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Multicentric investigations of the role in the disease severity of accelerated phospholipid changes in COVID-19 patient airway.

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Abstract

Context. The changes in host membrane phospholipids are crucial in airway infection pathogenesis. Phospholipase A2 hydrolyzes host cell membranes, producing lyso-phospholipids and free fatty acids, including arachidonic acid (AA), which contributes significantly to lung inflammation. **Aim.** Follow these changes and their evolution from day 1, day 3 to day 7 in airway aspirates of 89 patients with COVID-19-associated acute respiratory distress syndrome and examine whether they correlate with the severity of the disease. The patients were recruited in three French intensive care units. The analysis was conducted from admission to the intensive care unit until the end of the first week of mechanical ventilation. **Results.** In the airway aspirates, we found significant increases in the levels of host cell phospholipids, including phosphatidyl-serine and phosphatidyl-ethanolamine, and their corresponding lyso-phospholipids. This was accompanied by increased levels of AA and its inflammatory metabolite prostaglandin E2 (PGE2). Additionally, enhanced levels of ceramides, sphingomyelin, and free cholesterol were observed in these aspirates. These lipids are known to be involved in cell death and/or apoptosis, whereas free cholesterol plays a role in virus entry and replication in host cells. However, there were no significant changes in the levels of dipalmitoyl-phosphatidylcholine, the major surfactant phospholipid. A correlation analysis revealed an association between mortality risk and levels of AA and PGE2, as well as host cell phospholipids. **Conclusion.** Our findings indicate a correlation between heightened cellular phospholipid modifications and variations in AA and PGE2 with the severity of the disease in patients. Nevertheless, there is no indication of surfactant alteration in the initial phases of the illness.

1. Introduction

Clinical manifestations associated with SARS-CoV-2 infection range from asymptomatic to critically ill patients, with a mortality rate of approximately 2%. Most infected individuals exhibit limited symptoms. However, patients with specific risk factors, such as severe obesity, chronic lung, heart failure, diabetes mellitus, and immunosuppression, may develop more severe clinical patterns, such as respiratory failure, with a mortality rate approaching 50% [1, 2].

Alveolar epithelial cells expressing the ACE2 receptor are one of the primary cells targeted by aerosol-borne SARS-CoV-2 [3-5]. Infection with SARS-CoV-2 leads to fluid accumulation in the airways due to increased endothelial cell permeability and injury to type I and II alveolar cells. Furthermore, this infection causes an inflammatory immune response in the interstitial and alveolar space, impairs gas exchange. In the most severe cases, this can lead to acute respiratory distress syndrome (ARDS) [6]. ARDS is a common feature of COVID-19 and requires invasive mechanical ventilation [6]. The term “cytokines storm” describes the overwhelming and dysregulated immune response induced by SARS-CoV-2 infection, which is known to be involved in ARDS in lung infections and inflammatory diseases [6, 7]. The cytokine storm, a hyperactive immune response characterized by an excessive release of pro-inflammatory cytokines, has emerged as a crucial aspect of the pathogenesis of COVID-19. This dysregulated immune response can lead to severe lung inflammation and tissue damage and is believed to contribute to disease severity and mortality in affected individuals. The overproduction of cytokines such as interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-alpha), and interleukin-1-beta (IL-1-beta), among others, results in a cascade of events that further exacerbate the inflammatory response, leading to a self-perpetuating cycle of immune activation. This deregulation of cytokine expression is associated with the deregulation of lipid mediators, but this aspect has

been little studied in the airways of COVID-19 patients [8]. Although the features of respiratory failure observed in COVID-19 can meet the consensual definition of ARDS, only some of these features are consistent with those of typical ARDS [9].

The local concentrations of chemoattractants, such as chemokines [7] and leukotrienes [10, 11], which belong to the family of lipid mediators (LMs), provide crucial insight into the pathophysiological features of the disease. This is especially relevant as these mediators may be involved in infiltrating lung leukocytes, a critical process in lung inflammation among COVID-19 patients. Few studies have examined lung inflammation in severe COVID-19 patients, and even fewer have focused on LMs, in contrast to blood analysis studies [11-13]. Lipid mediators (LMs) are produced by converting free arachidonic acid (AA) through cyclo-oxygenase and lipoxygenase enzymes [10, 14]. Cleaving cell membrane phospholipids produce AA through phospholipases A2 (PLA2s) enzymes [14, 15]. PLA2s include secreted PLA2 (sPLA2) and cytosolic PLA2 (cPLA2) [14, 16].

Pulmonary surfactant is a lipid-protein complex synthesized by alveolar type II epithelial cells. It reduces surface tension along the alveolar epithelium, thus promoting alveolar stability [17]. Surfactant is composed of 10% proteins and 90% lipids. Unlike other phospholipids, it contains highly saturated fatty acids (such as palmitic acid) with a high proportion of dipalmitoylphosphatidylcholine (DPPC). Alterations in pulmonary surfactant, particularly through sPLA2-mediated hydrolysis of surfactant phospholipids, have been shown to play a prominent role in the etiology of ARDS [17, 18]. The destruction of DPPC increases surface tension at the air-liquid interface, leading to alveolar collapse and, ultimately, acute lung injury, such as ARDS [17, 18]. This study investigated the alterations in the levels of three phospholipid families in bronchoalveolar aspirates from COVID-19 patients after admission to the ICU and their potential role in disease severity. The first family consists mainly of cellular phospholipids

hydrolyzed by PLA2 during host infections to release free AA (Figure 1A). The second family consists of sphingolipids (Figure 1B) and cholesterol, which are involved in cell membrane fluidity, cell apoptosis, and virus entry into host cells. The third family comprises pulmonary surfactant phospholipids, which are known to play a role in alveolar stability and the etiology of ARDS. We discuss the potential roles and predictive values of these changes in disease severity in COVID-19 patients.

2. Materials and methods

2.1. Study design and participants

This study was a prospective multicenter non-interventional trial involving three French intensive care units (Groupe Hospitalier Paris Saint Joseph, Paris; Centre Hospitalier Victor Dupouy, Argenteuil; and Centre Hospitalier Andrey Mignot, Versailles).

2.2. Study population

The study was conducted from April 14, 2020, to September 30, 2021, during France's 3 first epidemic waves [19]. We screened adult patients (over 18 years old) who were admitted to the intensive care unit (ICU) for acute respiratory failure secondary to documented COVID-19 (laboratory-confirmed COVID-19 infection by RT-PCR assay for SARS-CoV-2). In this study, 89 patients were included. As the study began at the start of the pandemic and there was no consensus on treatment, some patients received corticoids, chloroquine or one of its derivatives, azithromycin, or IL-1 or IL-6 inhibitors (Tabel 1) as previously described [20]. No significant differences were observed between survivors and no survivors for the different parameters (age, sex, body mass index (BMI)).

All ventilated patients who met the Berlin definition of acute respiratory distress syndrome for less than three days were invited to participate in the study [21]. The inclusion of the patients occurred at the time of invasive mechanical ventilation.

2.3. Sample collection

Tracheal aspirates were made through the closed suction device connected to an external sterile trap without adding any saline instillation. If no secretion can be initially obtained, a new tracheal aspirate was attempted 3 hours later, as usual, until the first 24 hours. A volume of 1

to 5 ml of tracheal intubation was obtained from D1, D3, and D7 at each time point. No dilution, filtration, or viral inhibition was made before freezing. During the first week of mechanical ventilation, tracheal aspirates and blood samples were collected on day 1, day 3, and day 7. The samples were centrifugated, aliquoted, and stored at -80°C until analysis.

Before the first analysis, the samples were thawed on ice. If necessary, they were weighed and diluted $\frac{1}{4}$ in a sterile saline solution to reduce viscosity and homogenized with a pipette. The samples were not filtered or centrifuged to avoid bias in interpreting the results. The samples were then aliquoted according to the different analysis techniques, and the results were normalized following the dilution.

2.4. *Ethics*

Ethical approval was obtained from the Saint-Joseph Hospital Institutional Review Board (IRB n° 00012157) in March 2020 and registered on Clinicaltrials.gov (NCT04485364) on July 24, 2020 (see Table 1). The patient's non-opposition was obtained before inclusion in the study. If the patient could not express themselves initially, the non-opposition of the closest relative(s) was required.

2.5. *Sample preparations and lipid extractions*

300 μ L of aspirates were added to 1500 μ L of $\text{CHCl}_3/\text{MeOH}$ (1:1). Samples were vortexed twice for 30 s and incubated for 2 hours at room temperature with mixing. 225 μ L of H_2O was added, and samples were vortexed twice for 30 s before centrifugation at 1400 rpm for 10 min at 4°C. The upper phase (aqueous phase), containing gangliosides, lysoglycerophospholipids, and short-chain glycerophospholipids, was transferred into a glass tube. The protein disk interphase was discarded, and the lower lipid-rich phase (organic phase), containing other lipid classes, was pooled with the upper phase, and the mixture was dried using a speed vacuum

concentrator. All samples were resuspended in 100 μl of $\text{CHCl}_3/\text{MeOH}$ 2:1 (v/v), and 10 μl of each extract was hundred-fold diluted in a solution of $\text{MeOH}/\text{isopropanol}/\text{H}_2\text{O}$ 65:35:5 (v/v/v) before injection. A quality control (QC) sample was obtained by pooling 20 μl of diluted sample preparations. The QC sample was injected every 10 samples to evaluate signal variations of lipid species.

