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Interplay Between Pulmonary Membrane Properties and Lung Disease: A Study of Seven Bottlenose Dolphins

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eLife Assessment

The study presents data on the possible connection of respiratory pathologies like pneumonia in a cohort of dolphins with altered composition and concomitant perturbed biophysical properties of pulmonary surfactant complexes. Overall, it is a **valuable** contribution that could be of interest to scientists in the field. However, the study as it appears somewhat **incomplete** and additional clarification and discussions are required in order to explain a few methodological questions that may limit the impact of the work considerably.

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Abstract

Pneumonia is the leading cause of morbidity and mortality of bottlenose dolphins *Tursiops truncatus*. We investigate a series of rare and opportunistic samples of pulmonary surfactant membranes (PSMs) extracted from lungs of seven dolphins in the care of the U.S. Navy Marine Mammal Program. We found a striking correlation between PSM structure, lipidome, and mechanical properties with the severity of lung injury. Specifically, lipidomics reveals exacerbated contents of cardiolipins, confirming a result obtained for terrestrial mammals afflicted by experimental pneumonia. Employing a battery of X-ray scattering, atomic force, and electron microscopy, we evaluate how the altered lipid composition impairs the structural integrity of the PSM and leads to dehydration and enhanced rigidity. Our findings demonstrate that the function of pulmonary surfactant membranes goes far beyond lowering alveolar surface tension, regulating their biochemical and biophysical properties with lung pathology progression. This knowledge will be useful to the development of future diagnostics and therapeutics of respiratory diseases targeting lung membranes.

Introduction

The mammalian lung evolved to enable highly efficient gas exchange in the alveoli while mechanically undergoing repeated expansion and contraction during breathing. This process is particularly efficient in diving mammals that have cartilaginous airways extending to the mouth of the alveolar sacs and conforming lungs that allow: i) movement of air during lung compression,

ii) shutting down of N_2 adsorption and gas exchange, and iii) alveolar collapse. Denison et al. (1971) [\[1\]](#) While humans exchange about 20% of their lung capacity with each breath, dolphins take short and deep breaths, with an exchange of 75% to 90% of air in one-third of a second. Fahlman et al. (2015 [\[2\]](#), 2017 [\[3\]](#)) These breathing adaptations put them at increased risk of respiratory infections by enabling deep lung exposure to airborne threats at the marine surface. Pneumonia is an inflammatory respiratory disease that primarily affects alveoli and is the primary cause of morbidity and mortality in marine mammals. Venn-Watson et al. (2012) [\[4\]](#); Díaz-Delgado et al. (2018) [\[5\]](#)

Central to facilitating gas exchange, Olmeda et al. (2010) [\[6\]](#); Kim et al. (2020) [\[7\]](#) lung innate immunity, Chronopoulos et al. (2010) [\[8\]](#) and lung mechanics Prokop and Neumann (1996) [\[9\]](#); Hermans et al. (2015) [\[10\]](#) is the alveolar air/aqueous hypophase lining. This consists of a phospholipid-rich monolayer that reduces the surface tension in the alveoli to 5–20 mN/m, and that is why it's called *lung surfactant*. In reality, this interfacial monolayer directly connects to a complex and dynamic lipoprotein system occupying the whole volume of the alveolar hypophase. An updated squeeze-out model describes the monolayer interconnected to tubular membranous structures (tubular myelin, TM) Xu et al. (2020) [\[11\]](#); Keating et al. (2012) [\[12\]](#); Zuo et al. (2008) [\[13\]](#); Zhang et al. (2011b) [\[14\]](#) stemming from lipoprotein multilamellar bodies (MLBs) that are secreted by Type II pneumocytes. Lettau et al. (2022) [\[15\]](#); Ochs (2009) [\[16\]](#); Olmeda et al. (2017) [\[17\]](#); de la Serna et al. (2004) [\[18\]](#)

