



Glycerophosphocholine and Glycerophosphoethanolamine Are Not the Main Sources of the *In Vivo* ^{31}P MRS Phosphodiester Signals from Healthy Fibroglandular Breast Tissue at 7 T

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Purpose: The identification of the phosphodiester (PDE) ^{31}P MR signals in the healthy human breast at ultra-high field.

Methods: *In vivo* ^{31}P MRS measurements at 7 T of the PDE signals in the breast were performed investigating the chemical shifts, the transverse- and the longitudinal relaxation times. Chemical shifts and transverse relaxation times were compared with non-ambiguous PDE signals from the liver.

Results: The chemical shifts of the PDE signals are shifted -0.5 ppm with respect to glycerophosphocholine (GPC) and glycerophosphoethanolamine (GPE), and the transverse and longitudinal relaxation times for these signals are a factor 3 to 4 shorter than expected for aqueous GPC and GPE.

Conclusion: The available experimental evidence suggests that GPC and GPE are not the main source of the PDE signals measured in fibroglandular breast tissue at 7 T. These signals may predominantly originate from mobile phospholipids.

Keywords: MRSI, ^{31}P , relaxation time, 7 T, phosphodiester, breast, phospholipids

INTRODUCTION

The phosphomonoesters (PME), phosphocholine (PC) and phosphoethanolamine (PE), and the phosphodiesters (PDEs), glycerophosphocholine (GPC) and glycerophosphoethanolamine (GPE), are involved in cell membrane metabolism. From *ex vivo* studies, it is known that the PC/GPC ratio goes up on malignant transformation of cells (1, 2), while the decrease of the PC/GPC ratio was shown to be a marker in predicting cancer treatment response in *ex vivo* NMR studies (2–4). *In vivo*, the total choline signal, which can be obtained by localized ^1H MRS, has been shown to be a biomarker for malignancy and treatment response (5). In contrast to *in vivo* ^1H MRS, where only

Abbreviations: AMESING, Adiabatic Multi-Echo Spectroscopic Imaging; CSA, chemical shift anisotropy; DPPC, dipalmitoylphosphatidylcholine (1,2-dipalmitoyl-sn-glycero-3-phosphocholine); DMPC, dimyristoylphosphatidylcholine (1,2-dimyristoyl-sn-glycero-3-phosphocholine); GPC, glycerophosphocholine; GPE, glycerophosphoethanolamine; GPCr, (diacyl-)glycerophosphatidylcholine; GPTE, (diacyl-)glycerophosphatidylethanolamine; ILA, inter-lamellar attachment; MPL, membrane phospholipids; PC, phosphocholine; PE, phosphoethanolamine; Pi, inorganic phosphate; PCr, phosphocreatine.

a total choline signal can be observed, one can easily distinguish PME from PDEs with *in vivo* ^{31}P MRS and even PE from PC and GPE from GPC, with ultra-high field ^{31}P MRS (6). Besides higher spectral resolution, ultra-high field MRS comes with a higher signal-to-noise ratio that can be traded off for improving spatial resolution or to shorten scan time. High *in vivo* PME/PDE ratios, as measured with ^{31}P MRS, have been shown to be indicative of cancer, while treatment response is often accompanied by a reduction in PME/PDE (7–12). However, in contrast to some *ex vivo* methods, where extraction techniques are used to separate aqueous pools of metabolites from lipid pools, *in vivo* methods will also obtain signals from membrane phospholipids (MPL) (13, 14). Moreover, as these MPL have chemical shifts similar to GPC, e.g., glycerophosphatidylethanolamine (GPE) has almost identical chemical shift as GPC (15, 16) (the molecular structures and chemical shifts are shown in **Figure 1**) – *in vivo* distinction of these compounds is hampered.