2.6. *Untargeted lipidomics and data processing*

The lipidomic profiles were determined using an Ultimate 3000 liquid chromatography system (Thermo Fisher Scientific, San Jose, CA, USA) coupled to a high-resolution Thermo Orbitrap Fusion (Thermo Fisher Scientific, San Jose, CA, USA) equipped with an electrospray source (ESI). Chromatographic separation was performed on a Phenomenex Kinetex C8 column (150 x 2.1 mm, 2.6 μm) at 0.4 mL/min, 60 °C, using an injection volume of 10 μL . Mobile phases A and B consisted of $\text{H}_2\text{O}/\text{MeOH}$ 60:40 (v/v), 0.1% formic acid, and isopropanol/MeOH 90:10 (v/v), 0.1% formic acid in negative ionization mode, respectively. Ammonium formate (10 mM) was added to both mobile phases in positive ionization mode to detect glycerolipids and cholesteryl-esters in the $[\text{M}+\text{NH}_4]^+$ form. The gradient elution of solvent B was maintained for 2.5 min at 32%, from 2.5 to 3.5 min, it was increased to 45% B, from 3.5 to 5 min to 52% B, from 5 to 7 min to 58% B, from 7 to 10 min to 66% B, from 10 to 12 min to 70% B, from 12 to 15 min to 75% B, from 15 to 19 min to 80% B, from 19 to 22 min to 85% B, and 22 to 23 min to 95% B; from 23 to 25 min, 95% B was maintained; from 25 to 26 min solvent B was decreased to 32% and then maintained for 4 min for column re-equilibration. The mass resolving power of the mass spectrometer was 240,000 (FWHM) for MS experiments. Samples were analyzed in both positive and negative ionization modes. The ESI source parameters were as follows: the spray voltage was set to 3.7 kV and -3.2 kV in positive and negative ionization modes, respectively. The heated capillary was kept at 360 °C, and the sheath and auxiliary gas flow were set to 50

and 15 (arbitrary units), respectively. Mass spectra were recorded in full-scan MS mode from m/z 50 to m/z 2000. After LC-HRMS analysis of the samples and annotation of features, QC samples were re-injected for higher energy collisional dissociation (HCD) MS/MS experiments in positive and negative ion modes on the same instrument set in targeted mode using inclusion lists. The isolation width was set at m/z 0.8; the stepped normalized collision energy was set at $20\% \pm 10\%$, and the mass resolution was set at 17 500 FWHM at m/z 200. HCD mass spectra were manually inspected to confirm annotations. Without internal standards for all lipid species, the results are presented in a semi-quantitative form corresponding to comparing chromatographic peak areas for each robust annotated variable between day groups.

The ProteoWizard software converted raw files to mzML or mzXML format [22]. Mass spectra were processed using the *xcms* R package provided by the Workflow4Metabolomics Galaxy platform (<https://workflow4metabolomics.org>) [23]. The features were annotated using an *in-house* database that relied on accurately measured masses and several filtering steps, such as retention time windows depending on lipid classes and lipid species' relative isotopic abundance (RIA). This helps to limit false annotations for lipidomics.

2.7. Elisa PGE2, sPLA2, SP-D

Prostaglandin E2 ELISA Kit—Monoclonal (514010, Cayman Chemical), sPLA2 (human Type IIA) ELISA Kit (501380, Cayman Chemical), and Human SP-D ELISA Kit (Abcam, ab213827) were assayed in serum or/and aspirates using the ELISA method according to the manufacturer's instructions.

2.8. *Multiplex cytokine assay*

Serum and lung aspirate cytokine concentrations were determined using the multiplex approach (Bio-Rad, Marnes la Coquettes, France) on the CYBIO platform at Institut Cochin (Paris, France).

2.9. *Statistical analysis*

All figures are represented with the means+SEM. Differences in mean values between groups were determined by analysis of variance by a Mann-Whitney test and the sum-rank Wilcoxon test using Microsoft Excel and XLStat 2022.3.1. One-way ANOVA analysis was used with a post hoc Dunn's test for multiple comparisons. All results are expressed as mean \pm s.e.m. and corrected for multiple comparisons. $P < 0.05$ was considered statistically significant. Correlations between different factors were tested using Spearman's rank correlation coefficient ($p < 0.05$).

3. Results

3.1. *Changes in cellular phospholipids, AA, and PGE2 levels*

We first examined the presence and evolution of free fatty acids (FFA) in patient aspirates, including arachidonic acid (AA) and its inflammatory metabolite Prostaglandin E2 (PGE2). The total FFA levels did not change significantly during the study (Fig. 2A). However, we found an increase in AA levels, which started on day 3 and became significant on day 7 (Fig. 2B). This increase was accompanied by a parallel increase in PGE2 levels (Fig. 2C). We also detected docosahexaenoic acid (DHA) in the aspirates. However, the levels of DHA remained stable during the study period (Fig. 2D).

The levels of AA and PGE2 increased along with the phospholipid concentrations from day 3, which were sustained until day 7 (Fig. 3A-D (left panel)). The levels of lyso-phospholipids, including Lyso-PE and Lyso-PS, also increased according to the same kinetics as their parent phospholipids PE and PS (Fig. 3A-C (right panel)). However, the levels of Lyso-PC (Fig. 3B) reached their maximum values only on day 7, while those of Lyso-PI did not change significantly (Fig. 3D). On days 3 and 7, there was a parallel increase in the contents of diglycerides (DG) and triglycerides (TG), which are cellular precursors of phospholipids, compared to day 1 (Fig. 3E-F). The accumulation of lyso-phospholipids and FFAs resulted from the hydrolysis of cellular phospholipids by PLA2 enzymes (Fig. 1A) [10, 15, 17]. The PLA2 enzymes involved in this hydrolysis can act either on phospholipids secreted by host cells or intracellularly.

Therefore, we measured the concentration of sPLA2-IIA, the major member of the sPLA2 subgroup (known to hydrolyze secreted phospholipids), in the aspirates of patients. In the lung, sPLA2-IIA hydrolyzes cellular and surfactant phospholipids [24-27]. This enzyme is the major sPLA2 present in human biological fluids, whereas other sPLA2s are detected only at marginal levels [15, 17, 28]. We detected sPLA2-IIA in patient aspirates, but its concentration remained

unchanged during the study period (data not shown) and was not associated with the changes in AA levels at days 1, 3, and 7 (Sup Fig. 1). It is likely that an intracellular form of PLA2, such as cPLA2 [15, 16], could be implicated in the enzymatic breakdown of intracellular phospholipids in individuals afflicted with COVID-19. Regrettably, analysis of this intracellular enzyme was precluded in the collected samples owing to ethical constraints that prohibited the procurement of lung biopsies. Taken together, these findings indicate that SARS-CoV-2 infection leads to increased cellular phospholipid metabolism in the patient's airways, producing AA and PGE2, which may contribute to lung inflammation.

3.2. Increased ceramide, sphingomyelin, and cholesterol concentrations

We analyzed the evolution of ceramide (Cer), sphingomyelin (SM), and free cholesterol (FC) levels in patient aspirates (Fig. 4A-C). Cer and SM play a role in host cell death/apoptosis [29], and FC is involved in virus entry and replication in host cells [24, 25]. The lipidomic analyses revealed a significant increase in the levels of these lipids on day 3 after admission to the ICU, which remained elevated on day 7 (Fig. 4A-C). Significantly increased levels of cellular metabolites of Cer and SM, Hexa-ceramides, DiHexosyl-ceramides, and Gangliosides (Ga) were observed on days 3 and 7 compared to day 1 (Fig. 4D-F). Since Cer is produced through the hydrolysis of SM by sphingomyelinase enzymes (SMase), we examined the levels of one of these enzymes in patient aspirates, namely lysosomal acid Smase (LaSMase). This enzyme is considered one of the leading candidates for Cer production in the cellular response to stress, including environmental insults, pathogen infections, death receptor ligation, and chemotherapy drugs [26]. However, we could not detect LaSMase in the patient aspirates any day after ICU admission (data not shown), suggesting an absence of this enzyme leaking from the cells. Overall, these findings show that SARS-CoV-2 infection leads to increased pulmonary concentrations of FC and sphingolipids, which may potentially contribute to airway cell death

and virus replication. However, the enzymes responsible for these changes have yet to be identified.

3.3. *Analyses of pulmonary surfactant phospholipids and SP-D*

The most abundant component of pulmonary surfactant is dipalmitoyl-phosphatidylcholine (DPPC), which plays a major role in alveolar stability [18]. Therefore, we investigated the presence of this phospholipid and its evolution in the patient's lung aspirates. Our lipidomic analyses showed no changes in DPPC levels and its derivatives (palmitic acid and lyso-PC 16:0) throughout the study period (Fig. 5 A-C). We subsequently examined the evolution of typical surfactant protein-D (SP-D) in patient aspirates (Fig. 5D). SP-D was identified in these aspirates at all periods examined. Still, its levels moderately decreased on day 3 and remained very low on day 7. These findings suggest that pulmonary surfactant was not altered during SARS-CoV-2 airway infection, and this infection may not affect alveolar stability.

3.4. *Relationship between lipids changes and patient mortality*

Finally, we investigated the potential link between changes in lipid levels analyzed in this study in aspirates and the risk of mortality of patients (Fig. 6). On the first day of admission, free AA and cellular phospholipids PC, PE, and PS exhibited increased levels that were positively correlated with patient mortality (Fig. 6A-D). However, no correlations were found between PS, PI, and PGE2 levels and patient mortality (Fig. 6 E-F). We also investigated the correlation between patient mortality and phospholipid precursors TG and DG levels on day 1. The results indicated that TG levels positively correlated with mortality, while DG levels did not significantly correlate (Fig. 6G-H). Additionally, we found no correlations between changes in lipid levels examined in this study in aspirates and patient mortality risk on days 3 and 7 (sup Fig 2-3).