Lung surfactant irregularities have been associated with a variety of lung pathologies, and it should be expected that its composition, structure, and mechanical properties will change in response to lung injury. Milad and Morissette (2021) [\[19\]](#) Indeed, neonatal respiratory distress syndrome (NRDS) is characterized by insufficient pulmonary membrane production, whereas pulmonary alveolar proteinosis leads to pulmonary surfactant accumulation in the alveoli Milad and Morissette (2021) [\[19\]](#); Dushianthan et al. (2011) [\[20\]](#); Wang et al. (2021) [\[21\]](#) leading to impaired surface activity and difficulty breathing. Mutations in pulmonary membrane-related genes (i.e., biogenesis and regulation) have been identified as an important cause of severe pulmonary diseases Whitsett et al. (2015) [\[22\]](#); van Moorsel et al. (2021) [\[23\]](#) and even impact nonspecific lung pathologies such as lysosomal storage diseases. Paget et al. (2021) [\[24\]](#) Moreover, pulmonary membrane composition also plays a role in the diagnosis of lung diseases. Levels of specific surfactant proteins, for example, may have utility as prognostic and diagnostic indicators in patients with interstitial lung disease. Kaieda et al. (2020) [\[25\]](#); Takahashi et al. (2006) [\[26\]](#) Recently, two lung surfactant proteins (cetacean surfactant protein D and lipopolysaccharide-binding protein) were identified as potential biomarkers of pneumonia in bottlenose dolphins. Hamano et al. (2023) [\[27\]](#)

The pulmonary surfactant membrane lipidome, however, is less explored for hallmarks of lung disease and diagnosis, despite evidence that pneumonia affects levels of glycerolipids, glycerophospholipids, and phosphatidylglycerol. Shan et al. (2018) [\[28\]](#) Nancy Ray and co-authors Ray et al. (2010) [\[29\]](#) reported that pneumonia leads to a change in pulmonary membrane phospholipid composition in several terrestrial mammal species, specifically to an accumulation of a mitochondrial phospholipid, cardiolipin, in lung fluid. We have previously shown that excess cardiolipin compromises the structure and elasticity of pulmonary membranes, leading to gas exchange imbalance. Kim et al. (2020) [\[30\]](#); Porras-Gómez et al. (2022b) [\[31\]](#) Disruption of membrane and tissue elasticity affects alveoli compressibility Petty et al. (1979) [\[32\]](#) and increases alveolar surface tension, leading to atelectasis Günther et al. (2001) [\[33\]](#) and ultimately ineffective breathing. Günther et al. (2001) [\[33\]](#); Petty et al. (1979) [\[32\]](#) In this paper, we examine how the severity of respiratory diseases correlates with the lipidic composition, structure, and mechanics of pulmonary surfactant membranes extracted from Navy bottlenose dolphins (*Tursiops truncatus*) exhibiting a range of respiratory pathologies, including pneumonia. These rare, retrospective, and opportunistic samples come from a large biobank dating back decades and belonging to the U.S. Navy Marine Mammal Program. Lung exudates include pulmonary extracts and lung tissue. We found that cardiolipins are predominantly more abundant in abnormal dolphin lung exudates, leading to a loss of elasticity in membranes and lung tissue. We also noted that plasmalogen phosphocholines, a phospholipid class that protects against oxidative damage, appear more

abundant in lung membranes with a recent history of pneumonia compared to chronically diseased lungs and may elucidate an association of plasmatogen activity with early disease stages. We evaluate membrane fluidity and organization and present a link between lateral membrane heterogeneity and the physiological function of native pulmonary membranes under pathological conditions. In summary, we establish strong associations between the lung disease and the biophysical properties of pulmonary membranes extracted from bottlenose dolphins. This knowledge is central to the development of new prevention, diagnosis, and treatment strategies of pulmonary diseases, as well as the potential for repurposing and enhancement of pulmonary membrane replacement therapies. To the best of our knowledge, this is the first study that links pulmonary membrane homeostasis with pulmonary disease in marine mammals.

