

Abnormal liver phosphatidylcholine synthesis revealed in patients with acute respiratory distress syndrome

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Abbreviations: ARDS, acute respiratory distress syndrome; CDP, cytidine diphosphocholine; LPC, lysophosphatidylcholine; PC, phosphatidylcholine; PEMT, phosphatidylethanolamine N-methyltransferase; SAmE, S-adenosyl methionine.

Abstract

Acute respiratory distress syndrome (ARDS) is associated with a severe pro-inflammatory response; although decreased plasma cholesterol concentration has been linked to systemic inflammation, any association of phospholipid metabolic pathways with ARDS has not been characterized. Plasma phosphatidylcholine (PC), the major phospholipid of circulating lipoproteins, is synthesized in human liver by two biologically diverse pathways: the CDP:choline and phosphatidylethanolamine-*N*-methyltransferase (PEMT) pathways. Here, we used electrospray ionization–MS/MS both to characterize plasma PC compositions and to quantify metabolic fluxes of both pathways using stable isotopes in patients with severe ARDS and in healthy controls. Direct incorporation of *methyl*-D₉-choline estimated CDP:choline pathway flux, while PEMT flux was determined from incorporations of one and two methyl-D₃ groups derived from *methyl*-D₉-choline. Results of MS/MS analysis showed significant alterations in plasma PC composition in patients with ARDS versus healthy controls. In particular, the increased overall *methyl*-D₉-PC enrichment and—most important—the much lower *methyl*-D₃-PC and *methyl*-D₆-PC enrichments—suggest increased flux through the CDP:choline pathway and reduced flux through the PEMT pathway in ARDS. To our knowledge, this study is the first to demonstrate significant plasma PC molecular compositional changes combined with associated alterations in the dynamics of PC synthetic pathways in patients with ARDS.

Keywords

Phosphatidylcholine synthesis

Methyl-D₉-choline

Stable isotope

Introduction

Acute respiratory distress syndrome (ARDS) is a systemic pro-inflammatory condition associated with significant morbidity and mortality in the intensive care unit (1). Clinically, ARDS is characterised by severe hypoxic respiratory failure at least partly as a consequence of alterations in surfactant phospholipid metabolism leading to alveolar collapse and poor lung compliance (2). However, further questions remain, whether these phospholipid changes are only limited to the alveolar environment or if there are global alterations in phospholipid metabolism, in particular the hepatic handling of lipids have never been scrutinised before.

Phosphatidylcholine (PC) *de-novo* biosynthesis in humans is dependent on two distinct molecular pathways. While all nucleated mammalian cells are able to synthesise PC by the CDP: choline pathway, hepatocytes can convert phosphatidylethanolamine (PE) into PC by three sequential methylation of PE catalysed by PE-*N*-methyltransferase (PEMT) (3). The preference of PEMT selectively to synthesise polyunsaturated fatty acids (PUFA)-based PC species highlights the possibility of functional variation between these two synthetic pathways (4). In general, about 30% of hepatic PC is synthesised by PEMT and the remainder by the CDP: choline pathway. As lipid metabolism is highly orchestrated by the liver, alterations in hepatic PC synthesis may have clinical implications in ARDS that have not previously been investigated. Furthermore, phosphatidylcholines are precursors for several biosynthetic pathways of inflammation and resolution (5). Fatty acids esterified to phosphatidylcholines can be liberated by the actions of phospholipase-A₂ and subsequent generations of oxidative metabolites are regulators of inflammation and have been implicated at least in part in the pathogenesis of ARDS (6, 7). In contrast, docosahexaenoic acid (DHA; sn-2 22:6) can also generate mediators which are involved in resolution of inflammation (8). Patients with ARDS characteristically have reduced plasma concentrations of polyunsaturated fatty acids (9) and

mechanisms underlying these had not been elucidated. Furthermore, there may be clinical consequences of these alterations in phospholipid handling, influencing the degree of inflammation, initiation of resolution and progression into chronic inflammation.

PEMT knockout mice models exhibit a selective reduction in both plasma and hepatic PC species containing DHA (10). We postulate, that one possible reason for such reductions in plasma PUFA-PC species in ARDS may be a consequence of alterations in hepatic PEMT mediated PC synthesis. Traditional lipid quantification techniques using gas chromatography require several multi-step processes including separation, derivatisation and saponification, which are labour intensive and are not informative regarding molecular species. Stable isotope labelling with *methyl-D₉*-choline chloride combined with electrospray ionisation tandem mass spectrometry (ESI-MS/MS) enables assessment of the molecular specificity of PC synthesis via both PEMT and CDP:choline pathways (4). By quantifying the fractional enrichment of single, two and three deuterium labelled methylated PC species, it is possible to characterise *methyl-D₃*-S-adenosyl methionine enrichment (*methyl-D₃*-S-AMe) in liver with subsequent estimation of overall PC flux through PEMT pathway.

Materials and Methods

National ethics committee and University Hospital Southampton research and development approval was obtained (10/WNo01/52) (11/SC/0185). Non-smokers without any prior medical history were enrolled as controls. ARDS patients were identified according to the American European Consensus Conference diagnostic definition (11). This was based on the following criteria; degree of hypoxemia, bilateral infiltrates on chest radiograph in the absence of clinically raised left atrial pressure. Once assent was obtained, patients were enrolled within 72 hours of onset of ARDS. While patients did not receive a controlled diet, they all received enteral feeding (Nutrison™, Nutricia, Trowbridge, UK) through a nasogastric tube with 24 hours of admission to ICU. This provided a goal of achieving 25 kcal/kg body wt/day within 48 to 72 hours and aimed to provide 9.25 mg/kg body wt/day of enteral choline by this time and 5 and 3.4 mg/kg body wt/day respectively of the n-3 polyunsaturated fatty acids eicosapentaenoic and docosahexaenoic acids by this time. Both patients and controls were infused with 3.6mg/kg *methyl-D₉*-choline chloride (Cambridge Isotopes) over 3 hours. Blood samples were collected in ethylenediaminetetraacetic acid specimen bottles at 0, 6, 12, 24, 48, 72 and 96 hours for patients, and at 0, 8, 24, 48, 72 and 96 hours for healthy controls after *methyl-D₉*-choline chloride infusion. The collected samples were then centrifuged at 400×g×15 minutes at 20°C. The plasma supernatant was aspirated and stored at -80°C.

Phosphatidylcholine analysis

Internal standards (Avanti Polar Lipids) of 10nmol of dimyristoyl- PC (PC14:0/14:0), 1nmol of LPC17:0 and 50pmol of D₄-choline were added to 100µl of plasma. Phospholipid fraction was extracted by chloroform: methanol: water (v/v2:2:1) (12). The lower chloroform rich layer was dried under nitrogen gas at 37°C and was directly infused through mass spectrometry with an electrospray interface after dissolving with methanol: butanol: H₂O: 25% NH₄OH (6:2:1.6:0.4 v/v) at a rate of 8µl/min. Collision induced decomposition resulted in protonated

phosphocholine head group with m/z of +184 for PC, m/z +187 for the incorporation of one deuteriated-*methyl*-PC group, m/z +190 two and m/z +193 for three deuteriated-*methyl*-PC groups. Application of specific precursor ion scans (P184, P187, P190 and 193) resulted in quantification of endogenous and one, two and three deuterated-*methyl*-PC molecular species compositions and concentrations and hence enrichment.