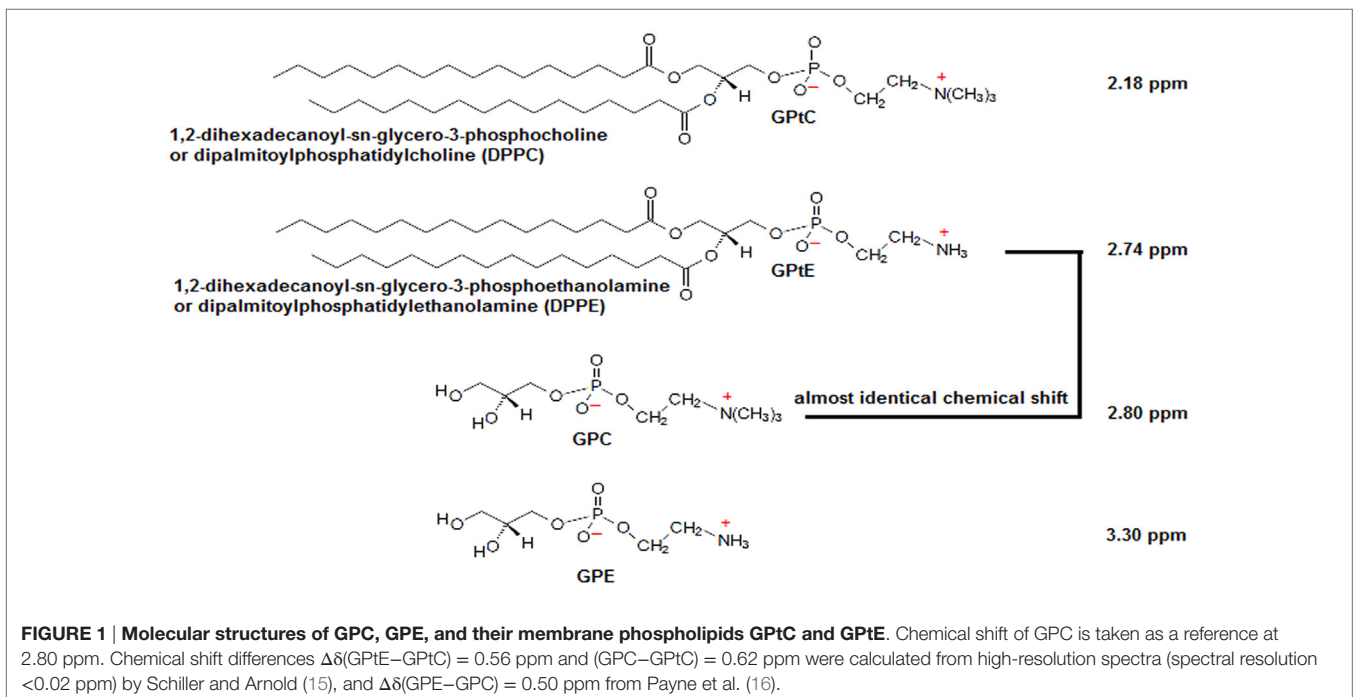
At lower field strength (<2.5 T), *in vivo* ^{31}P spectra of various tissues, e.g., breast (17, 18), brain (19–24), liver (25–28), and kidney (29, 30), show a large signal in the PDE chemical shift range, with its top between 2 and 3 ppm with respect to phosphocreatine (PCr) at 0 ppm. The full width at half max of this signal is dependent on the field strength, and the delay between excitation and acquisition, as, for instance, caused by phase encoding. It has been suggested (21, 25) that this membrane peak disappears almost completely at high-field strength due to enhanced relaxation by chemical shift anisotropy, leaving only the signals of the aqueous-soluble metabolites GPC and GPE. Nowadays, with high magnetic field human MRI systems becoming available, the origin of the PDE ^{31}P MRS signal – whether GPC and GPE, and/or GPC and GPE – is of renewed interest.

Here, we show that the PDE signals measured *in vivo* in fibroglandular tissue of the human breast (^{31}P signal from breast

fatty tissue is below the detection limit) at 7 T are possibly signals from MPL, although their line widths suggest aqueous small molecules, such as GPC and GPE. Measurements are performed at 7 T to distinguish GPE from GPC and GPC from GPE. Adiabatic multi-echo spectroscopic imaging (AMESING) (31) and progressive saturation are used to identify the mobility of the molecules as reflected in the T_2 - and T_1 -values, respectively, to enable a distinction between the aqueous GPE and GPC from the more restricted MPL (GPC and GPE). Data are obtained in breast glandular tissue and compared to GPC and GPE metabolite signals as measured in liver tissue, all in healthy human volunteers *in vivo*.