We studied the possible implications of changes in cholesterol and the sphingolipid family on patient mortality. Our findings indicate that increased levels of SM and FC are positively correlated with mortality (Fig. 6 J- K). However, it should be noted that Cer, an SM metabolite, exhibited an inverse correlation with patient mortality (Fig. 6 I-J). The levels of other metabolites of SM, namely HexCer, DiHexCer, and Ga, were increased and positively correlated with patient mortality (Fig. 6 L-N). These findings suggest that changes in cholesterol and sphingolipids levels are associated with patient mortality. Finally, our analyses showed that the evolution of SP-D levels, changes in surfactant phospholipid concentrations, and their ratios to SP-D did not correlate to patient mortality (data not shown).

4. Discussion

The major aim of this prospective multicenter non-interventional study was to examine the evolution of phospholipids concentration in lung aspirates from COVID-19 patients and whether they correlate with the severity of the disease. The studies were conducted from April 14, 2020, to September 30, 2021, by focusing on three French intensive care units: Groupe hospitalier Paris Saint Joseph in Paris, Centre hospitalier Victor Dupouy in Argenteuil, and Centre hospitalier Andrey Mignot in Versailles. Adult patients (over 18 years old) admitted to the intensive care unit (ICU) were screened for acute respiratory failure resulting from laboratory-confirmed COVID-19 infection by RT-PCR assay for SARS-CoV-2.

The study found a time-dependent increase in the levels of AA and its inflammatory metabolite PGE₂. Accelerated AA metabolism is known to play a key role in the pathogenesis of several airway inflammatory and infectious diseases (13, 15). Our findings extend those of previous studies [11, 30] and show that the increase in AA levels was accompanied by elevated lysophospholipid concentrations, suggesting the involvement of PLA₂-dependent hydrolysis of airway phospholipids. Previous studies have measured LMs in bronchoalveolar lavages (BALs) of COVID-19 patients, which reflect alveolar contents, whereas the present study used bronchial sections that predominate in aspirates. However, our studies report the presence of surfactant components in the aspirates, indicating that they are a mixture of bronchial and alveolar secretions.

PLA₂ enzymes are commonly found in mammalian tissues, including intracellular and secreted PLA₂ (sPLA₂) [14-16]. While the type of PLA₂ responsible for AA release in our studies is yet to be identified, our results show that sPLA₂-IIA, the predominant member of the sPLA₂ group, is

not involved in AA release. Therefore, we can hypothesize that an intracellular PLA2, such as cPLA2 [15, 16], may play a role in AA release through the hydrolysis of cellular phospholipids in patients with COVID-19. Unfortunately, this intracellular enzyme could not be analyzed in the collected samples due to ethical reasons preventing lung biopsies. Recent studies suggest that cPLA2 α plays a critical role in coronavirus replication, most likely by producing lysophospholipids necessary to form specialized membrane compartments where viral RNA synthesis occurs [31]. Although the mechanisms by which SARS-CoV-2 infection activates cellular PLA2 are still unknown, so the interaction of the virus with ACE2 may play a role in this activation. A previous study demonstrated that stimulation of the ACE2 receptor results in the activation of intracellular PLA2 [32], leading to the hydrolysis of cellular phospholipids and subsequent production of LMs. In theory, hydrolysis should decrease the levels of phospholipids in airway aspirates. However, our data showed increased levels of these phospholipids. Therefore, it is likely that PLA2 hydrolyzed cellular phospholipids at the early stage of SARS-CoV-2 infection, followed by a re-synthesis of these phospholipids to offset their hydrolysis. Our studies showed that increased PE, PS, and PC concentration and AA release positively correlated with the risk of patient mortality. These metabolic changes may be predisposing factors to pulmonary complications in SARS-COV-2-infected patients.

Our results also showed a time-dependent increase in SM and Cer levels in patient aspirates, which may have significant pathophysiological implications in COVID-19 disease. SM and Cer are crucial in modulating host cell death and apoptosis in various pathophysiological conditions [29, 33]. The data showed a clear correlation between the increased SM level (the precursor of Cer in host cells) and the mortality risk of SARS-CoV-2-infected patients. Apoptosis is an early process that may be involved in initiating lung injury, which can lead to loss of pulmonary function and respiratory failure. Several studies have reported the major roles of SM and Cer

alterations during apoptosis and death in multiple infectious and inflammatory diseases and animal models of organ infection and injury [34-36]. For instance, programmed cell death is a crucial cellular response commonly observed in Influenza virus infection and associated lung injury [37].

A key observation of our study is that cholesterol levels in the aspirates of COVID-19 patients increased in a time-dependent manner and correlated with patient mortality. This increase may play a significant role in the SARS-CoV-2 infection process. Indeed, FC has been suggested to facilitate the entry of numerous viruses into host cells. Recent studies indicate that SARS-CoV-2 requires cholesterol to enter pulmonary epithelial cells and form pathological syncytia [24]. Consistent with this hypothesis, depletion of cell membrane cholesterol by Cholesterol 25-Hydroxylase inhibits membrane fusion of SARS-CoV-2, potentially reducing viral replication [38].

Finally, we investigated the possible changes in the levels of pulmonary surfactant phospholipids, which are known to play a key role in the etiology of ARDS (16, 17). "Our results showed no changes in the levels of DPPC, the main surfactant phospholipid, or of its derivatives, palmitic acid and Lyso-PC (16:0) in the aspirates of COVID-19 patients, which matches with previously quoted studies. Palmitic acid and Lyso-PC (16:0) are specific components of surfactant lipids, present in very low amounts in host cells. Additionally, it suggests an increase of non-surfactant lipids over time in the samples. This result is also consistent with our data showing no changes in sPLA2-IIA, known to cleave surfactant phospholipids, levels in these aspirates. "

This enzyme plays a role in surfactant phospholipid hydrolysis in ARDS patients [39]. However, our data differ from a recent study [40] that showed a significant depletion of DPPC in the BALF of COVID-19 patients. It is important to note that in the study above, the median age of the healthy control subjects (23 years old) was matched by the age of the COVID-19 patients (65

years old). Therefore, it is difficult to determine whether the observed differences between these patient groups are due to SARS-CoV-2 infection or age differences.

Furthermore, unlike our present study, these studies did not investigate the kinetics of lipid changes during the disease. The strength of our research is that we were able to examine the kinetic phospholipid changes in the lung aspirates of patients after ICU admission, assuming that these patients were their own controls. The absence of surfactant alteration reported in our studies is consistent with the observed clinical pattern during the early phase of ARDS. In the early stages of the disease, there appears to be a relationship between “happy hypoxemia” [41] and vascular diseases (microthrombi, vascular shunt, compromise of lung perfusion) rather than alveolar edema. The published CT-scan images highlight the presence of a low abundance of plasma leakage in alveolar spaces rather than a large amount of liquid in the alveoli [42]. Therefore, the absence of altered lung compliance during the initial phase of mechanical ventilation (L phase) strongly suggests the lack of edema or surfactant alterations in the alveolar space at this stage of the disease [43].

In conclusion, the present studies demonstrate an accelerated lipid increase in the airways associated with the severity observed in patients with COVID-19. The production of free AA and its inflammatory metabolite PGE₂ may play a key role in this severity. Studies currently underway in our laboratory examine the evolution of lipid concentration and its kinetic differences from those of inflammatory cytokines in the airways of COVID-19 patients (Manuscript in preparation). These results suggest that cytokines and LMs may have distinct roles in inducing/modulating airway inflammation in COVID-19 patients. In contrast to previous reports, our study investigated the evolution of lipid concentration in patients' airways and identified the first three days after admission to the ICU as the likely peak of this metabolism. Recent studies suggest that cPLA₂ α plays a critical role in coronavirus replication, most likely by

producing lyso-phospholipids necessary to form specialized membrane compartments where viral RNA synthesis occurs [31]. We can speculate that SARS-CoV-2 viruses may reprogram cellular metabolism upon infection to remodel lipid membranes and fuel the production of new virions. This can lead to a vicious circle between viral infection and host cell lipid remodeling.

SARS-CoV-2 viruses may reprogram cellular metabolism upon infection to remodel lipid membranes and fuel the production of new virions. This can lead to a vicious circle between viral infection and host cell lipid remodeling.

Table n°1: population

	Total population (n=89)	survivors (n= 67)	non-survivors (n= 22)	P
Age, median [IC]	63,0 (34-83)	62,7 (34-83)	67,4 (51-78)	0.117
Sex, (% male)	54 (60,6)	41 (61,2)	13 (59,0)	0.841
BMI (kg/m ²), median [IC]	29,35 (18,8-47,4)	28,8 (19,8-47,4)	31,2 (18,8-45,4)	0.54
0-25	26%	26%	25%	
25-30	28%	32%	15%	
30-35	29%	26%	40%	
35-40	12%	12%	10%	
40-45	6%	5%	10%	
Co-morbidities, n (%)				
Hypertension	47 (52.8)	33 (49.3)	14 (63.6)	0.24
Ischemic heart disease	4(4.5)	1 (1.5)	3 (13.6)	0.017
Heart failure	1 (1.1)	0 (0)	1 (4.5)	0.08
Diabetes mellitus	29 (32.5)	21 (31.3)	8 (36.3)	0.66
smokers	22(24.7)	17 (25.4)	5 (22.7)	0.80
Number of co-morbidities, (%)				0.077
0	30%	36%	9%	
1	29%	26%	36%	
2	27%	25%	36%	
3	9%	10%	5%	
4	2%	1%	5%	
5	2%	1%	5%	
6	1%	0%	5%	
Chronic treatment, n (%)				
Corticosteroids	6 (6.7)	5 (7.5)	1 (4.5)	0.64
Angiotensin receptor blocker	13(14.6%)	10 (14.9)	3 (13.6)	0.88
ACE inhibitors	7(7.8%)	4 (6.0)	3 (13.6)	0.246
Current disease, median [IC]				