Results

Lung extracts reproduce the length-scale and structural diversity of native pulmonary membranes

Bronchoalveolar lavages (BALs) were performed as a minimally invasive routine procedure to diagnose Navy dolphins affected by a range of naturally occurring respiratory pathologies, i.e., illness and pathology were not artificially induced. We performed organic extractions to separate the lipid material from BAL lung exudates. [Fig. 1A](#) depicts the reconstitution of BAL extraction from a healthy animal. To validate that the extraction procedure enabled the study of membrane suspensions, we performed an organic extraction, which led to the collection of crude amphiphilic molecules (lipids and surfactant proteins). Subsequently, we subjected the samples to ultra-centrifugation cycles that separate debris and remaining cells, including neutrophils, leukocytes, and other immune cells that could have infiltrated the hypophase, especially because pneumonia is characterized by increased neutrophil infiltration. We then rehydrated (schematics of [Fig. 1A](#)) the organic phase tagged with a fluorescence lipid probe and inspected the sample by laser confocal fluorescence microscopy ([Fig. 1B](#)). We observe that lung lipid extracts reassemble upon rehydration into small, large, single, and multilamellar vesicles. These aggregate forms reproduce the expected membrane microstructures observed in native alveolar hypophase. Higher resolution transmission electron microscopy (TEM) negative staining images of rehydrated BAL lipids show the presence of elongated and multivesicular (MVS) structures ([Fig. 1C](#)) consistent with previous studies of native BALs. [Lettau et al. \(2022\)](#); [Ochs \(2009\)](#); [Olmeda et al. \(2017\)](#). This indicates that pulmonary membranes can be successfully extracted and reconstituted from BALs of Navy dolphins. No evidence of cells or cell debris could be detected by confocal, optical, or electron microscopy.

Lipidome signatures characterize lung injury

Previous studies have compared bronchoalveolar lavage (BAL) lipids between terrestrial mammals (including humans) to those of marine mammals [Gutierrez et al. \(2015\)](#); [Spragg et al. \(2004\)](#) such as diving pinnipeds (seals). In general, phospholipid concentrations are greater in BAL from pinnipeds than in humans, with a greater percentage of phosphatidylcholine (PC) compared to other phospholipid species. Pinniped surfactant was significantly enriched in the fluidic PC species. This indicates that diving mammals may have larger surfactant pool sizes and larger aggregates with anti-adhesive properties than surfactant from terrestrial mammals. Comparison of odontocete—dolphins and porpoises—lipids to that of pinnipeds found that odontocetes have comparable PC composition to that of pinnipeds. [Gutierrez et al. \(2015\)](#). The difference in fluidic PC levels in marine mammal lung surfactant constituents is likely to facilitate film respreading during rapid alveoli collapse. [Spragg et al. \(2004\)](#); [Denison et al. \(1971\)](#); [Miller et al. \(2006\)](#).

We categorized lung exudates from dolphins with acute/chronic lung disease or no lung disease at the time of sample collection ([Table 1](#)) per diagnosed by the team of U.S. Navy Marine Mammal Program veterinarians. To study the lipidic composition of lung exudates, we performed structural lipidomics and identified ~490 lipid species ([Fig. 2](#)). Lipidomic analysis shows that global lipid