Metabolite analysis

Plasma choline and betaine were analysed from the upper aqueous phase of the lipid extraction after removal of the lower dichloromethane-rich organic layer. Aqueous phase (2 ml) was freeze dried and subsequently reconstituted with 100 μ l of 1:1 mixture of acetonitrile and water. Separation was achieved by a Acquity UPLC-Xevo TQ triple quadrupole mass spectrometer system (Waters, Withenshaw, UK) using a BEH-Hilic column (2.1mm \times 100mm \times 1.7 μ m) with a column temperature of 30°C. The flow rate was 200 μ l/min with a starting mobile phase composition of 1% solvent A (ammonium formate 0.5M/L, pH 4.1 and H₂O) and 99% solvent B (0.5% formic acid in acetonitrile). A linear gradient to 98% solvent A at 4.1 minutes was followed by re-equilibration to starting conditions at 9 minutes. MS/MS was used for the mass determination in positive ionisation MRM mode with following conditions; source temperature 150°C, capillary voltage 3.8kV, cone voltage 50V, collision energy 28eV, desolvation gas (N₂) flow of 800L/h at 200°C, collision gas (Ar) flow 0.15L/min, interscan delay 0.02s and dwell time 0.03s. Labelled and unlabelled fraction of choline and betaine was quantified by multiple reaction monitoring (MRM) mode using m/z transitions 103.8 \rightarrow 59.8 (choline), 112.9 \rightarrow 68.9 (D₉-choline), 117.9 \rightarrow 58.9 (betaine) and 126.9 \rightarrow 67.9 for D₉-betaine.

Plasma cholesterol and triacylglycerol analysis

Plasma lipid profile (HDL-Cholesterol, LDL-Cholesterol and triacylglycerol) was quantified by automated Konelab 20 Autoanalyser at NIHR Southampton Biomedical Research Centre. Lipotrol (ThermoFisher) was used as quality control sample.

Data analysis and statistics

The PC ion peaks were quantified using Masslynx software and dedicated in-house Excel spread sheets programmed in Visual Basic. Molecular compositions of major PC species for ion peaks were identified in separate analyses by MS/MS fragmentation of $[M-16]^-$ ions in negative ionisation and are presented as, for example, PC16:0_20:4, making no assumption about positional distributions of acyl groups (results not shown). Choline and betaine concentrations were calculated by Quanlynx software, corrections for recovery using the D₄-choline internal standard. The data are presented as mean \pm standard error of mean (SEM). Comparison of groups was made by unpaired two tailed student's T-tests or two-way analysis of variance for multiple comparisons using Graph Pad Prism (Version 5.04).

Results

Demographics

Ten healthy volunteers who are non-smokers without any prior medical problems and ten patients with severe ARDS ($PaO_2/FiO_2 < 100$ mmHg) were recruited. Patients were older than the control group ($P < 0.0001$). Choline dose infused per weight was similar between groups ($P = 0.865$). The subjects' characteristics are listed in Table 1.

Plasma lipids, choline and PC

Plasma choline concentration remained relatively constant over the study period, without any significant difference between patient and control groups. Infusion of *methyl*-D₉-choline chloride did not alter total choline concentration for either group (Figure 1A). Plasma PC concentration at enrolment tended to be lower in patients (1.45 ± 0.13 mmol/l) than in controls (1.94 ± 0.19 mmol/l) and remained persistently lower throughout the investigative period, but the difference was only significant at certain time points (0, 6, 72 and 96 hours) after *methyl*-D₉-choline chloride infusion (Figure 1B). In contrast, there were significant reductions for

patient compared with control groups in baseline values of total cholesterol ($P<0.0001$) (Figure 1C), HDL-cholesterol ($P<0.0001$) (Figure 1D) and LDL-cholesterol levels ($P<0.0001$) (Figure 1E). However, triacylglycerol concentrations were similar between both groups ($P=0.736$) (Figure 1F).

In contrast to the relatively small difference in total plasma PC concentration, ARDS patients exhibited highly significant deficits of selected individual PC species. For instance, plasma concentrations of PC16:0_18:2, PC18:0_18:2, PC18:1_18:2 and PC16:0_20:4 were decreased in the patient group by respectively 42%, 34%, 47% and 36%, while concentrations of the monounsaturated species PC16:0_18:1 and PC18:0_18:1 were unchanged (Figure 2). Patients had significantly lower concentrations of total di-unsaturated and polyunsaturated PC species with a slight non-significant increased concentration of total monounsaturated PC species (Figure 2B-E).

Methyl-D₉-Choline and methyl-D₉-betaine enrichment

Following *methyl-D₉*-choline infusion, the enrichment of label in plasma choline was similar at the earliest time point for patients (9%) and controls (8%) and was $<2\%$ of total plasma choline for both groups by 96 hours. Despite these similarities, there was a more rapid decay of labelled choline in patients ($t_{1/2}=7$ hours) compared to controls ($t_{1/2}=34$ hours) ($P<0.05$), indicating an increased turnover of plasma choline presumably to meet increased metabolic requirements of the patient group. Choline is oxidised to betaine in the liver and betaine enrichment followed a similar pattern to that of choline. Conversion of choline to betaine was rapid, such that label enrichments were identical at the earliest time point for both groups, there was again a trend for a more rapid decay of *methyl-D₉*-betaine in patients ($t_{1/2}=7$ hours vs 16 hours; not significant) and enrichment was $<2\%$ after 96 hours in both groups (Figure 3).

Methyl-D₉, methyl-D₃ and methyl-D₆ phosphatidylcholine enrichment

Unlabelled PC composition (P184) was monitored by precursor scan of m/z 184 (P184) and enrichments of one-*methyl* (D₃) and two-*methyl* (D₆) groups, synthesised by the PEMT pathway, and of three-*methyl* groups (D₉), synthesised by the CDP-choline pathway (Figure 4), were determined by precursor scans respectively of m/z 187 (P187), m/z 190 (P190) and m/z 193 (P193). There is a theoretical possibility of three *methyl*-D₃ groups being incorporated into PC by the PEMT pathway, but this would be indistinguishable from direct incorporation of *methyl*-D₉-choline and would be statistically negligible. In healthy controls, *methyl*-D₉-PC enrichment was 0.2% of total PC at the earliest time point and subsequently increased to a maximum value at 24 hours of 0.51% of total PC. The enrichment pattern of *methyl*-D₉-PC in patient's plasma was identical to that of controls, but with a significantly higher proportional enrichment at and after 24 hours (Figure 5A).

In contrast to this increased enrichment of the *methyl*-D₉-choline in patients' plasma PC, enrichments of the *methyl*-D₃ and *methyl*-D₆ labels were significantly lower compared to the healthy controls. The maximal enrichment of *methyl*-D₃-PC was 0.13% of total PC for the patient group compared with 0.32% for the control group ($P < 0.001$) (Figure 5B). Time courses of label incorporation were comparable for both groups and delayed compared with *methyl*-D₉ incorporation, being maximal at 48 hours. The maximal *methyl*-D₆-PC enrichment was 0.0014% of total PC for the patient group compared to 0.0063% for the control group (Fig 5C). The plateauing of *methyl*-D₃ and D₆ enrichment from 24 hours is more likely suggestive of breakdown of *methyl*-D₉ labelled PC than slow *methyl*-D₃ or D₆ incorporation. The lower *methyl*-D₃ and *methyl*-D₆ enrichments in patients indicate that the overall flux through the PEMT pathway was significantly reduced compared to healthy controls.

Quantification of methyl-D₃-S-Adenosyl-Methionine enrichment (SAME)

S-adenosylmethionine is the principle methyl-donor for various catalytic reactions including phosphatidylcholine biosynthesis in the liver via PEMT pathway (Figure 4). Direct assessment of dynamic biosynthesis of SAME requires hepatic tissue sampling and is not feasible to study in humans *in-vivo*. For this reason, hepatic *methyl-D₃-SAME* enrichment was estimated from the plasma incorporation results using precursor scans of P187 for one methyl group and P190 for two methyl groups from $\text{SAME} = \text{P190} / \Sigma(\text{P187} + \text{P190})$. The derivation of this calculation for the quantification of hepatic *methyl-D₃-SAME* enrichment has been discussed elsewhere (4). In controls, *methyl-D₃-SAME* was maximal at the initial time point (2.92%) and subsequently showed an exponential decay with a half-life of 18.3 hours. Although in patients the *methyl-D₃-SAME* enrichment was also maximal (4.98%) at the earliest time point, there was a rapid decline with much shorter half-life of 3.4 hours compared to the healthy controls (Figure 5D; $P < 0.05$). This suggests that the *methyl-D₃-SAM*, which is an intermediary for several synthetic pathways is rapidly metabolised in patients compared to controls.