Length before hospital admission (days),	6[4 - 9]	6[4 - 9]	5 [4 – 8.8]	0.94
Length before ICU admission (days),	8 [6 - 11]	8.5 [5.8 - 11]	8 [6 – 10.8]	0.69
length between hospital and ICU admission	1[0 – 3.25]	1 [0 – 3.8]	1.5 [0 – 3.0]	0.47
Acute treatment, n(%)				
Corticosteroids	42 (47.2)	30 (44.8)	12 (54.5)	0.43
Chloroquine or Hydroxychloroquine	10 (11.2)	9 (13.4)	1 (4.5)	0.26
Azithromycin	9 (10.1)	8 (11.9)	1 (4.5)	0.32
Anakinra	5 (5.6)	4 (6.0)	1 (4.5)	0.80
Anti-IL-6R	5 (5.6)	4 (6.0)	0 (0)	0.24
During ICU stay				
Catecholamines, n (%)	34 (38,6%)	24 (36,4%)	14 (63,6%)	0.006
Dialysis, n (%)	21 (23.6)	10 (14.9)	11 (50.0)	0.0007
Invasive Mechanical ventilation (%)	100%	100%	100%	1
Length of mechanical ventilation, median [IC]	13 [9 – 24.5]	11.5 [7.8 – 30.0]	18 [13.0 – 19.0]	0.56
Neuromuscular blockers	100%	100%	100%	1
SAPS II, median [IC]	41 [34.7 – 48.2]	41 [35.0 – 46.0]	41 [32.0 – 56.8]	0.56
Length of ICU stay (mean; SD)	17,4 (0-95)	17,8 (0-95)	16,3 3-51)	0.741
ICU survival, n (%)	67 (75.3)	-	-	-

ACE: Angiotensin-converting enzyme; Anti-IL-6R: inhibitors of interleukin-6 receptor (i.e., Tocilizumab or Sarilumab); BMI: body mass index; ENT: Ear, Nose, Throat, and neck ICU: intensive care unit; NAIDS: non-steroidal anti-inflammatory drug; SAPS II: Simplified acute physiology score n°II; VAP: ventilator-associated pneumonia.

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Abbreviations

Covid-19, Coronavirus disease; ARDS, acute respiratory distress syndrome ; SARS-CoV-2, Severe acute respiratory syndrome coronavirus 2; ACE2, Angiotensin-Converting Enzyme 2; LM, Lipid mediators; AA, arachidonic acid; PC, phosphatidyl-choline; PE, phosphatidyl-ethanolamine ; PS, phosphatidyl-serine ; PI, phosphatidyl-inositol ; PLA2, phospholipases A2 ; sPLA2, secreted PLA2; cPLA2, cytosolic PLA2; DPPC, dipalmitoyl-phosphatidylcholine; PG, phosphatidyl-glycerol; PGE2, prostaglandin E2 ; DOPG, dioleoyl-phosphatidyl-glycerol ; FFA, free fatty acids; DHA, docosahexaenoic acid ; DG, diglycerides; Lyso-PL, lyso-phospholipids ; TG, triglycerides; IL, Interleukin; IFN-g, interferon-gamma ; IP-10, Interferon gamma-induced protein 10; MCP-1, monocyte chemoattractant protein 1 ; MIP-1a, Macrophage Inflammatory Proteins ; Cer, Ceramides; SM, Sphingomyelin; FC, free cholesterol; SpD, surfactant protein-D.

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LEGENDS OF FIGURES

MAIN FIGURES

Figure 1: Phospholipid concentration in host cells

Part A of the figure shows the cleavage by PLA2 enzymes of membrane and surfactant phospholipids. This leads to the release of fatty acids, including AA, which COX and LOX enzymes convert to lipid mediators (such as PGE2 and LTB4) that regulate inflammation. The release of fatty acids is concomitant to the accumulation of lyso-phospholipids, which are re-converted to phospholipids by cellular Acyl-transferase (AT) to limit the cytotoxic effects of lyso-phospholipids. Part B of the figure shows, in a simplified manner, the main pathways involved in Sphingolipid metabolism in host cells. The nature of the substituent (R) in the CH₂-OR moiety of Sphingosine determines the nature of the metabolite produced. For example, if the substituent R is "Phosphocholine," sphingomyelin is produced.

Figure 2: Quantification of free fatty acids concentrations and PGE2 in the airways of Covid-19 patients.

A) shows the levels of total free fatty acids (FFA), B) the levels of arachidonic acid (AA), C) the levels of PGE2, and D) those of DHA in the patient's aspirates at day 1, 3, and 7 after ICU admission. Statistical significance was calculated using ANOVA one-way analysis and Dunn's post hoc test for multiple comparisons with a significance set at $p < 0.05$. (* $p < 0.05$).

Figure 3: Analyses of phospholipids and corresponding Lyso-phospholipids concentrations in airways of Covid-19 patients.

A) shows the levels of PE and Lyso-PE, B) shows the level of PC and Lyso-P, C) shows the level of PS and Lyso-PS, D) shows the levels of PI and Lyso-PI, E) shows the level of DG and F) shows the levels of TG in aspirates of the patients 1, 3 and 7 days after ICU admission. Statistical

significance was calculated using ANOVA one-way analysis and Dunn's post hoc test for multiple comparisons with a significance set at $p < 0.05$. (* $p < 0.05$), ** $p < 0.001$, *** $p < 0.0001$).

Figure 4: Quantification of Ceramides (Cer), Sphingolipids (SM), Free cholesterol (FC), HexCeramides (HexCer), Di-HexCeramides (DiHexCer), and Gangliosides (Ga) levels in the airways of Covid-19 patients and correlations to patients' mortality.

A) shows the Cer level, B) shows the SM, C) shows the FC, D) shows the level of HexCee, E) shows the level of DiHexCer, and F) shows the level of Ga. in aspirates of the patients 1, 3 and 7 days after ICU admission. Statistical significance was calculated using ANOVA one-way analysis and Dunn's post hoc test for multiple comparisons with a significance set at $p < 0.05$. (* $p < 0.05$).

Figure 5: Quantification of the concentrations of the surfactant phospholipids (DPPC and LPC 16:0), derived fatty acids (FA 16:0), and the protein surfactant D (SP-D) in the airways of Covid-19 patients.

A) shows the DPPC levels, B) the LPC 16:0 levels, C) the PA 16:0 levels, and D) the levels of SP-D in aspirates of the patients 1, 3, and 7 days after ICU admission. Statistical significance was calculated using ANOVA one-way analysis and Dunn's post hoc test for multiple comparisons with a significance set at $p < 0.05$. (* $p < 0.05$).

Figure 6: Correlations between the airway concentrations of AA, phospholipids, PGE2, TG, DG, Cer, SM, FC, HexCer, DiHexCer, and Ga and patients' mortality at day 1.

A-F) show the correlations between the airway AA, PC, PE, PS, and PI levels and patient mortality at day 1; G and H) show the correlations between the airway TG and DG levels and patient mortality at day 1; I-N) show the correlations between the airway Cer, SM, FC, HexCer, DiHexCer, and Ga levels and patient mortality at day 1. Statistical significance was calculated by a Mann-Whitney test with a significance set at $p < 0.05$. (* $p < 0.05$; **** $p < 0.0001$).

SUPPLEMENTARY FIGURES

Figure 1: Analyses of the correlations between the airway's sPLA2-IIA concentrations and AA levels.

A-C) Shows the sPLA2-IIA aspirate levels and AA levels on days 1, 3, and 7, respectively. Statistical significance was calculated by a Pearson correlation test with a significance set at $p < 0.05$.