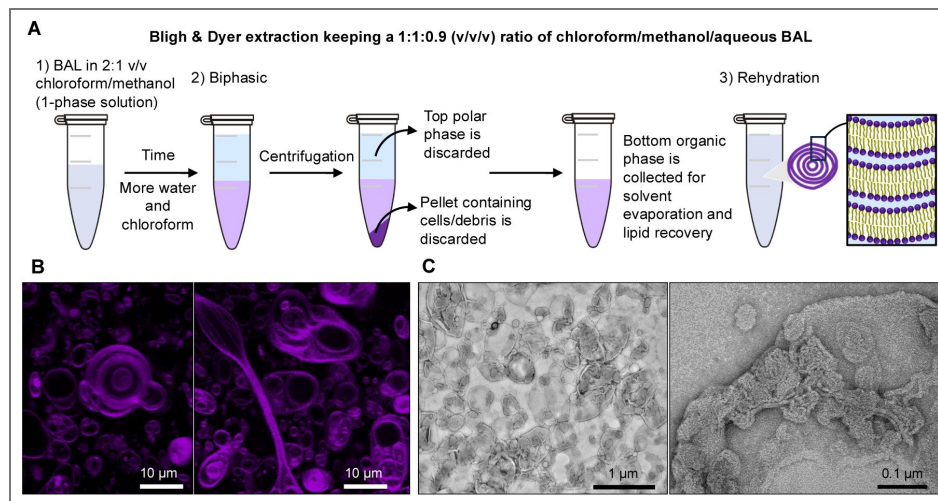


Figure 1. Lung exudate extraction procedure and visualization.

A) Schematics of organic extraction to obtain the lipidic phase in the form of a dry film after solvent evaporation and subsequent lipid self-assembly upon rehydration of the dry film in a capillary tube. B) Laser confocal fluorescence microscopy images of fluorescently labeled pulmonary lipid extracts in water. C) Transmission electron microscopy images of BAL lung extracts from a dolphin with clinically healthy lungs exhibiting multivesicular structures (MVS) and tubular structures.

profiles help distinguish samples by presence or absence of lung disease, as depicted through the principal linear discriminant analysis (Fig. 2A). The multivariate analysis demonstrates that over 70% of lipid variations can be explained by two components that support disease clustering. More-over, among the top discriminant lipid species for lung severity are several cardiolipins (CL), phosphatidylserines (PS), phosphatidylcholines (PC), and plasmalogens (Fig. 2B). To identify the potential involvement of lipids in biophysical and biochemical processes, we performed a lipid ontology (LION) analysis. Compared to lung extracts with no lung disease, we identified a significant enrichment within components of membranes in the endoplasmic reticulum (ER) and mitochondria, as well as plasmalogens and glycerophospholipids in lungs with acute/chronic disease (Fig. S1). Here we show marine mammals also accumulate excess cardiolipins during pneumonia (Fig. 2C). Alterations in the degree of saturation are also observed with disease, yielding more acyl chains at lower unsaturation levels. Lipidomic profiling confirmed that lung fluid from dolphins with chronic/acute lung disease (Fig. 2D) contains higher levels of a wide variety of cardiolipin species, compared to lungs from dolphins with no lung disease. Lung disease significantly alters lipid signatures in lung exudates.

Lung pathology disrupts lateral membrane domain organization