MATERIALS AND METHODS

^{31}P MRS measurements of glandular breast tissue were obtained from healthy volunteers using a dedicated breast coil (MR Coils BV, Druen, The Netherlands) interfaced to a 7-T MRI system (Philips, Cleveland, OH, USA). Pulse-acquire and multi-echo acquisitions [AMESING (31)] were obtained with adiabatic RF pulses. Excitation was done with an adiabatic half passage (AHP) of 2 ms. For refocusing 4 ms B_1 insensitive rotation pulses (BIR-4 180°) were used. The AHP excitation pulse had a frequency sweep of 10.0 kHz and the BIR-4 refocusing pulses a frequency sweep of 20.0 kHz. Both pulses had tangent frequency modulation and hyperbolic tangent amplitude modulation as described in Garwood and Ke (32). Pulses were driven with $\gamma B_{1\text{max}} = 1700$ Hz. Transmitter offset on the ^{31}P channel was set to 600 Hz with respect to the resonance of PCr. All chemical shifts reported here are referenced to PCr as standard at a chemical shift of 0.0 ppm, which is -2.48 ppm compared to 85% phosphoric acid. Frequency calibration of the scanner is done based on the water signal (the MR system uses a fixed ratio between ^{31}P and ^1H carrier frequency



such that the proton signal for water corresponds to the ^{31}P PCr signal). Measurements with the AMESING sequence were performed with a T_R of 6 s, $8 \times 8 \times 8$ spherical acquired MRSI, $2 \text{ cm} \times 4 \text{ cm} \times 4 \text{ cm}$ voxel sizes for the breast on five volunteers.

Both FID and symmetric echoes were acquired with 256 data points, and the spectral bandwidth for the acquisition of the FID was 17.0 kHz and for the echoes 8.5 kHz (echo spacing 45 ms) to maintain equal acquisition durations for FID and each half echo. Acquired data were spatially Hamming filtered and zero filled in the time domain to 8192 data points. To obtain high SNR spectra of the breast, the datasets of five volunteers (age range 24–30 years) were pooled and Pi-weighted based on the FID signal. Phosphorus metabolite T_1 -values in the breast were measured for five volunteers by means of progressive saturation with an adiabatic AHP pulse-acquire 1D MRSI sequence with T_R values in the range of 0.5–8 s, where the scan time was kept identical for each T_R . The FID data were acquired with 512 data points and a spectral bandwidth of 8.2 kHz. A 1D MRSI encoded in the anterior–posterior direction was chosen to effectively suppress signals from the underlying pectoral muscles. Data were spatially Hamming filtered and subsequently zero filled in the time domain to 8192 data points. To obtain high SNR datasets for T_1 -fitting, the volunteers were measured two or three times and the data per volunteer were averaged. Before averaging, all spectra were aligned for Pi. Averaged spectra were spectrally fitted in JMRUI (33) using the AMARES algorithm (34), chemical shifts for the GpTE + GPC and GpTC resonances were fixed with a soft constraint to $2.77 \pm 0.1 \text{ ppm}$ and $2.18 \pm 0.1 \text{ ppm}$ and free but equal line width.