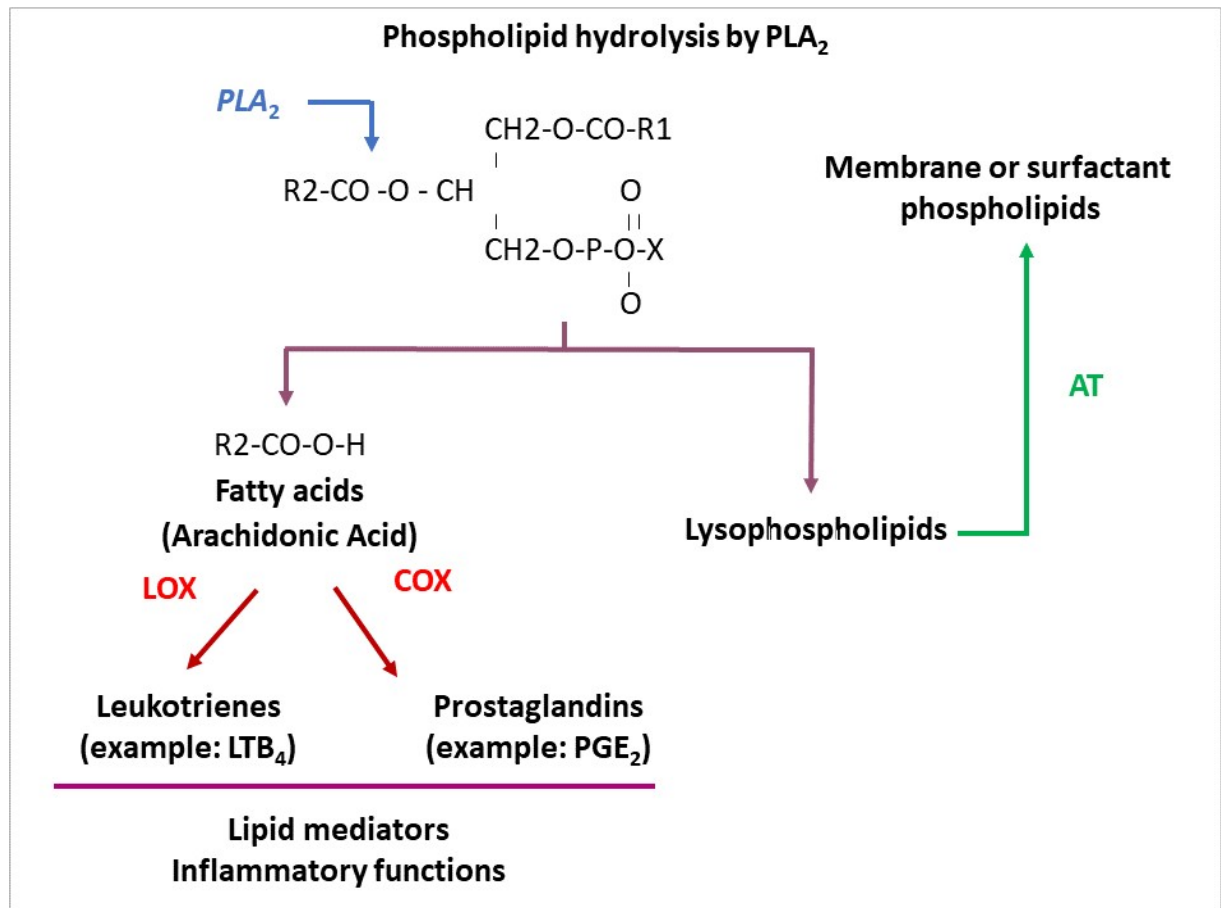
Figure 2: Correlations between the airway concentrations of AA, phospholipids, PGE2, TG, DG, Cer, SM, FC, HexCer, DiHexCer, and Ga, and patients' mortality at day 3.

A-F) show the correlations between the airway AA, PC, PE, PS, and PI levels and patient mortality at day 3; G and H) show the correlations between the airway TG and DG levels and patient mortality at day 3; I-N) show the correlations between the airway Cer, SM, FC, HexCer, DiHexCer, and Ga levels and patient mortality at day 3. Statistical significance was calculated by a Mann-Whitney test with a significance set at $p < 0.05$.

Figure 3: Correlations between the airway concentrations of AA, phospholipids, PGE2, TG, DG, Cer, SM, FC, HexCer, DiHexCer, and Ga and patients' mortality at day 7.

A-F) show the correlations between the airway AA, PC, PE, PS, and PI levels and patient mortality at days 7; G and H) show the correlations between the airway TG and DG levels and patient mortality at day 7; I-N) show the correlations between the airway Cer, SM, FC, HexCer, DiHexCer, and Ga levels and patient mortality at day 7. Statistical significance was calculated by a Mann-Whitney test with a significance set at $p < 0.05$.