Molecular specificity of Methyl-D₉-PC, Methyl-D₃-PC and Methyl-D₆-PC enrichment

The profiles of label incorporation by both the CDP: choline and PEMT pathways masked a considerable variation in terms of individual molecular species and between patients and control. While the rank order of enrichment of individual species by both pathways were similar for patients and controls, there were significant differences both in maximal enrichments achieved and in the kinetics of label incorporation. The individual species identified in Figure 6 were selected as representative of general patterns of incorporation. As reported previously for human volunteers (4), there was preferential incorporation of *methyl-D₉* choline into palmitoyl-containing PC species, with maximal enrichments of PC16:0_18:1,

for instance, observed at 24 hours for controls ($0.77 \pm 0.04\%$) and patients ($0.99 \pm 0.08\%$) (Fig 6 A and B). By contrast, label incorporations into stearyl-containing PC species (PC18:0_20:4, PC18:0_22:6) were both lower and delayed, especially for patients for whom these enrichments were maximal at 48 hours. This result was consistent with previous studies that reported initial hepatic synthesis of palmitoyl-containing PC species by the CDP: choline pathway, followed by subsequent acyl remodelling into stearyl-containing species (4).

In direct contrast to the profile of *methyl*-D₉-choline incorporation, incorporations into both *methyl*-D₃-PC and *methyl*-D₆-PC showed very different molecular specificities (Fig 6 C-F). This was most apparent for *methyl*-D₃ incorporation into a group of PUFA-containing species (PC16:0_22:6, PC18:0_20:4, PC18:0_22:6) with maximal enrichments at 24 hours (Fig 6D), with incorporations into the other three species being lower and maximal at 72 hours. We interpret this result as initial incorporation of *methyl*-D₃ by the PEMT pathway into PUFA-containing PC species, followed by recycling of the labelled choline and its re-incorporation into the second groups of species by the CDP: choline pathway. This distinction between pathways was also apparent for patients, although *methyl*-D₃-PC and *methyl*-D₆-PC enrichments of the PUFA-containing species were both decreased and delayed for the patient compared with the control group (Fig 6C and E).

Fractional PC synthesis by PEMT pathway

The total PC synthesis through the PEMT pathway was estimated by using values of *methyl*-D₃-SAM enrichment corrected for the corresponding *methyl*-D₃-PC enrichment (Figure 7). In controls, the maximal PEMT mediated PC synthesis was $23.2 \pm 2.2\%$ at 96 hours. The total rate of synthesis was 0.3%/h up to 8 hours and was maximal at 24 hours 0.56%/h. In patients, the PC synthesis through PEMT pathway was globally reduced for all species. The maximal total PC synthesis via PEMT pathway was $17.28 \pm 4.47\%$ at 96 hours. This is an absolute reduction

of 25% in the PC synthesis by PEMT pathway in ARDS patients. In controls, the fractional PC synthesis via PEMT pathway was highest for PC18:0_22:6 (77% of total PC at 48 hours) followed by PC16:0_22:6 (53% of total PC at 48 hours). Mono and di-unsaturated PC species showed a slow steady increase in fractional PC synthesis (Figure 8).

Lysophosphatidylcholine composition and molecular specificity of Methyl-D₃PC and Methyl-D₆PC labelling

The concentration of lysophosphatidylcholine (LPC) relative to PC was relatively high in plasma (controls $4.8 \pm 0.18\%$, patients $4.3 \pm 0.11\%$), in contrast to most tissues where LPC amounts are low to minimise its lytic potential. The composition of LPC reflected that of plasma PC, with the fractional concentration of LPC18:1 being increased in patients by 40% and that of LPC18:2 being decreased by 20% (**Fig. 9A**). Our results support animal studies that suggest circulating LPC is actively secreted from the liver rather than being generated by extra-hepatic activity of phospholipase A₂ (13). *Methyl-D₉* and *methyl-D₃* total LPC fractional enrichments (Fig 9) followed similar temporal patterns to those of plasma PC (Fig 5A), with comparable maximal values at 24h and 48h respectively. Similar to the plasma PC synthesis results, incorporation into total LPC of the *methyl-D₉* label was higher and that of the *methyl-D₃* label lower in the patient compared with the control group. This result suggests that enrichment of LPC is in equilibrium with that of PC, without the delay that would have been inherent in any significant extra-hepatic hydrolysis. The incorporation patterns of labels into individual molecular species of LPC exhibited a more rapid and greater enrichment of *methyl-D₉* in the 16:0, 18:1 and 18:0 species and significantly higher enrichment of *methyl-D₃* in LPC22:6 (Fig 10).

Discussion

ARDS is a multi-organ disease process characterised by a severe form of hypoxic respiratory failure and the ARDS patients in this study were recruited on the basis of their respiratory not hepatic symptoms, with only one patient reported with severe liver failure. The decreased concentration of plasma cholesterol in the patient group (Figure 1) was comparable to previous reports of patients with systemic inflammation and sepsis (14-16), which also showed decreased concentrations of HDL-C and LDL-C with maintained concentrations of VLDL and triacylglycerol. The underlying mechanism for this negative temporal association is not clear. In contrast to the cholesterol results, plasma total PC concentration was only marginally decreased throughout the study in the ARDS patient group, suggesting that PC synthesis and secretion was a relatively well-maintained hepatic function in this condition. The lack of change to plasma choline concentration between patient and control groups was expected as this metabolite is under tight homeostatic control in the relatively short term except in response to severe nutritional deprivation (17-19).

This is the first study to demonstrate selective alterations to the composition of plasma PC molecular species in patients with ARDS (Figure 2A), the details of which suggest nutritional restriction of unsaturated fatty acids. For instance, monounsaturated fatty acids such as oleate can be synthesised *de novo*, and consequently plasma concentrations of oleate-containing PC species were unchanged in ARDS patients (Figure 2C). By contrast, n-6 and n-3 fatty acids containing two or more unsaturated double bonds cannot be synthesised *de novo* in the body and plasma concentrations of virtually all PC species containing these fatty acids were decreased in ARDS patients. The marked decrease in linoleate-containing PC species in ARDS patients (Figure 2B) is consistent with previous fatty acid analyses of diminished plasma phospholipid linoleic acid concentration in ARDS patients (9). Our results do not support the

suggestion in this study that the decrease was due to increased lipid peroxidation, resulting from oxidative stress during systemic inflammation as we did not observe accumulation of either long or short-chain PC products of lipid peroxidation in the mass spectrometry analysis ((20), results not shown). Additionally, the extent of the decreased concentration was selective depending on the precise molecular species composition, which would not have been expected if this was due solely to peroxidation of plasma PC. For instance, Figure 2A details greater decreased concentrations of unsaturated PC species containing palmitate at the *sn*-1 position (PC34:2, PC16:0_18:2; PC36:4, PC16:0_20:4; PC38:6, PC16:0_22:6) than of their stearate-containing counterparts (PC36:2, PC18:0_18:2; PC38:4, PC18:0_20:4; PC40:6, PC18:0_22:6). This pattern of change strongly suggests a metabolic origin, as the CDP:choline pathway in the liver preferentially synthesises *sn*-1-palmitoyl-PC species (21, 22).

In contrast to the unchanged plasma concentration of choline (Figure 1A), turnover of *methyl*-D₉-choline (Figure 3A) and its oxidation product *methyl*-D₉-betaine (Figure 3B) were both significantly increased in ARDS patients, with very similar temporal kinetics. This observation demonstrates the extent of the homeostatic regulation of plasma choline concentration even with increased turnover. As no additional choline was provided in the diet above recommended daily requirement of 550mg/day, constant choline concentration in the face of increased turnover implies release of unlabelled choline from tissue stores, probably from hydrolysis of PC in muscle and other tissues. Such a metabolic adaptation to maintain constant the concentration of a metabolite essential for preservation of neuronal activity may be one contributory cause to the rapid and extensive loss of muscle tissue in ARDS patients (23).