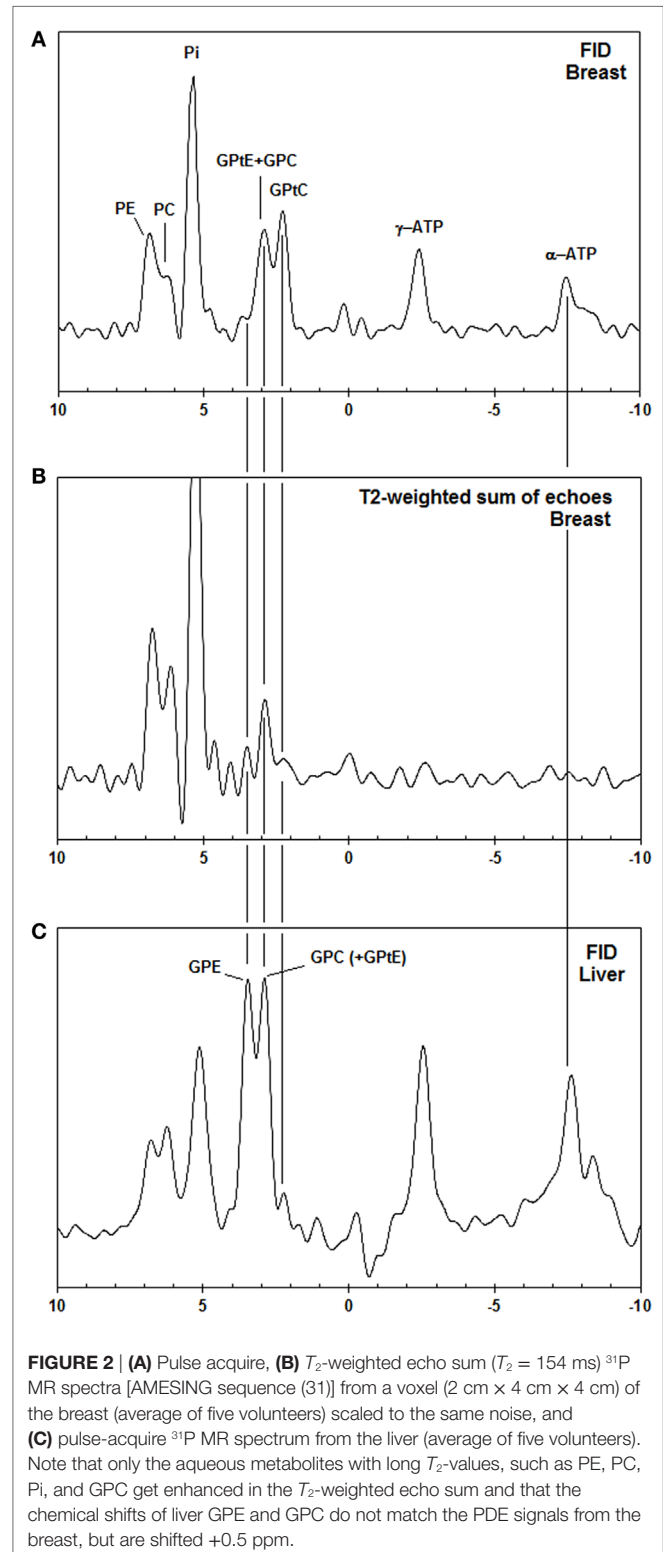
^{31}P MRS liver measurements were also done with the AMESING sequence with 32×10 2D MRSI (feet–head direction unlocalized) voxel sizes $1 \text{ cm} \times 1 \text{ cm}$ (echo spacing 40 ms) on five volunteers for the liver [data included from earlier study (35)], using a half volume coil (MR Coils BV, Drunen, The Netherlands). Other parameters and data post-processing and analysis were equal to those used in the breast measurements. Here, a 2D scheme with small AP and LR dimensions of the voxels was chosen to be able to exclude signal from muscle tissue, whereas the FH dimension is unlocalized but constrained by the coil sensitivity to encompass the liver but not beyond.

The study was approved by the local medical ethics review board (METC UMC Utrecht) and written informed consent was obtained from all volunteers.

RESULTS

In **Figure 2**, the spectra of a voxel of the breast (average spectrum of five volunteers) and the liver (five volunteers) are shown. Due to limited bandwidth of the adiabatic pulses only the spectral range from +10 to -10 ppm is shown. The chemical shifts of the GPC and GPE signals in the liver (**Figure 2C**) do not correspond to the signals observed in the breast spectra (**Figures 2A,B**), which are usually labeled GPC and GPE, while the chemical shifts of the other metabolites (PE, PC, Pi, γ -ATP, α -ATP) in liver and breast do match. **Figures 2A,B** show, for a voxel of breast glandular tissue, the average FID and the average T_2 -weighted

echo-sum spectra using a fixed T_2 weighting of $154 \pm 5 \text{ ms}$ (36), scaled to the same noise. Note that the signal intensities of the peaks labeled PE, PC, Pi, and (GpTE + GPC) increase, or at least do not decrease, in the echo-sum spectrum as compared