A



B

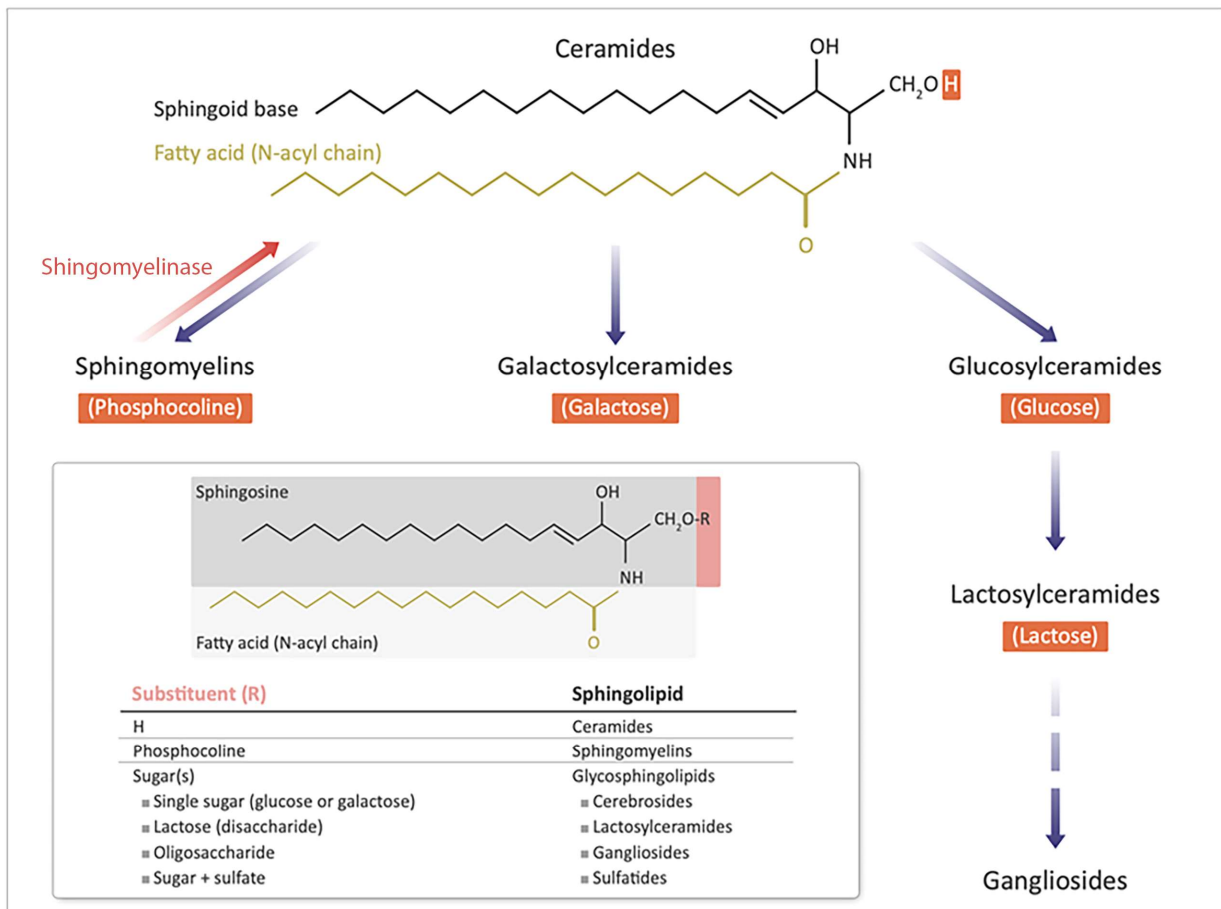


Figure 1

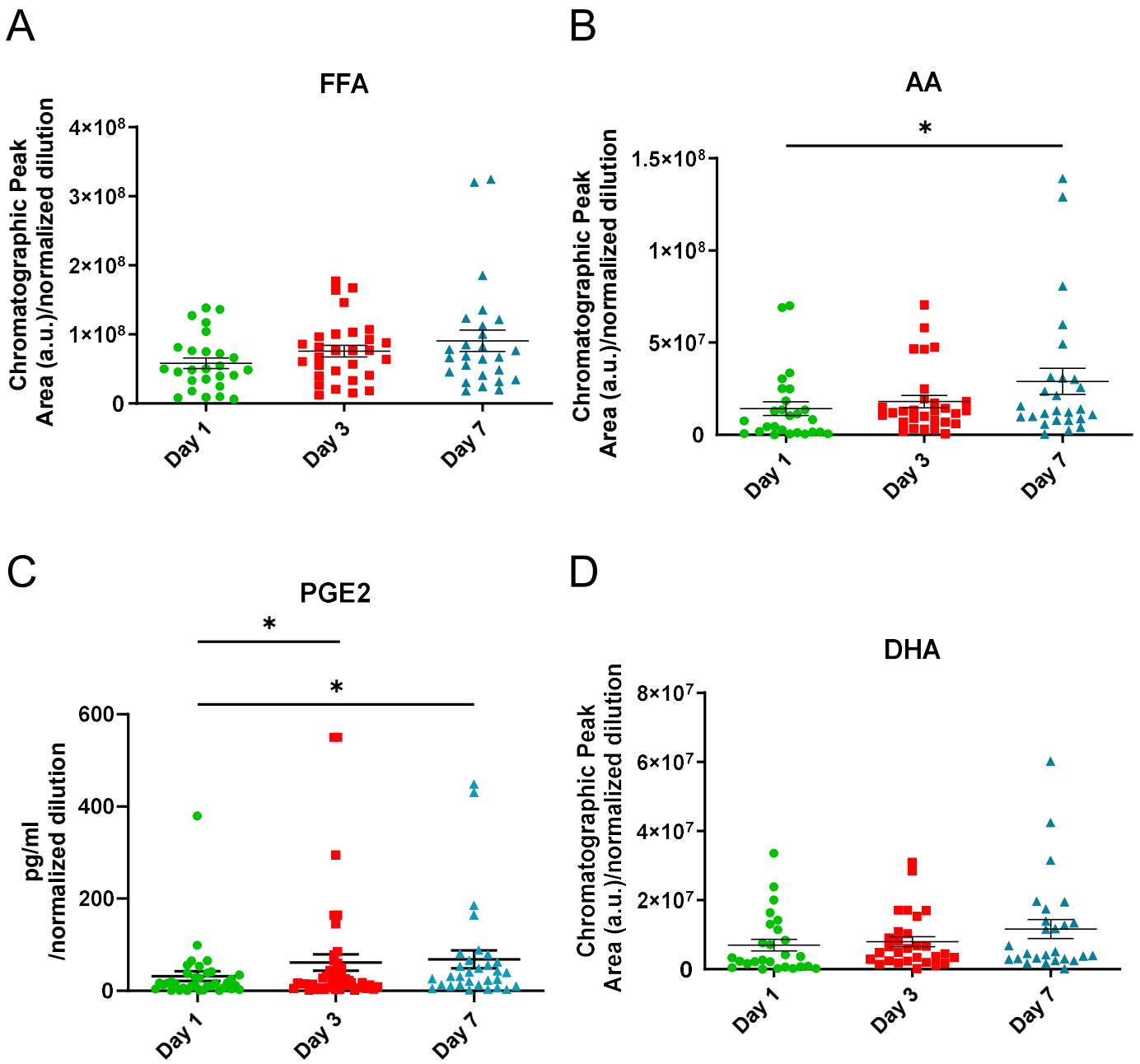


Figure 2

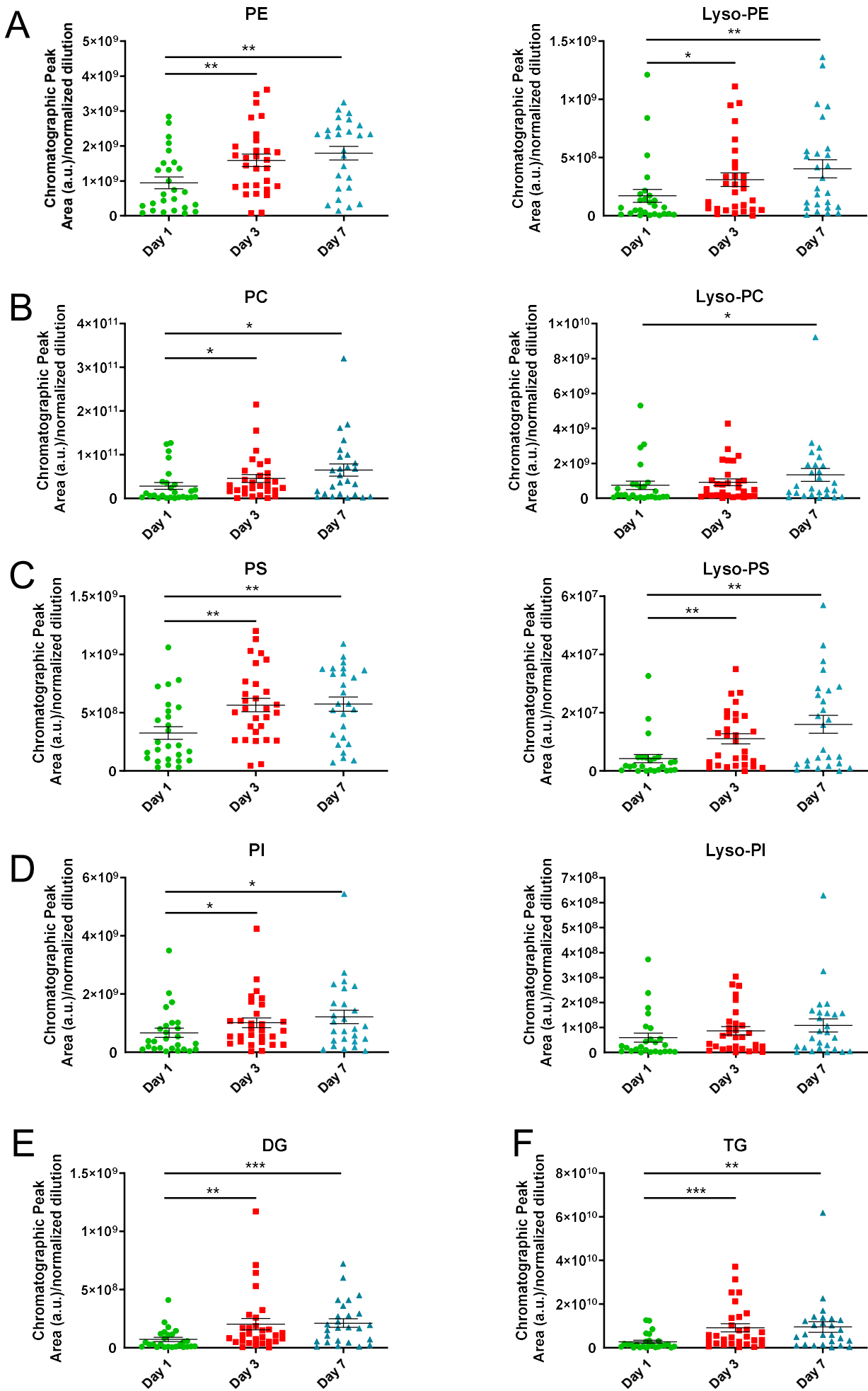


Figure 3

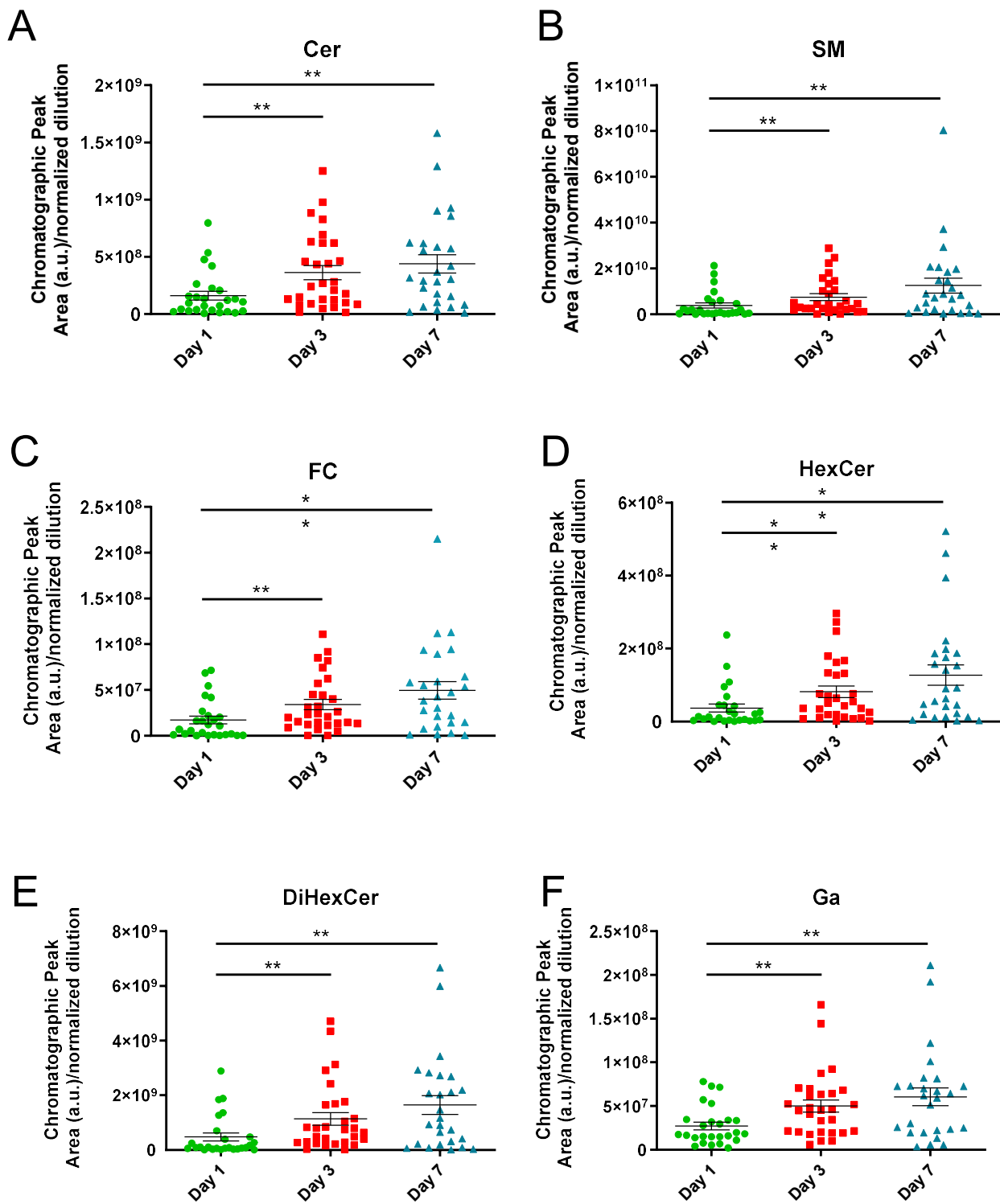