The increased fractional enrichment of *methyl*-D₉-choline incorporation into total plasma PC in ARDS patients (Figure 5A) further suggests that PC synthesis *de novo* by the CDP:choline is a preserved pathway in the liver under these conditions of metabolic stress. Increased synthesis and secretion of plasma PC (Figure 5A) combined with decreased plasma PC concentration (Figure 1B) possibly indicates minimal disruption of plasma PC secretion in ARDS patients compared with healthy controls. As rates of secretion and catabolism must be balanced at an equilibrium concentration, this suggestion is supported by the lack of increased catabolism and turnover of plasma PC in patients (Figure 5A). Alternatively, increased uptake of *methyl*-D₉-choline into the liver in patients, resulting in increased enrichment of label in the hepatic choline metabolite pool supporting PC synthesis, could explain both the rapid turnover of *methyl*-D₉-choline (Figure 3A) and increased enrichment of *methyl*-D₉-choline PC in plasma from ARDS patients. This possibility could not be addressed in the current study in the absence of liver biopsy. Interestingly, however, we have previously reported enrichment of *methyl*-D₉-choline label in surfactant PC from lung lavage samples from the same ARDS patients, combined with significantly decreased total PC recovery (2). The similarity of results from two different organs, liver and lungs, where synthesis and secretion of PC is a major function, supports the concept that one mechanism to preserve this critical pathway may be tissue sequestration of plasma choline to support PC synthesis.

In contrast to the CDP:choline pathway, enrichment of plasma PC with the *methyl*-D₃ (Figure 5B) and *methyl*-D₆ (Figure 5C) demonstrates no comparable preservation of the PEMT pathway in the livers of ARDS patients. PEMT is an intracellular organelle membrane bound enzyme that catalyses sequential methylations leading to the conversion of PE to PC, and accounts for about 30% of PC production in control hepatocytes. Importantly, it is the only mechanism for synthesis of choline in the body (3), and decreased PEMT activity indicates an

increased reliance on dietary choline intake. Such reduced choline synthesis is another potential contributor to the increased plasma choline turnover in ARDS patients (Figure 3A). The importance of the PEMT pathway has been studied in detail in a variety of mouse models, including the PEMT^{-/-} mouse (10, 24) and mice fed a choline/methionine deficient diet (25). These animal studies highlight not only the significance of PEMT activity in states of choline deprivation, but also provide supporting evidence for the requirement of this pathway for VLDL secretion and the pathophysiological consequence of steatohepatitis.

To our knowledge, the quantification of *methyl*-D₃-SAME enrichment, based on the MIDA analysis of the *methyl*-D₃ and *methyl*-D₆ PC enrichments (4), is the first report of increased hepatic SAME turnover *in vivo* in a disease state characterised by metabolic dysregulation (Figure 5D). Whereas previous studies have reported plasma SAME concentration as a surrogate for its concentration in liver (26), estimation of metabolite flux in liver is a more sensitive indicator of SAME status. Although direct analysis of liver SAME concentration was not feasible in the current study, our observation has considerable implications for the whole range of methylation reactions in the body in addition to PEMT activity.

Analysis of the molecular specificity of plasma PC synthesis by CDP:choline and PEMT pathways in healthy volunteers confirms our previous report (4). *Methyl*D₉-choline was incorporated directly initially into *sn*-1 palmitoyl species, followed by a slower incorporation into *sn*-1 stearoyl species (Figure 6B). Conversely, turnovers of *sn*-1 stearoyl species were considerable slower than those of *sn*-1 palmitoyl species. Plasma PC synthesised by the CDP:choline pathway in ARDS patients followed very similar patterns in terms of individual molecular species (Figure 2A). Intriguingly, these dynamic changes differ in detail from the compositional analyses presented in Figure 2, with no enhanced incorporation into

PC16:0/18:1 and relatively comparable incorporations into unsaturated PC species. Moreover, the pattern of synthesis of plasma PC species by the PEMT pathway was preserved in ARDS patients (Figures 2C-F), despite their lower rate of label incorporation (Figures 5B, C). Incorporation of the *methyl*-D₃ and *methyl*-D₆ labels was bi-phasic for both ARDS patients and healthy controls. This incorporation pattern was consistent with an initial synthesis of selected PUFA-containing PC species (PC18:0_22:6, PC16:0_22:6, PC18:0_20:4) by the PEMT pathway, followed by plasma PC recycling back to the liver, release of *methyl*-D₃-choline and *methyl*-D₆-choline and their subsequent delayed incorporation into *sn*-1 palmitoyl species (PC16:0_18:1, PC16:0_18:2, PC16:0_20:4) characteristic of CDP:choline pathway synthesis (Figures 5A, B). This molecular selectivity for the initial synthesis of polyunsaturated PC species by the PEMT pathway is consistent with the major contributions of PE38:4, PE38:6, PE40:6 and PE36:4 reported for liver PE from healthy volunteers (27). The altered molecular specificity for the CDP:choline pathway in the context of unchanged specificity for the PEMT pathway is probably due to differential compositional modulation of their respective substrate pools of diacylglycerol (DAG). Although all located on the endoplasmic reticulum, the enzymes for the CDP:choline and PEMT pathways are spatially segregated, the former being associated with the Golgi and the latter with the mitochondria (28). We have shown previously, by incubation of isolated rat liver endoplasmic reticulum with either CDP:¹⁴C-choline or CDP:¹⁴C-ethanolamine, that compositions of DAG for synthesis of PC and PE are also distinct and differentially regulated (22). Consequently, it is likely that the composition of the DAG substrate pool for CDP:cholinephosphotransferase, the last enzyme of the CDP:choline pathway, was altered in the ARDS patients with no change to the composition of the DAG pool that determined the composition of the mitochondrial microsomal PE used for PC synthesis by PEMT. The decreased PEMT flux in the patient group may possibly be due to a decreased ratio of PE to PC, which is reported to regulate PEMT activity in rat liver (29).

The plasma LPC composition generally reflected acyl remodelling mechanisms inherent in hepatic PC synthesis and secretion, containing fatty acids characteristic of both *sn*-1 and *sn*-2 positions (Figure 9A), with decreased LPC18:2 and increased LPC18:1 in ARDS patients. The dynamic flux of LPC synthesis (Figure 9B) was very similar to that of PC (Figure 2), with enhanced incorporation *methyl*-D₉-choline into plasma PC of ARDS patients, with decreased incorporation of *methyl*-D₃-choline. The suggestion of secretion of 20:4-containing LPC species by rabbit liver for selective transport to peripheral tissues is not supported by our results, which again showed patterns of label incorporation into plasma LPC (Figure 10) that broadly mirrored the synthetic patterns of the intact PC species in plasma rather than selective enhanced labelling of LPC20:4.

Our findings suggest that patients with ARDS had significantly lower concentrations of total plasma PC, particularly PC16:0_18:2 and PUFA-based species, with a global reduction in total PC flux through PEMT pathway. This could be due to several reasons. Firstly, ARDS patients tend to have multi-organ involvement including hepatic dysfunction and this reduction in synthesis may be an indication of the degree of hepatic synthetic dysfunction endured. However, only one patient in our study had clinically overt liver failure and this patient had dramatically reduced PEMT PC synthesis. Other patients with reduced PEMT PC synthesis had no significant evidence of liver enzyme abnormalities in their plasma. We can hypothesise that this reduction in PEMT activity may reflect early hepatic synthetic dysfunction prior to biochemical evidence of liver damage. Other possibility is the lack of available SAmE for optimal hepatic PC synthesis. The *methyl*-D₃-SAmE enrichment in patients was higher at the initial time point and showed a rapid decline until 24 hours followed by a steady state decay, which was lower than that of controls, indicating that

SAMe may be prioritised to maintain methyl balance or used for other methyl dependant pathways instead of PEMT-mediated hepatic PC synthesis. While no direct evidence is available that increased hepatic SAMe turnover contributes to the pathogenesis of ARDS, increased SAMe-dependent histone methylation is an important component of the inflammatory response in sepsis and cancer (30). Alternatively, enhanced SAMe turnover could have been partly due to decreased synthesis, and inhibition of liver SAMe synthetase has been reported in an endotoxic rat model of septic shock (31).