to the FID spectrum of the breast, while the signals of GPC and ATP, with known short T_2 -values, do decrease in the echo-sum spectrum. Unlike ATP, the short apparent T_2 for GPC (and GPtE) is not the result of homonuclear coupling. In **Figure 3**, a comparison is made between T_2 fits obtained for the PDE signal at 2.2 ppm (labeled GPtC) from the breast (a) and the 2.8 ppm signal from the liver (labeled GPC), showing almost a factor 3 lower T_2 for the GPtC signal from the breast. **Figure 4** shows the T_1 -fits for the average signals of (GPtE + GPC) and GPtC from fibroglandular breast tissue as measured in the five volunteers. The (GPtE + GPC) signal is fitted bi-exponentially with a short T_1 component for GPtE (taken equal to GPtC, $T_1 = 1.2$ s) The T_1 -value that is fitted for the long T_1 component GPC is 3.2 s.

DISCUSSION

Phosphorous spectra from the breast and liver as shown in **Figure 2** do not match to the chemical shift of the PDE signals. As the chemical shift of GPC and GPE are hardly pH sensitive, but Pi and to a lesser extent also PE, PC, and γ -ATP are (37), pH differences between liver and breast may influence chemical shift. If we would shift the breast spectrum by +0.5 ppm to match the PDE signals between breast and liver, this would correspond to

a shift in pH of +0.4 units based on Pi chemical shift. This pH difference is unreasonably large, moreover expressing the change in chemical shift of +0.5 ppm for PE, PC, and γ -ATP in pH units is either not possible, or goes beyond any physiologic condition. Therefore, it seems likely that the metabolite signals in the breast that do not match those in the liver are (GPtE + GPC) and GPtC. Healthy liver is known to show high signals of GPC and GPE in ^{31}P MRS *in vivo* and also in *ex vivo* perchloric acid extracts (38, 39). The peak labeled GPtC in the liver spectrum of **Figure 2C** is sometimes referenced to as (potentially) phosphoenolpyruvate (28, 40). However, it does not show up in ^{31}P MRS perchloric acid liver extract studies (38, 39), even though phosphoenolpyruvate is sufficiently soluble in an aqueous phase.

The most likely reason for the nearly constant (GPtE + GPC) signal over FID and echo sum (**Figures 2A,B**) is that aqueous GPC, with a relatively long T_2 , increases in the echo sum, while GPtC, just like GPtE, decreases in intensity due to short T_2 .

A recent lipidomic profiling study on healthy mammary epithelial and breast cancer cells (41) has shown that in the membranes of healthy mammary epithelial cells the concentration of GPtC is approximately twice the concentration of GPtE. If we assume that the T_2 -weighted echo-sum signal at the chemical shift of GPtE + GPC in **Figure 2B** is indeed all GPC and we assume

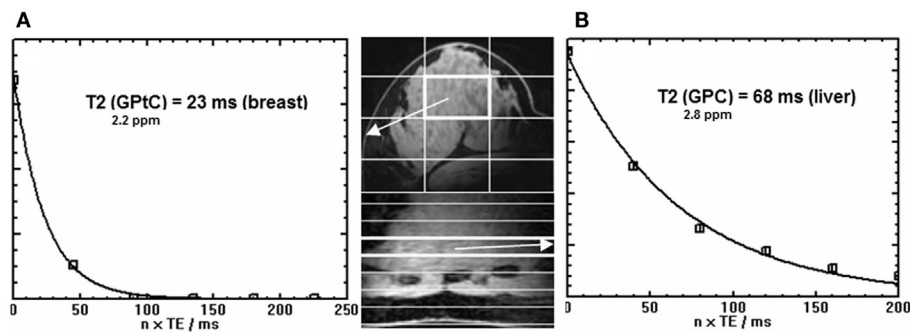


FIGURE 3 | Signal decay [as quantified by spectral fitting of FID and echoes with JMRUI (33)] as a function of time of the GPtC peak at 2.2 ppm obtained from (A) the breast (36) (echo spacing 45 ms) and (B) the true GPC peak from the liver (35) at 2.8 ppm (echo spacing 40 ms). Data were obtained by the AMESING sequence (31) and are averaged values for the group of volunteers. Note the threefold reduced T_2 of the ^{31}P spins of GPtC in the breast as compared to GPC in the liver. Images shown are (fast field echo) examples of breast and liver for one volunteer.