Figure 4

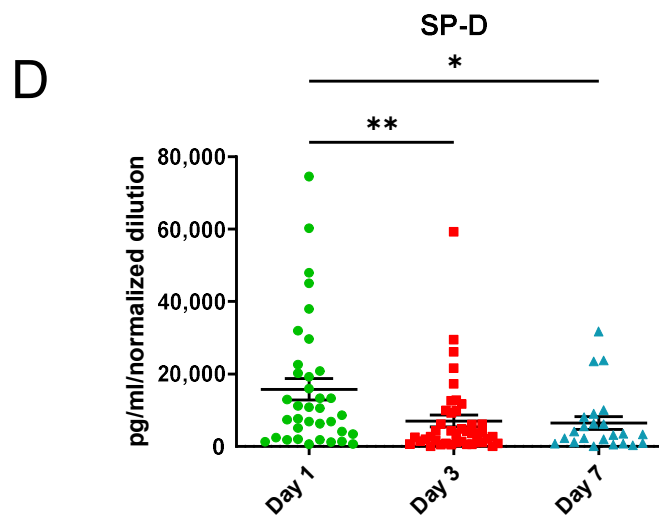
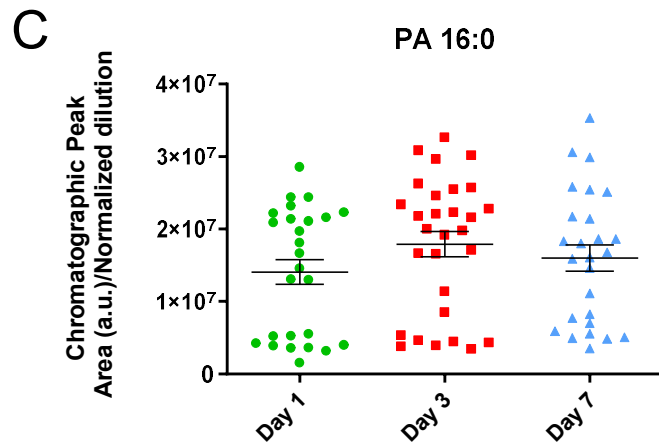
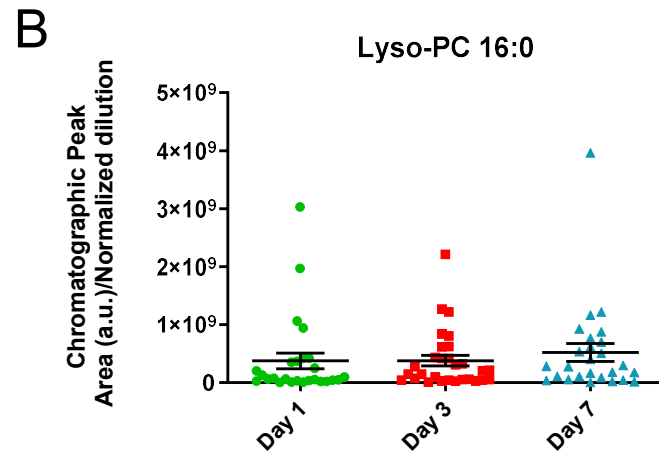
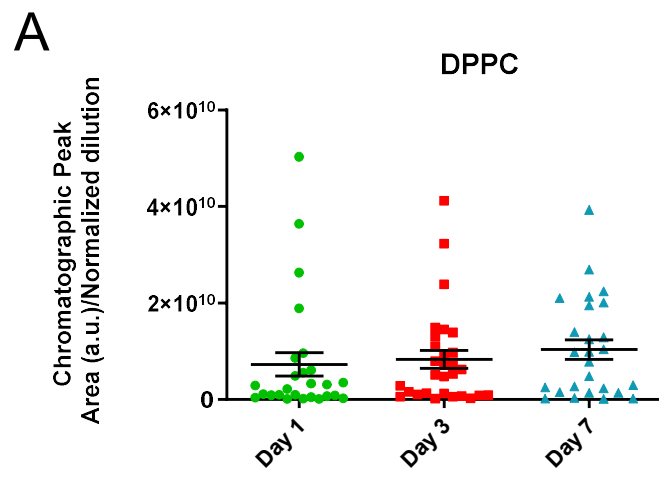