Pulmonary membranes comprise a fine balance of saturated and unsaturated lipids that laterally phase-separate into membrane domains de la Serna et al. (2004), 2013; Dhar et al. (2012); Alonso et al. (2004) that maintain mechanical robustness and rapid re-spreading to reduce surface tension during breathing. Postle et al. (2001) The observed alterations in lipid composition and saturation (Fig. 2) are likely to impact the lateral organization of lung membrane domains. We analyzed extracted multilayered membranes by Atomic Force Microscopy (AFM) to evaluate morphological changes indicative of phase separation. It is important to emphasize that the samples studied are not adsorbed monolayers, as is most common for AFM studies of lung surfactants, but rather multilayered bilayer stacks (≈ 100 nm in thickness) of lung membrane extracts that were supported onto solid substrates by spin-coating at near 100% RH. AFM analysis conveys information about the morphology and spatial distribution of phase-separated domains such as lipid *gel*-phase (L_β) and the *liquid*-phase (L_α) domains (Fig. S2). The L_β phase comprises thicker bilayers with well-packed saturated lipids forming stiffer domains vs. (L_α) domains, which are thinner and more *fluid*-like. AFM fast force mapping is a valuable complement to topography analysis as it quantitatively evaluates the interaction forces between the probe and the sample. The combination of topography and force mapping AFM demonstrates that locally L_β domains are indeed stiffer than the surrounding L_α phase and lower adhesion forces. In a similar fashion, local domain mechanical properties have been mapped for DPPC:DOPC supported bilayers with AFM. Saavedra V et al. (2020) Our data suggests that lung pathology significantly disrupts the morphology of phase-separated L_β domains, including size and shape. Quantitative AFM analysis shows that lung disease leads to a reorganization of the immiscible L_β - L_α membrane domains. The optimization of L_β domain area/perimeter (A/P) ratio is indicative of the ability to modulate line tension between the L_β and the surrounding L_α phases. Lin et al. (2007); de la Serna et al. (2004) Lung exudates from dolphins with lung disease conditions display larger and more elongated L_β domains (Fig. 3A, right). In contrast, in membranes coming from healthy lungs, the L_β phase coalesces into more circular domains (i.e., a wider population around circularity index of 0.8) covering larger surface areas (Fig. 3B-C), indicating the ability to sustain higher line tension.