Studies investigating phospholipid biosynthesis and molecular variations between these synthetic pathways are lacking and are not fully investigated in humans. This is due to the lack of accessibility of human hepatic tissue and methodological limitations in determining *in-vivo* PEMT activity. Since hepatic PC synthesised from PE by PEMT activity is predominantly polyunsaturated, plasma PC-DHA concentration has been proposed as a surrogate for hepatic PEMT activity (32). In our study, however, albeit of limited cohorts of subjects, there was no correlation between concentration of DHA-containing PC species and PEMT flux (results not shown). Stable isotope labelling of choline with deuterium and analytical methods using tandem mass spectrometry (MS/MS) has enabled assessment of hepatic PC molecular synthetic patterns in isolated rat primary hepatocytes and more recently in healthy human volunteers (4), cystic fibrosis patients (33) and pregnant women (34). The study of pregnant women used continuous oral *methyl-D₉* choline chloride labelling as opposed to our pulsed infusion. Pulsed infusion of stable isotopes has the advantage of providing information about metabolism of individual molecular species of PC, enabling characterisation of the molecular specificity of PEMT activity. In conclusion, this is the first study to apply this dynamic labelling methodology to investigate PC molecular synthetic patterns in a specified disease cohort characterised by systemic inflammatory response and severe hypoxic respiratory failure.

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Tables

	Patients	Controls	P value
Age	61±5	26 ±2	<0.0001
M:F	5:5	6:4	
Weight (Kg)	76±8	78±4	P=0.798
Choline infused (mg)	271±30	277 ± 15	P=0.865

Table 1: Patients and control characteristics. Data presented as mean ± SEM.

Plasma PC	Patients	Controls	Difference
PC16:0_18:2	0.344 ± 0.031	0.593 ± 0.058	Significant
PC16:0_18:1	0.321 ± 0.038	0.276 ± 0.033	Not significant
PC16:0_20:4	0.149 ± 0.018	0.233 ± 0.021	Significant
PC18:1_18:2	0.092 ± 0.008	0.174 ± 0.019	Significant
PC18:0_18:2	0.209 ± 0.026	0.316 ± 0.042	Significant
PC18:0_18:1	0.058 ± 0.010	0.051 ± 0.008	Not significant
PC16:0_22:6	0.050 ± 0.010	0.083 ± 0.007	Significant
PC18:1_20:4	0.038 ± 0.005	0.060 ± 0.006	Significant
PC18:0_20:4	0.095 ± 0.014	0.117 ± 0.013	Not significant
PC18:0_22:6	0.022 ± 0.005	0.024 ± 0.003	Not significant

Table 2: Fractional plasma PC levels at enrolment (mmol/L). Data presented as mean ± SEM. Students t- test, P value <0.05 is significant.

Figures

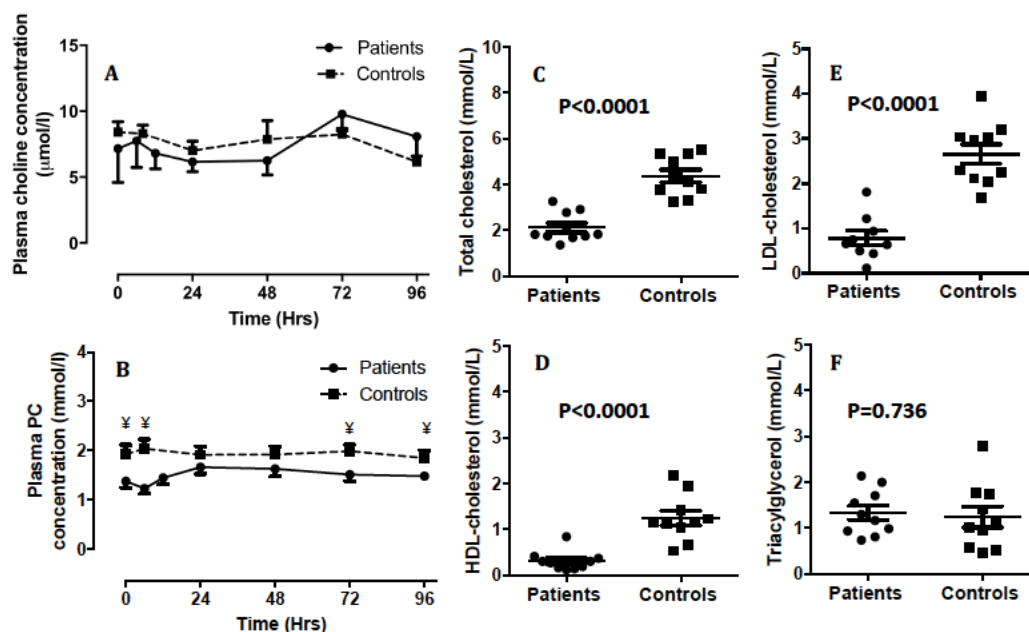


Fig. 1. Plasma concentrations (mmol/l) of (A) choline, (B) phosphatidylcholine(PC), (C) total cholesterol, (D) HDL-cholesterol, (E) LDL- cholesterol and (F) triacylglycerol between ARDS patients and healthy controls. Plasma choline and PC was measured for the duration of the investigative period (4 days). Cholesterol and triacylglycerol concentrations are at time t=0 on day 1. Error bars indicate standard error of mean. Statistical analysis unpaired Student's T- test with ¥- P<0.05.