Figure 5

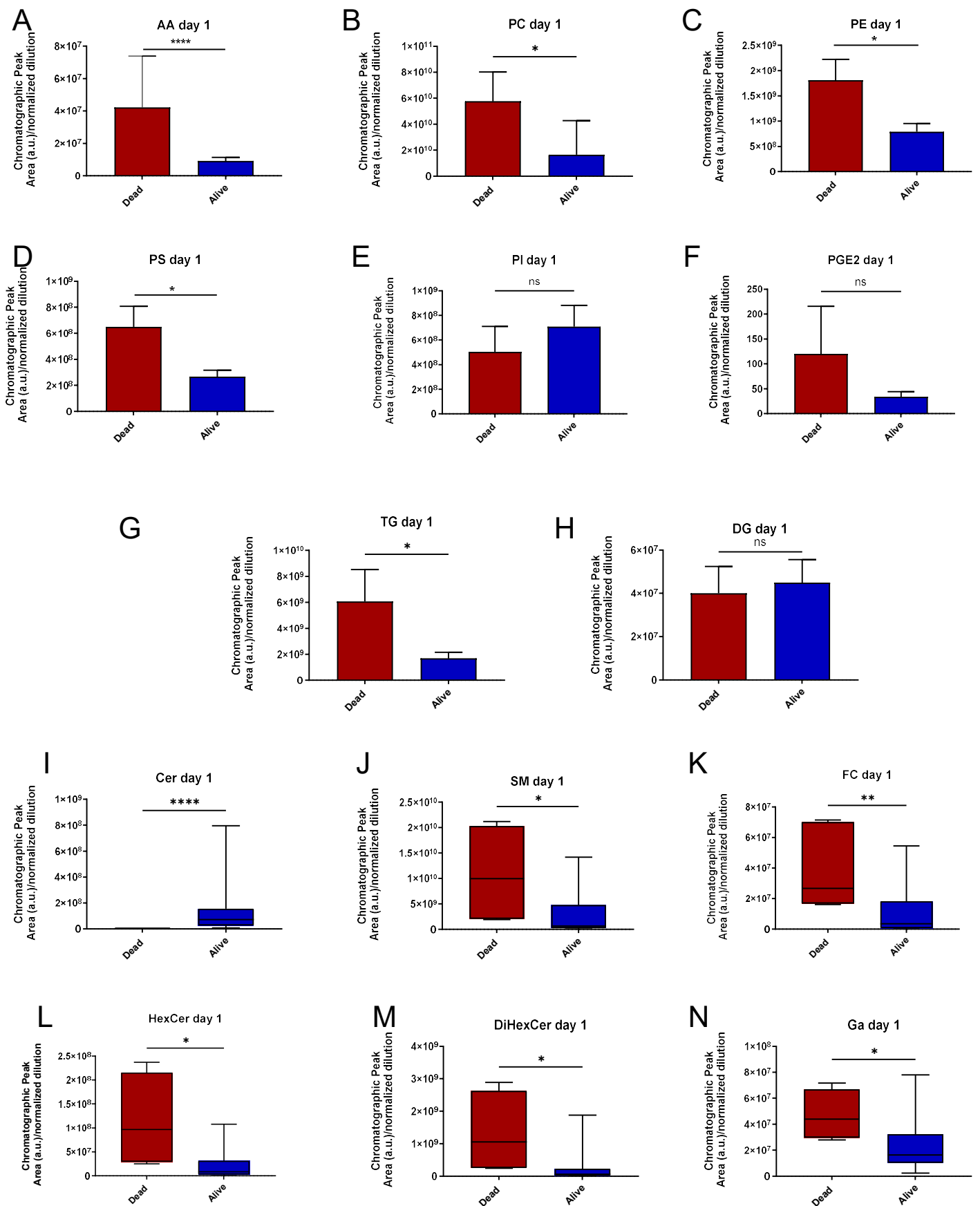
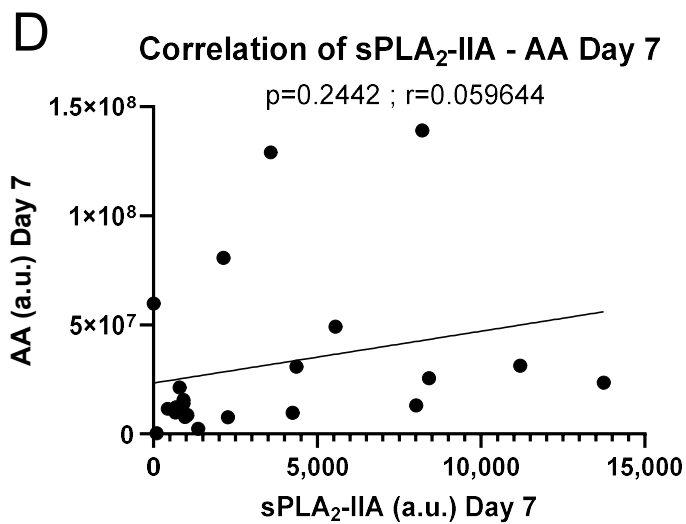
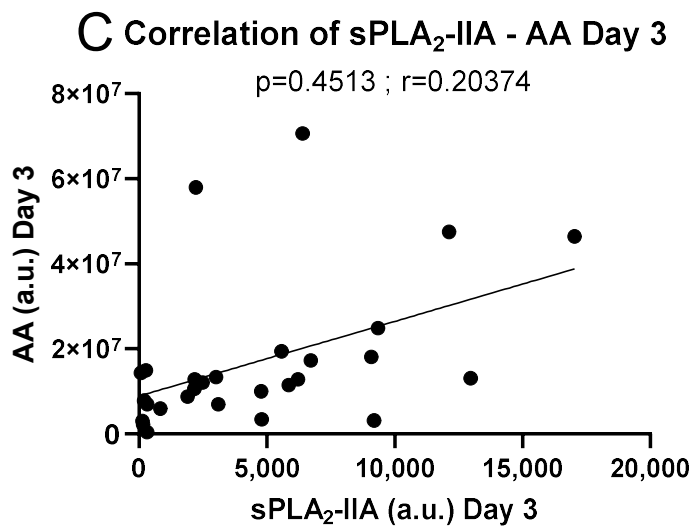
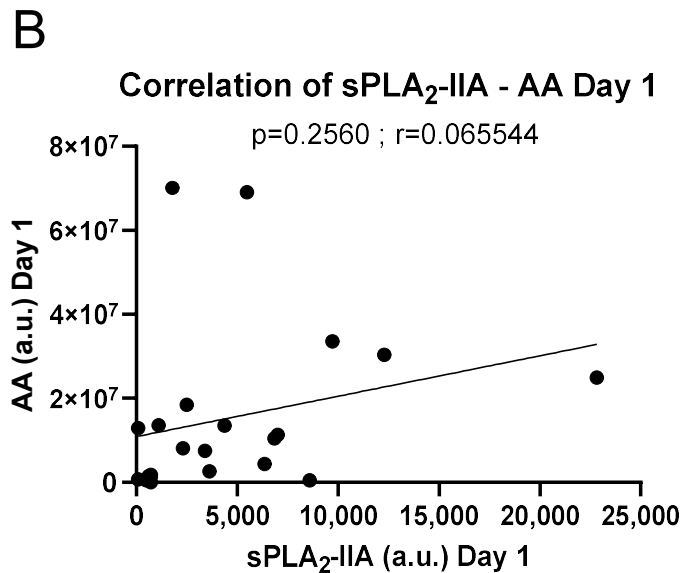
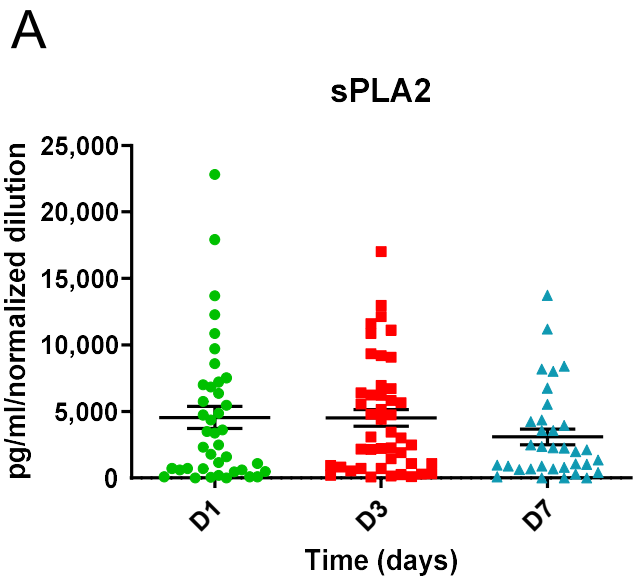
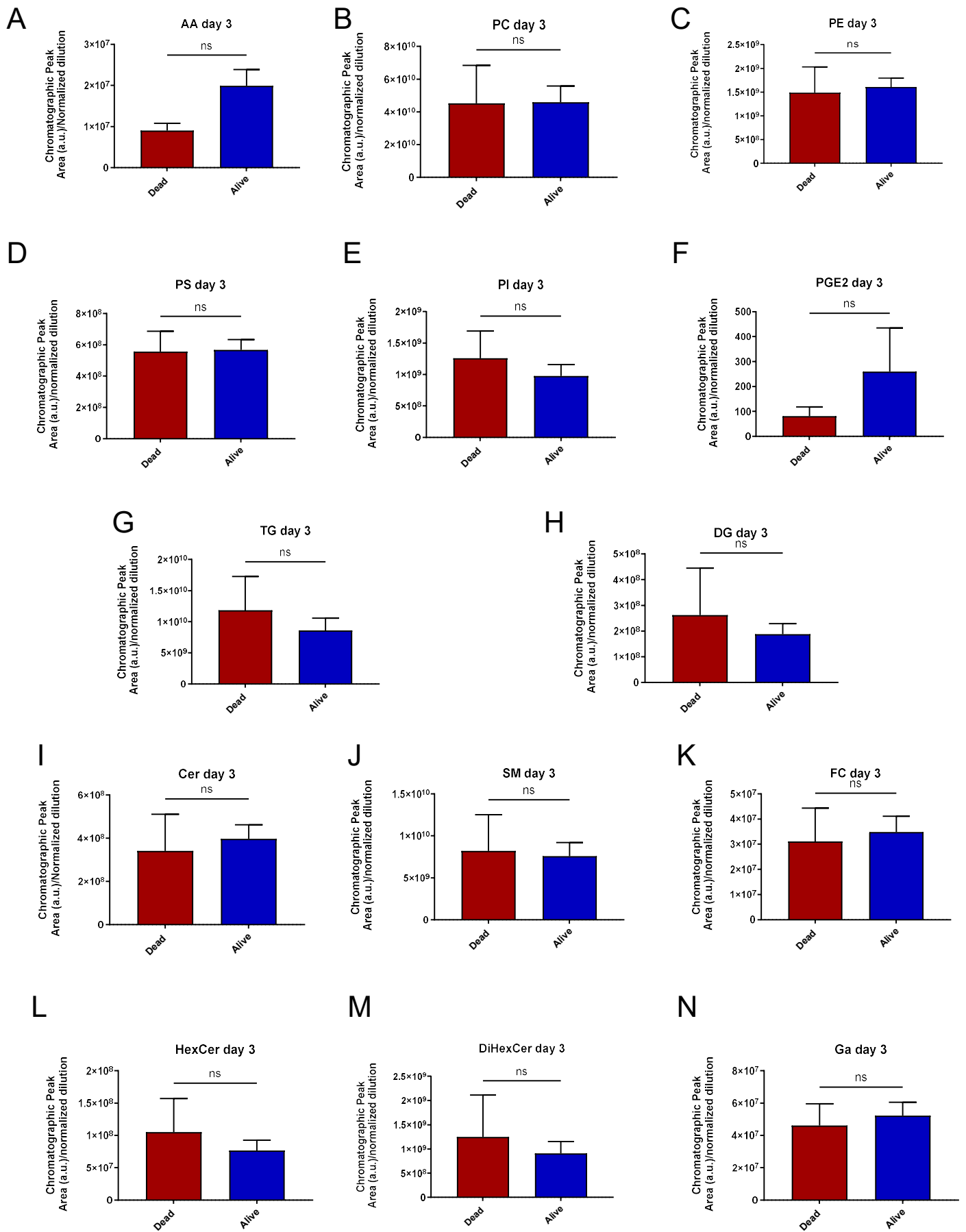
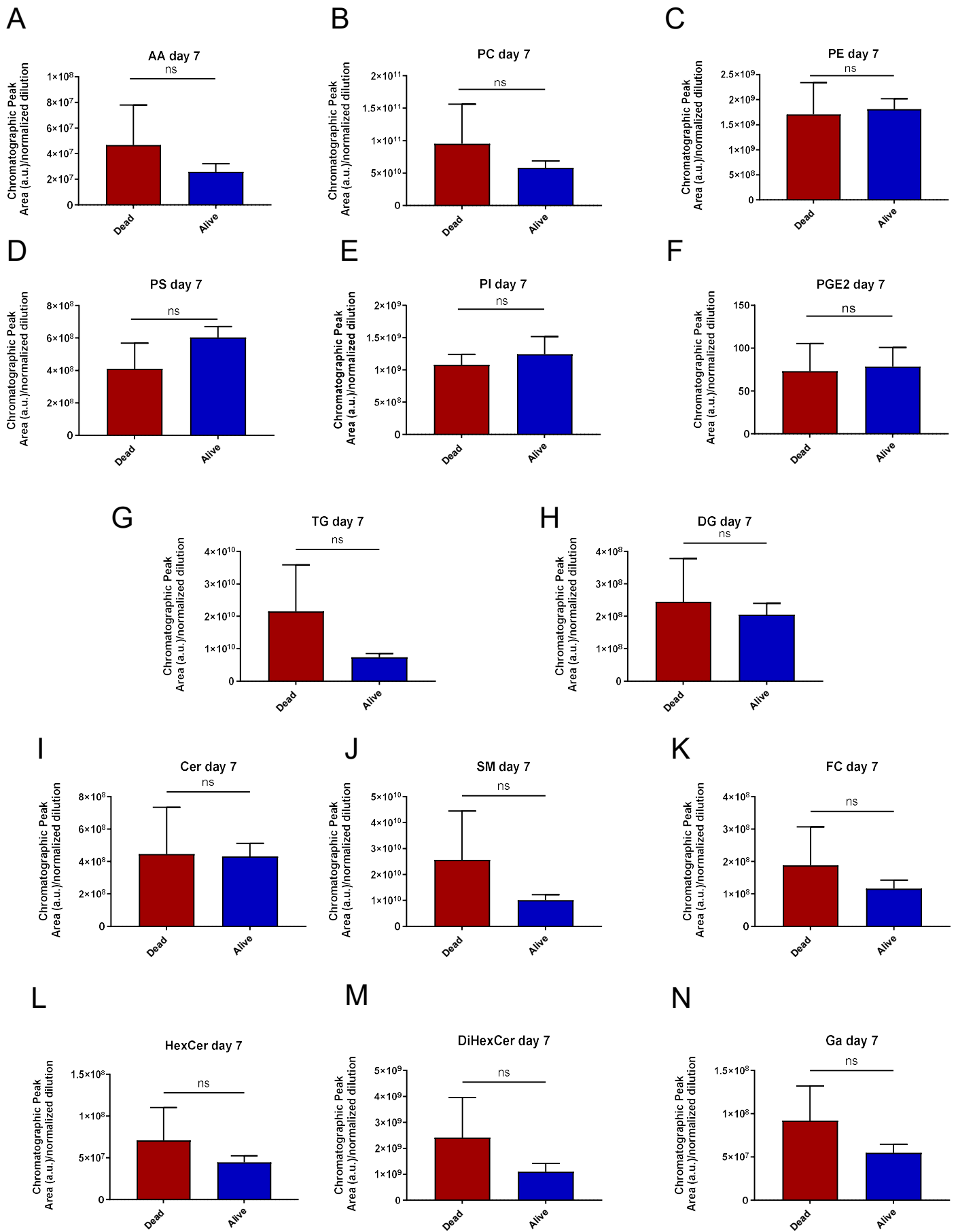


Figure 6





Sup Figure 2



Supp Figure 3