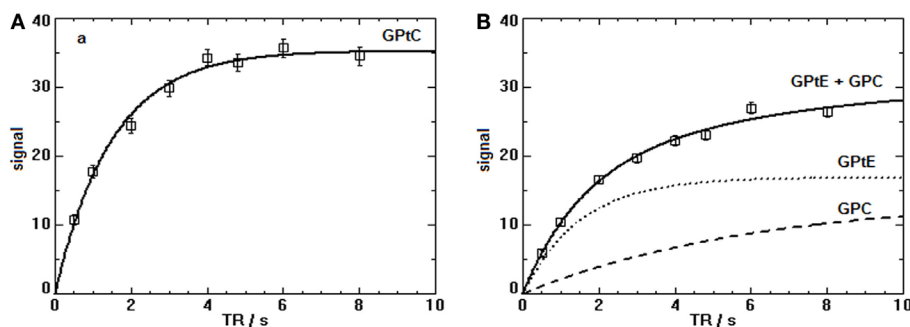


FIGURE 4 | T_1 -fits of the progressive saturation measurements for the PDE signals (sum of five volunteers) in the breast at 7 T. (A) The T_1 -value of the GPtC signal (mono-exponential decay) is 1.2 ± 0.3 s. (B) The (GPtE + GPC) signal for the five volunteers was fitted bi-exponentially with a fixed short T_1 component for GPtE taken equal to GPtC leading to a T_1 for GPC of 3.2 ± 0.6 s.

a similar T_2 for GPC and PE and PC, then we can calculate the GPC to GPC ratio by subtraction of the GPC contribution in **Figure 2A**. This leads to a GPC to GPC ratio of 2, in close agreement with the lipidomic profiling study. Minor contributions from sphingomyelin and glycerophosphatidylserines, seen in the lipidomic profiling study will probably add to the *in vivo* ^{31}P MR signal of GPC and glycerophosphatidylinositol to the *in vivo* ^{31}P MR signal of GPC, not altering the ratio substantially. Chemical shift differences for these different phospholipids are reported by Schiller and Arnold (15).

The ^{31}P T_2 values in liver can be low due to the presence of iron, for instance, in the form of ferritin, which is known to increase the relaxivity of water (42). *In vivo* values for T_2 of ^{31}P metabolites (PE, PC, Pi, GPE, GPC) in the liver ranging between 37 and 71 ms have recently been measured at 7 T in our hospital in a group of five healthy volunteers (35). For the breast, however, we measured that the T_2 of the ^{31}P spins at the chemical shift of GPC (36) is even a factor 3 shorter than the T_2 of the ^{31}P spins of GPC in the liver (35), as shown in **Figure 3**. In fact, compared to the reported T_2 values – measured at 7 T – of PDEs in calf muscle [$T_2 = 314$ ms (43), 375 ms (31)] or of PC and PE in the breast, the T_2 value of GPC we measured is almost an order of magnitude lower. Spectral fitting of the FID spectrum of **Figure 2A**, simplified by equal line widths for PE, PC, Pi, and equal linewidths for the PDE signals shows an additional linewidth for the PDE signals of 9 Hz, which is close to the calculated value of 12 Hz when considering the measured T_2 of 23 ± 1 ms (35) and a T_2 for the PME and Pi of ~ 160 ms.

The low signals for GPE and GPC in the echo-sum spectra of the breast are corroborated by an *in vitro* extract study on breast tumors by Smith et al. (14), where it was shown that GPE and GPC concentrations are low in non-necrotic breast tumors and that, at low field, PDE signals observed *in vivo* are mainly from phospholipids. A recent LC MS study by Mimmi et al. (44) showed a very low average concentration of only 0.04 mmol/kg GPC in three healthy fibroglandular breast tissue samples.