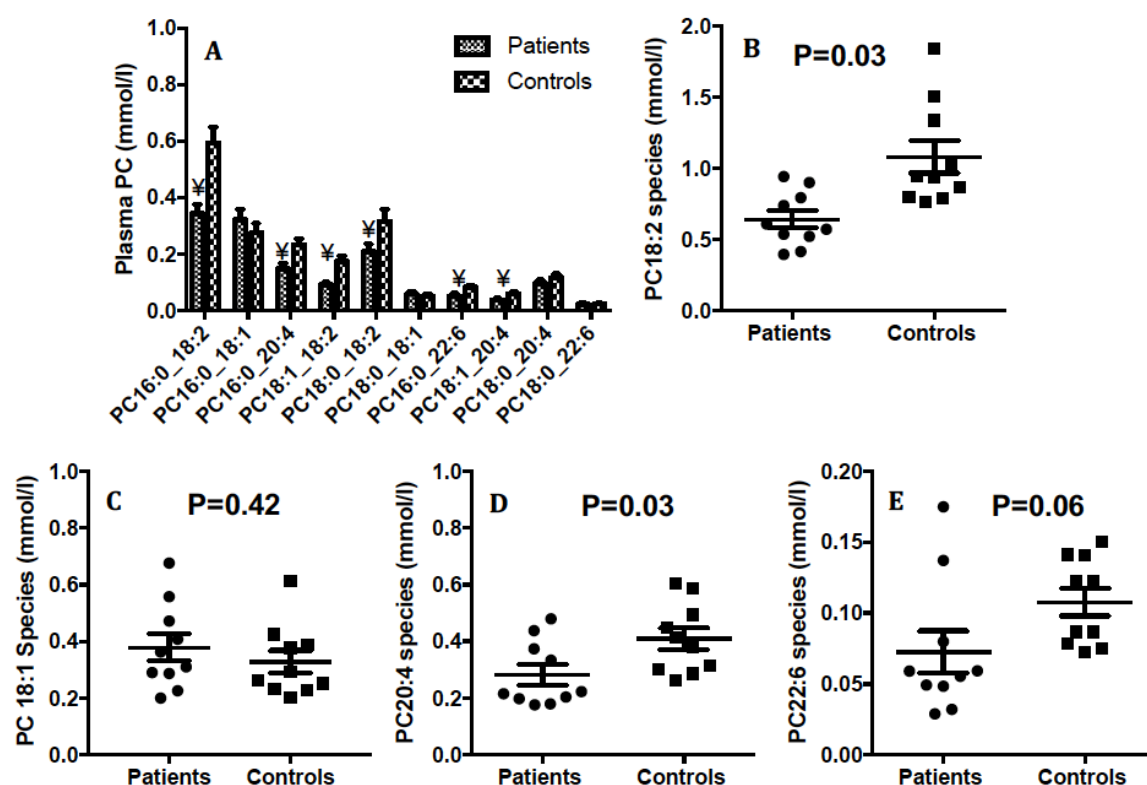


Fig. 2. Concentration of selected plasma PC molecular species at enrolment (t=0). (A) Mean distribution of PC species. Error bars indicate standard error of mean. Statistical analysis unpaired Student's T- test with \forall - $P < 0.05$. (B-E) Scatter plots to illustrate the variation and differences between patient and control groups for selected major ($>2\%$ presence) PC species containing 18:2 (B), PC18:1 (C), PC20:4 (D) and PC22:6 (E).

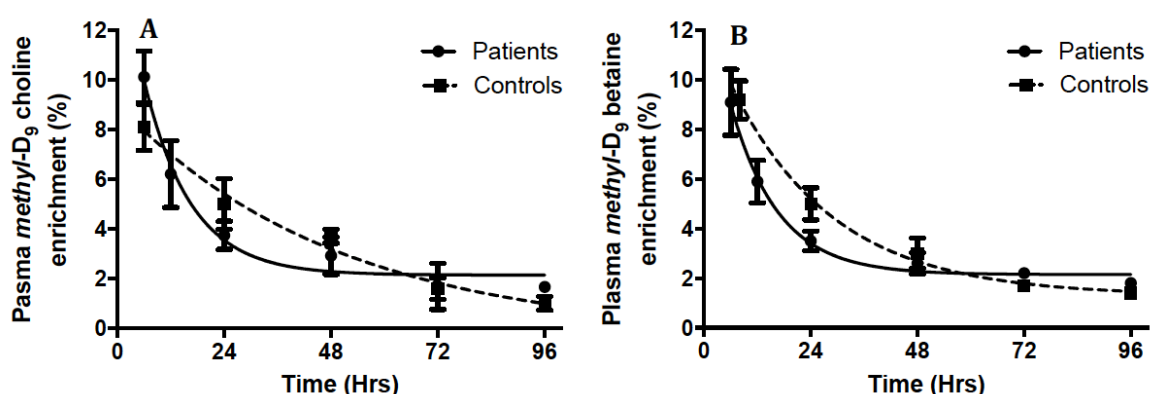


Fig. 3. Plasma *methyl-D₉*-choline (A) and *methyl-D₉*-betaine (B) enrichments over the total investigative period (4 days). Earliest measured *methyl-D₉*-choline enrichment, expressed as a percentage of total plasma choline, was 10% and 8% for patients and controls respectively, with a corresponding value of 9% for *methyl-D₉*-betaine from both groups. Turnovers of *methyl-D₉*-choline ($P < 0.05$) enrichment was faster for patient (solid line) compared with control (dashed line) groups (mean \pm SEM). A similar trend was observed for *methyl-D₉*-betaine although this was not statistically significant. Half-lives were calculated from exponential fits to the data.

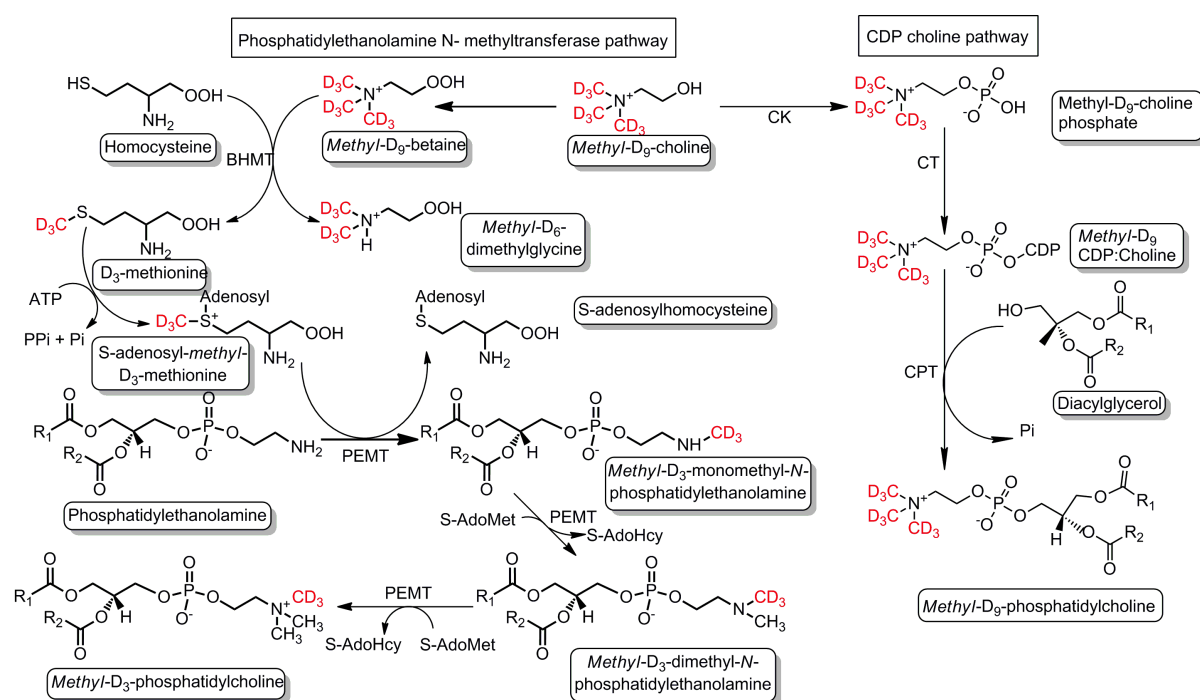


Fig. 4. Schematic illustration of phosphatidylcholine synthesis and incorporation of labelled *methyl-D₉*-choline via CDP: choline and phosphatidylethanolamine N-methyltransferase pathways. Deuterated methyl groups are highlighted as red. The fate of each deuterated methyl groups can be traced in individual PC species synthesized by both pathways. CK, choline kinase; CT, phosphocholinecytidyltransferase; CPT, choline phosphotransferase; PEMT phosphatidylethanolamine-*N*-methyltransferase.