Lung disease modulates membrane and tissue elasticity

We previously employed AFM to demonstrate that pneumonia-associated phospholipids (i.e., cardiolipins) significantly increase the Young modulus of stacked bilayers in both ternary lipid membrane models and bovine lung extracts. Porras-Gómez et al. (2022b) This stiffening arises because cardiolipin promotes fusion of apposed bilayers within the stacks, which reduces interlayer water content and concomitant higher inter-bilayer adhesion. Consistent with this mechanism, our AFM measurements showed higher adhesion forces in samples with acute or chronic lung disease (Fig. 4A, right), and these effects correlate with elevated cardiolipin

Figure 2. Lipidomic analysis.

A) Principal linear discriminant analysis of lipids from lung exudates. Replicates are represented by similar symbols. The values for components 1, 2, and 3 are shown. B) Top 30 species that discriminate between groups and account for variation within component 1. Cardiolipin species are indicated by red triangles. C) Cardiolipin abundances depicted as a Z-score. D) Cardiolipin species analysis in samples from acute/chronic pulmonary infection. Cardiolipin fatty acyl-chain length is shown on the x-axis, and cardiolipin fatty acyl unsaturation level (double bonds) is shown on the y-axis.

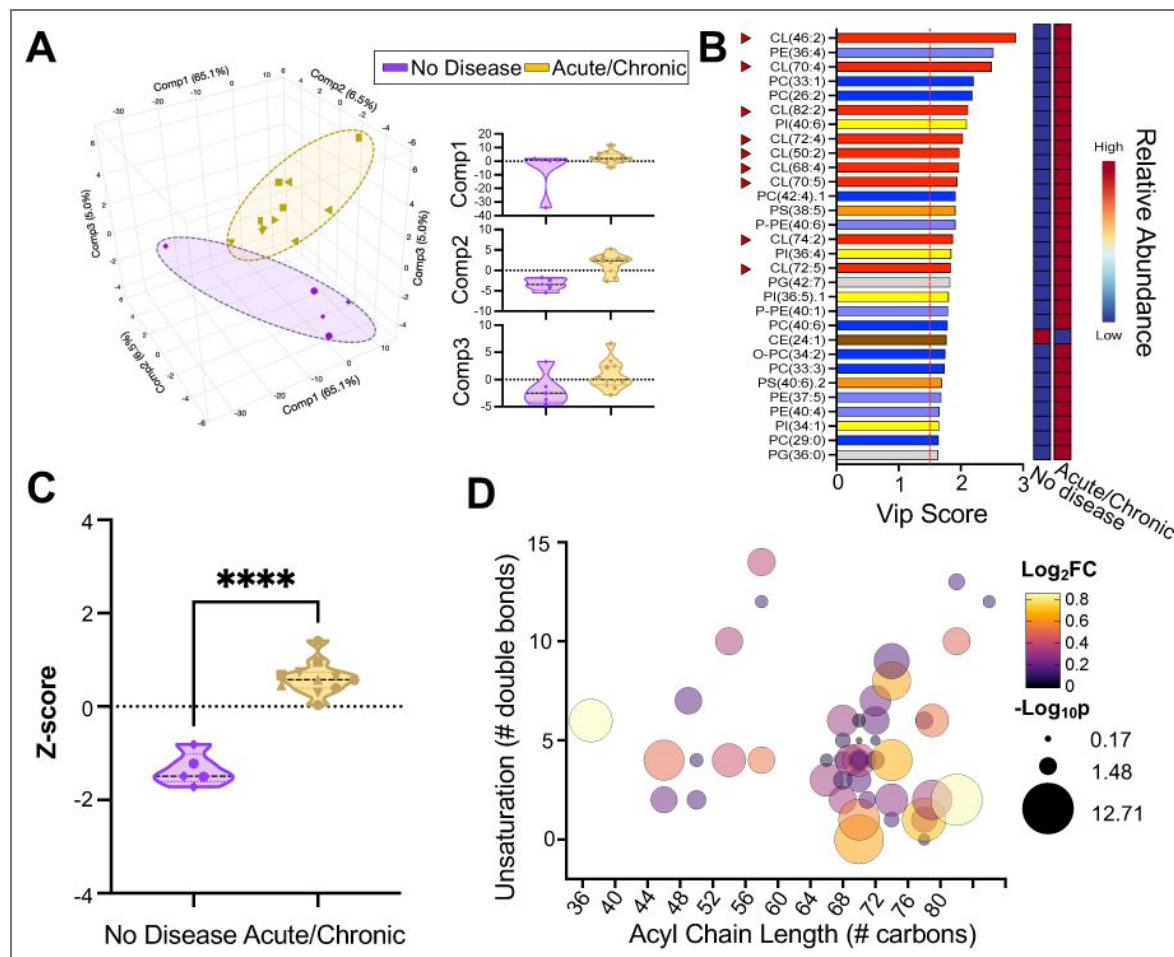


Table 1. Categories assigned to *Tursiops truncatus* lung exudates with corresponding number of animal samples (*n*)

Pulmonary condition category	Description
Chronic or acute lung disease (<i>n</i> =5)	History of chronic or acute pulmonary infection such as bacterial or fungal pneumonia, mild alveolar interstitial syndrome, or recent history of pneumonia and recovery after treatment
No lung disease (<i>n</i> =2)	No history of pulmonary disease at the time of sample collection