Another reason to suspect that the dominant PDE signal we observe in the breast at 7 T originates from mobile lipid structures is based on the results of the T_1 -measurements of the PDE signal in the breast, as depicted in **Figure 4**. Here, the T_1 of GPC was fitted mono-exponentially leading to 1.5 ± 0.1 s and the signal of GPC + GPC was fitted bi-exponentially with the T_1 of the GPC component fixed equal to the T_1 of GPC. For the signal of the aqueous GPC, this leads to 6 ± 2 s. The fitted T_1 of mobile GPC is three- to fivefold lower than that reported for GPC and GPE in calf muscle and brain at 7 and 3 T (43, 45, 46). A value of 1.4 s for the T_1 of the ^{31}P MRS signal of dipalmitoylphosphatidylcholine (DPPC) vesicles with an average diameter of 100 nm has been measured by Klauda et al. (47) above the phase transition temperature. For multi-lamellar dispersions of dimyristoylphosphatidylcholine (DMPC), a value around 1 s has been measured just above the phase transition temperature by Dufourc et al. (48). The T_1 -value of 6 s fitted for the GPC component, **Figure 4B**, agrees well with the T_1 -values for GPC reported at 3 T and 7 T for calf muscle and brain ranging from 4 to 7.8 s (43, 45, 46).

All presented data throughout this paper is based on average spectra of the group of volunteers. This has the advantage that it maximizes signal to noise and enables the most reliable relaxation

time fitting. A short coming is that individual physiological differences between volunteers are averaged out.

The fitted linewidth of the two overlapping PDE signals of the breast spectrum depicted in **Figure 2A** is 58 Hz (i.e., 0.5 ppm). Bulk phospholipid bilayers show broad asymmetrical lineshapes (several tens of ppm) caused by large chemical shift anisotropy (49). Therefore, if the very sharp PDE resonances that we observe are from MPL, then these MPL must be highly mobile phospholipids, for which chemical shift anisotropy and dipolar couplings are sufficiently averaged out. Especially at ultra-high field, relaxation by chemical shift anisotropy that goes with the square of the field causes additional line broadening as compared to spectra recorded at lower field strength. Highly mobile phospholipids can be found in small-sized vesicles (<50 nm) (50), and in large arrays of lipidic particles (51), inter-lamellar attachments (52), and inverted cubic structures (52–54) within the lipid bilayer.

A rough estimate of the percentage of *in vivo* visible mobile phospholipids at 7 T can be made as follows. The ratio of PE to PC is ~ 2 and the PME to PDE ratio is ~ 1 (**Figure 2A**). A weighted average of PC concentrations measured in healthy breast tissue by Mimmi et al. (44) is 0.08 mmol/kg, with PE/PC = 2 (**Figure 2A**), this leads to a PME concentration of ~ 0.2 mmol/kg. Most of the PDE signal is from mobile phospholipids (**Figures 2A,B**). The total concentration of phospholipids in human tissues is in the range of 17–83 mmol/kg (55). With a signal ratio of PDEs to PMEs in breast glandular tissue of 1.4 at $T_R = 6$ s (35) and a T_1 of PMEs of 5 s (56) and a T_1 of mobile phospholipids of 1.5 s, the total concentration of *in vivo* visible mobile phospholipids in the human breast at 7 T is also of the order of ~ 0.2 mmol/kg. This leads to a crude estimate of the visible mobile phospholipid fraction at 7 T of 0.2–1.2%.

CONCLUSION

The PDE signals from the breast, as measured with MRSI techniques at 7 T *in vivo*, show aberrant behavior from aqueous GPC and GPE. The T_1 and T_2 relaxation values for these PDE signals are too short to represent true aqueous GPC and GPE. In addition, the chemical shifts of these PDE signals do not correspond to GPE and GPC, but are shifted -0.5 ppm with regard to these, and correspond to chemical shift values of GPC and GPC. These PDE signals could originate from mobile lipid structures such as small vesicles with diameters ≤ 50 nm, large arrays of ILAs or large domains of inverted cubic phases within the lipid bilayer. As the PC over GPC ratio is used as a biomarker in breast cancer research, the *in vivo* obtained value will be contaminated with signal from GPC – having a similar chemical shift as GPC – or the GPC peak may be erroneously assigned as GPC.

AUTHOR CONTRIBUTIONS

WK: study design, MR measurements, data analyses, first draft of typescript; BS: MR measurements, critical revision of typescript; JR: MR measurements, data analysis, critical revision of typescript; JW: study design, critical revision of typescript; AN: study, design, critical revision of typescript; PL: study design, critical revision of typescript; DK: study design, critical revision of typescript. All authors approved the final version of the manuscript.

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