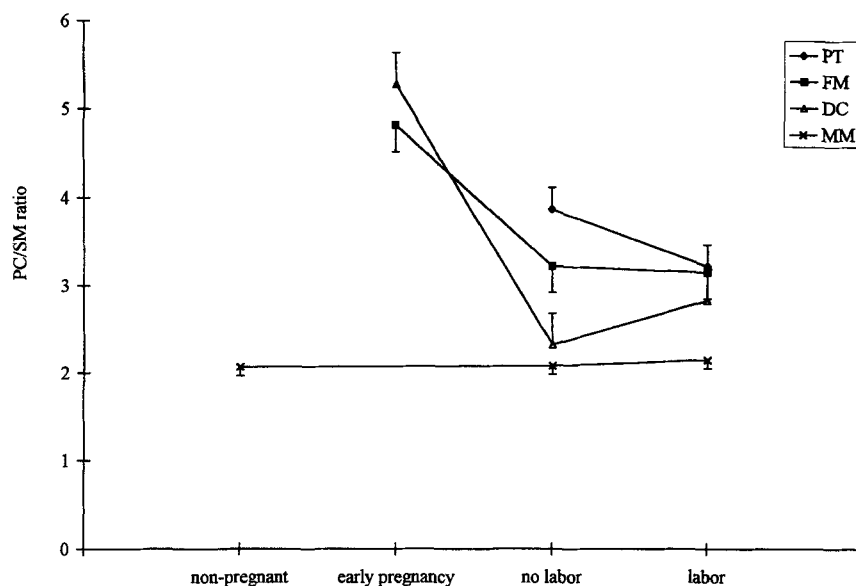
The PL composition was identical in all patients, irrespective of whether or not they were in labor (Table 5). There was no evidence that in cases when there was an obstetric indication for cesarean section tissue PLs were in any way different. The PC/SM ratio decreased during the course of pregnancy in the decidua, placenta and fetal membranes, but not in the myometrium (Fig. 3).

### DISCUSSION

The <sup>31</sup>P NMR technique employed indicated the same level of PLs in human muscle as did the natural abundance <sup>13</sup>C NMR, about 10 mmol/kg wet tissue.<sup>16</sup> The chemical phosphorus determination also correlated reasonably with the actual results when <sup>31</sup>P NMR was used. The findings of the present study concerning the content and composition of PSs appear to be valid, with 0.05 mmol/kg as a detection limit.

The composition of the main pools of myometrial PL (PC, SM, PE, PI) remained constant during pregnancy. When using water soluble extracts and <sup>31</sup>P NMR, Phoenix and Wray have documented a significant fall in the phosphomonoester phosphoethanolamine in the human, but not in the rat myometrium during the course of pregnancy.<sup>17</sup> Rat myometrial tissue showed an increase of phosphoethanolamine on day 1 postpartum. The composition and fluidity differences observed can be affected by the method used.<sup>18,19</sup>

Pregnancy-associated tissues, especially the placenta, contain a wide variety of the PLs needed for fetal development, but not LPC. In one study based on 300 kg of human placenta, PA, LPC and LPE were still below the detection limit.<sup>20</sup> The findings of the present study strengthen the opinion that by using <sup>31</sup>P NMR, the artefacts arising with other methods can be eliminated. No detectable amounts of lysocompounds were found in pregnancy-associated tissues. This is contrary to earlier findings when different extraction and analyzing processes were used,<sup>7,8</sup> but accords with more recent findings that by avoiding acidic solutions and employing NMR it is possible to prevent the occurrence of artificial lysocompounds, which otherwise increase during the analysis.<sup>9,12</sup> We were also able to demonstrate LPC in the <sup>31</sup>P NMR spectrum when employing a different extraction method.<sup>7</sup> A tissue known to contain LPC is the lens.<sup>12</sup> The compound was detectable with our methods in human lenses thus confirming that with the present method it is possible to detect it in tissue samples. Lysophospholipids are found when there is necrosis,<sup>21</sup> but detectable amounts are not found in the



**Figure 3.** PC/SM ratios of the tissue extracts during the course of pregnancy. The bars indicate S.E. In the pregnancy-specific tissues placenta (PT), fetal membranes (FM), and decidua (DC) the ratio changed, but not in the myometrium (MM).

normal liver,<sup>22</sup> nor in the myometrium, decidua, placenta and fetal membranes (present study). Tissue lysophospholipids could appear in pathological conditions of the fetomaternal unit and mediate the pathophysiological process to the fetus, as in the case of the heart or brain.<sup>4-6, 10, 21</sup> The total quantity of PLs in the placenta and fetal membranes was large compared to that in the maternal tissues, the myometrium and the decidua. This supports the dynamic supposition that autacoids, which originate from high fetal PLs, affect the myometrial function.<sup>23</sup>

Lysocompounds are one factor in the regulation of membrane fluidity, which was found to increase during pregnancy.<sup>8, 24, 25</sup> The detection limit in this study (0.05 mmol/kg) was less than the level of amphiphils (1.5 mol%) needed to produce an increase in membrane fluidity.<sup>26, 27</sup> Since no lysophospholipids were detected in the different tissues studied, nor in the uterine smooth muscle under two different physiological conditions (i.e. clinical contractions vs no contractions), it is probable that the increase in myometrial fluidity during late pregnancy

depends on other variables, such as double bonds (unsaturation), short hydrocarbon chains or cholesterol.<sup>27</sup> PC saturation, cholesterol enrichment and membrane fluidity can be regulated by estradiol 17 $\beta$ -D-glucuronide or by influencing PE-metabolism.<sup>18</sup> In our pilot study with crude myometrial PL extract we were able to find a clear peak in <sup>1</sup>H NMR for PLs containing double bond(s). Further pilot experiments will be necessary in order to determine whether the isolation of different cell compartments is relevant for future studies of uterine function and pregnancy maintenance, which depends on estrogen/progesterone action<sup>2</sup> in several myometrial cell compartments at connexin 43 levels and trafficking,<sup>28</sup> or on the fluidity itself.<sup>29</sup>

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