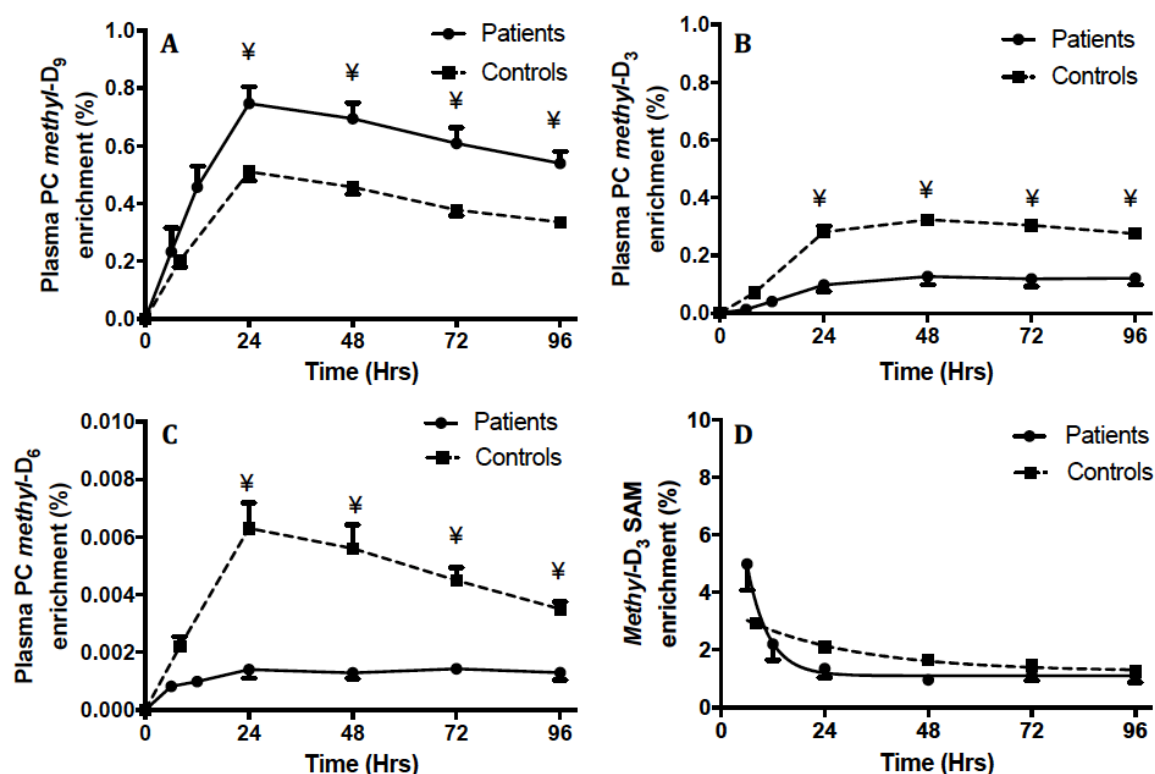


Fig. 5. Methyl-D₉-PC (A), methyl-D₃-PC (B), methyl-D₆-PC (C) and methyl-D₃-SAMe (D) enrichment between patients and controls. The PC enrichment was calculated as percentage of total plasma PC pool. Methyl-D₃-SAMe was estimated by $P190/\Sigma(P187+P190)$. Continuous line represents patients and dotted line corresponds to controls, mean \pm SEM, ¥-P<0.05. Half-lives for SAMe were calculated from exponential fits to the data and were significantly different (P<0.05) for patient (3.4 hours) and control (18.3 hours) groups.

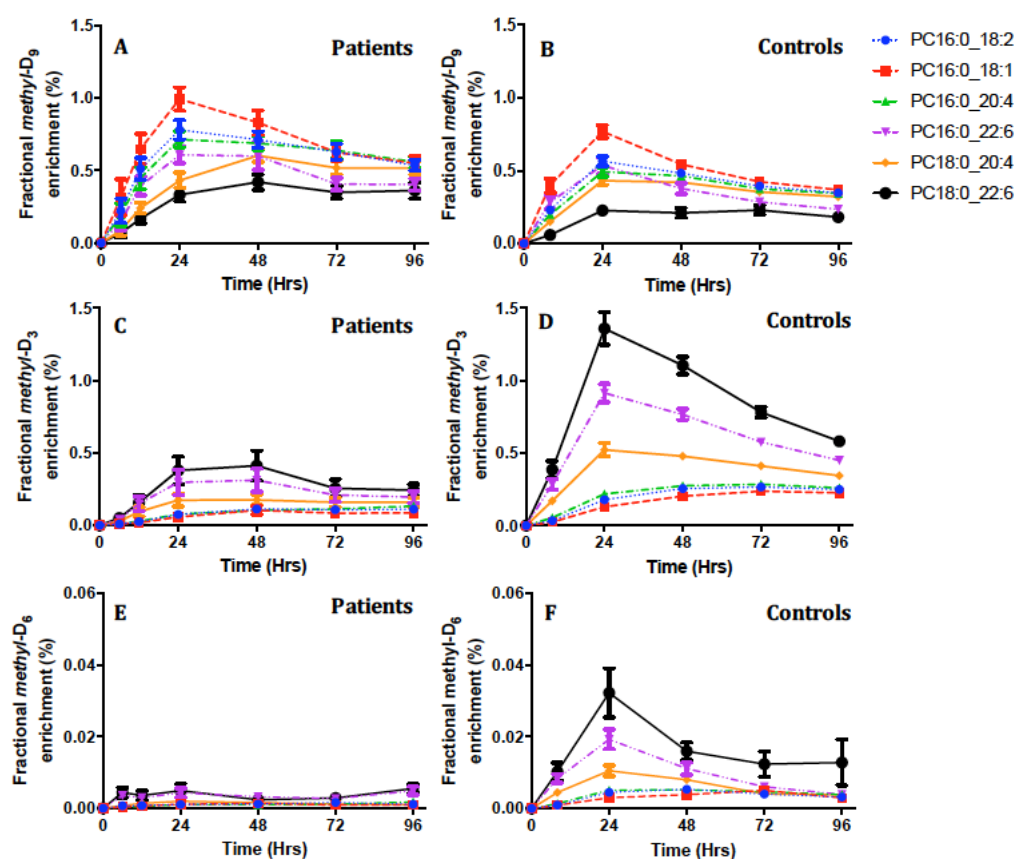


Fig. 6. Fractional methyl- D₉-PC (A, B), methyl-D₃-PC (C, D) and methyl-D₆-PC (E, F) enrichment for individual PC species. Results are presented as the labelled fraction in the total plasma PC pool for that individual PC species, compared between patients and control groups over the investigative period for 4 days. Each line colour represents individual PC species; blue, PC16:0_18:2; red, PC16:0_18:1; green, PC16:0_20:4; purple, PC16:0_22:6; yellow, PC18:0/20:4; black, PC18:0/22:6. (Mean \pm SEM).

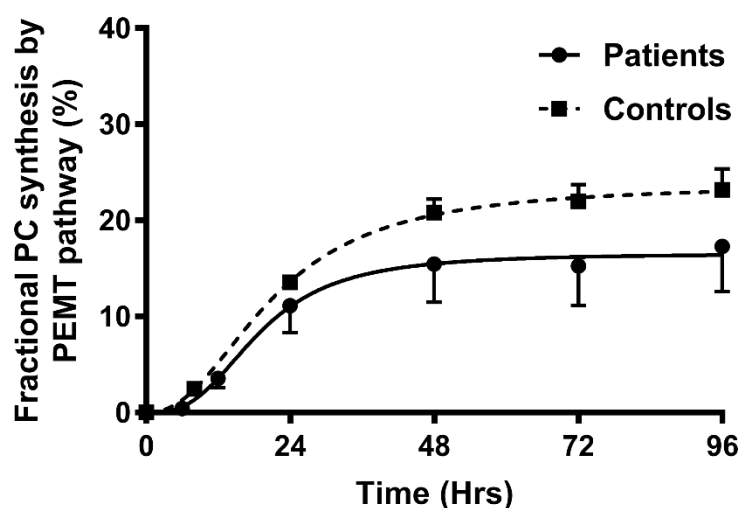


Fig. 7. Fractional synthesis of phosphatidylcholine via phosphatidylethanolamine N-methyltransferase pathway. This was estimated by using total *methyl*-D₃-SAME corrected for the corresponding *methyl*-D₃-PC enrichment and presented as the percentage of total PC synthesis. Continuous line represents patients and dotted line corresponds to controls, both calculated as sigmoidal curve fits ($P < 0.05$)

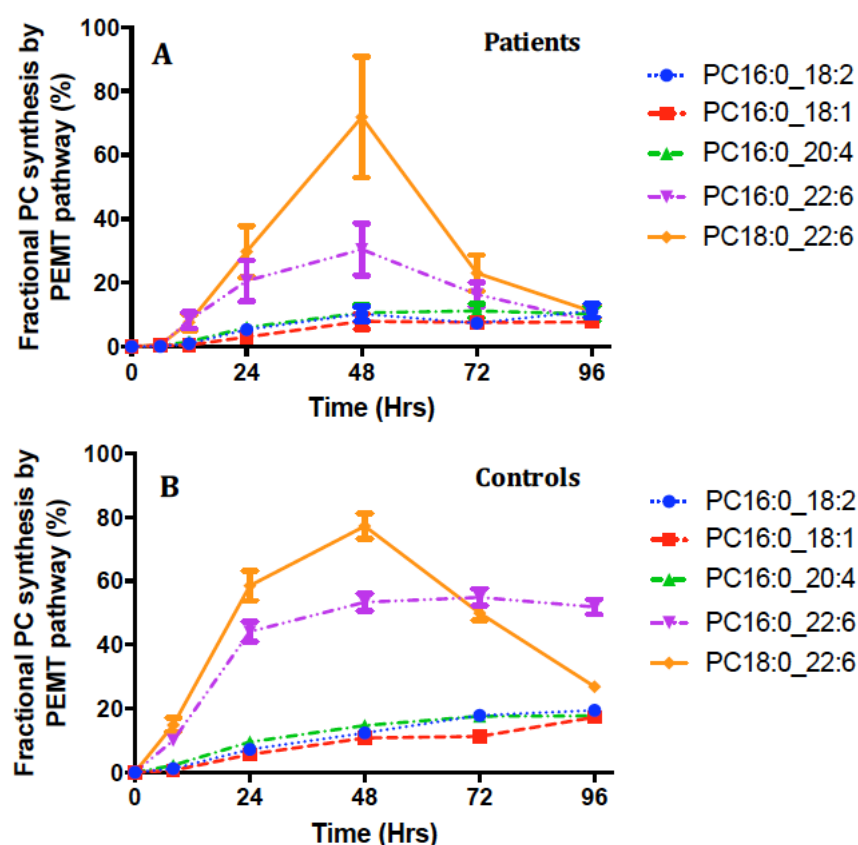


Fig. 8. Molecular specificity of PC fractional synthesis via PEMT pathway. This was estimated by using *methyl*-D₃-SAME corrected for the corresponding *methyl*-D₃-PC enrichment for that particular PC species of interest. Among the selected PC species, PC16:0_22:6 followed by PC16:0_22:6 were the major PC species synthesized by this pathway. Each coloured line represents individual molecular PC species; blue, PC16:0_18:2; red, PC16:0_18:1; green PC16:0_20:4; purple PC16:0_22:6 and yellow, PC18:0_22:6 (mean \pm SEM).

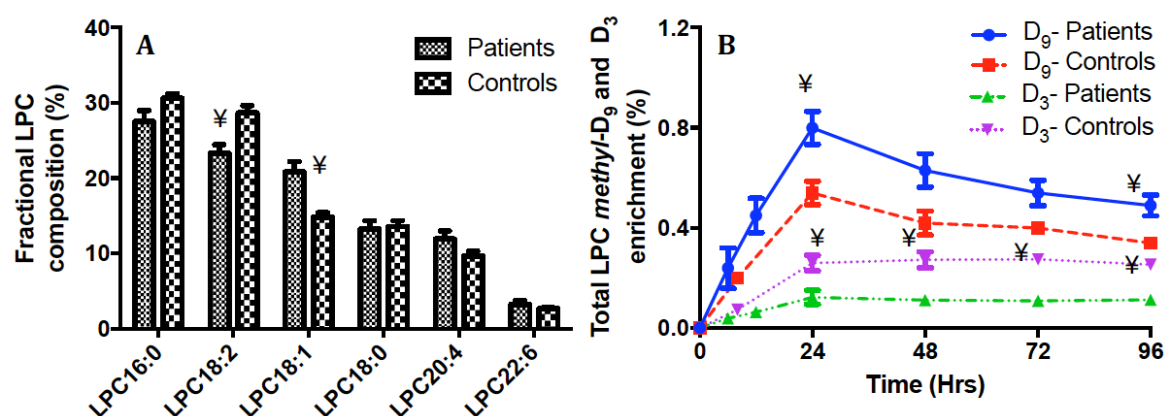


Fig. 9. Plasma lysophosphatidylcholine composition at enrolment (A) and *methyl-D₉*-LPC and *methyl-D₃*-LPC enrichments (B) over the investigative period (4 days) for ARDS patients and controls. Significant difference in LPC composition was noted for LPC18:2 and LPC18:1. The LPC enrichment was calculated as percentage of total plasma LPC pool. Blue, *methyl-D₉*-LPC enrichment for patients; red, *methyl-D₉*-LPC enrichment for controls; green, *methyl-D₃*-LPC enrichment for patients; purple, *methyl-D₃*-LPC enrichment for controls (mean \pm SEM, ¥- $P < 0.05$).

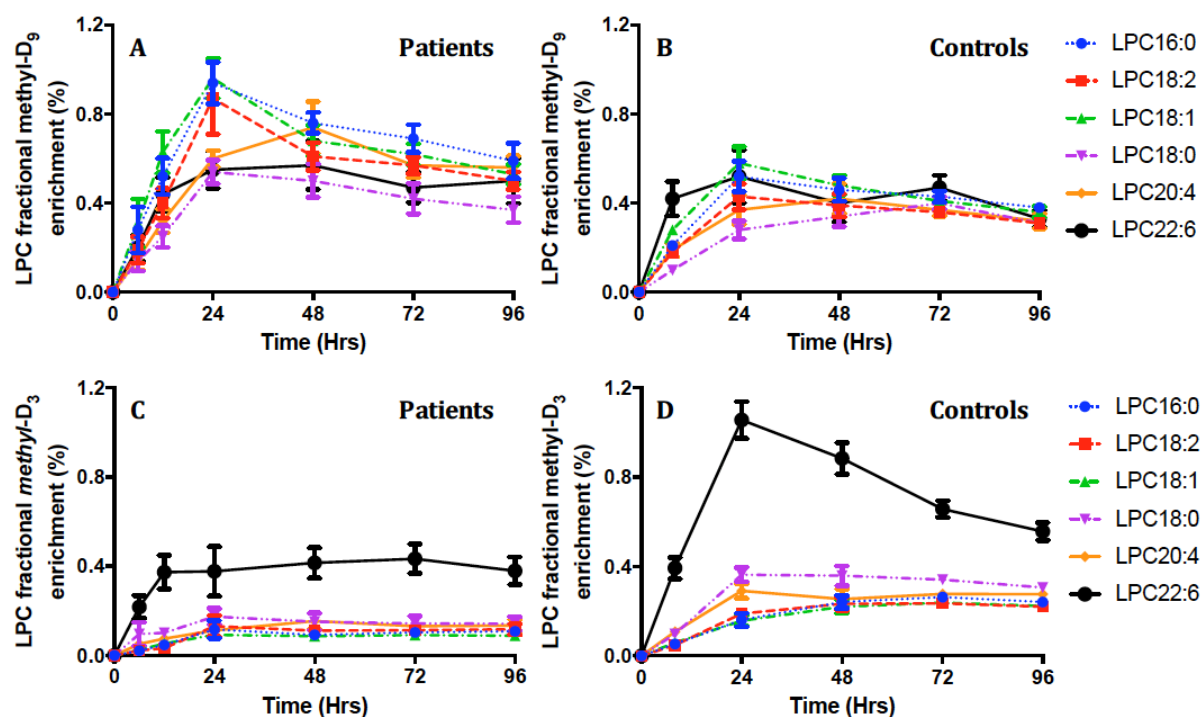


Fig.10. Molecular specificity of fractional *methyl-D₉*-LPC (A, B), *methyl-D₃*-LPC (C, D) enrichment for individual LPC species. Results are presented as the labelled fraction in the total plasma LPC pool for that individual PC species, compared between patient and control groups over the investigative period for 4 days. Each line colour represents individual LPC species; blue, LPC16:0; red, LPC18:2; green, LPC18:1; purple, LPC18:0; yellow, LPC20:4; black, LPC22:6 (mean \pm SEM).