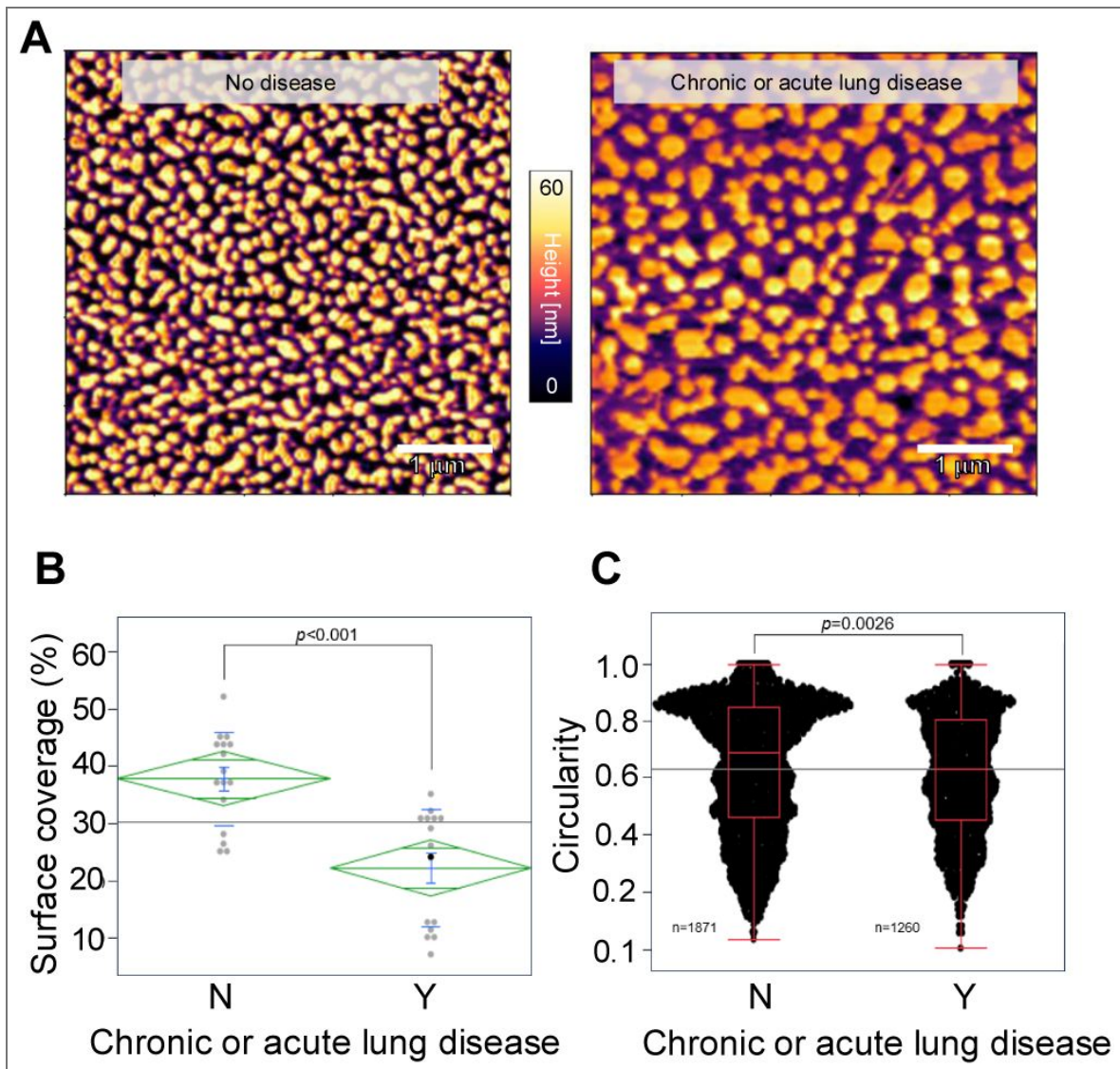


Figure 3. Microscopy analysis of lung extracts and lung tissue.

A) AFM topography maps of multilayered lung fluid extracts from dolphins with no disease and with lung disease. B) One-way ANOVA analysis of surface coverage with a significant p-value. C) Oneway ANOVA analysis of domain circularity by lung disease condition, resulting from topography map analysis with a significant p-value. The number of domains analyzed is shown.

abundance detected in samples from acute and chronic lung disease (Fig. 4B). Moreover, a strong positive correlation was observed between cardiolipin abundance and the Young modulus ($R^2 = 0.7732$; Fig. 4C), indicating induced stiffening of the pulmonary membranes in chronic or acute pulmonary conditions. Notably, stiffening is also observed in lung tissue affected by lung disease (Fig. S3A). Two lung tissue samples from dolphins with lung disease and one from a healthy dolphin postmortem were analyzed with fast force mapping (Fig. S3B-D). In the same way, the Young modulus was higher in diseased tissue. In addition to AFM force mapping, we utilized transmission electron microscopy (TEM) negative staining imaging to investigate the nanoscale morphology of pulmonary membranes in lung tissue (Fig. S3E-G). Lung tissue from dolphins with no lung disease present well-hydrated multilamellar bodies as well as vesicles and well-defined elongated membranes. In contrast, chronic/acute lung disease samples also display well-hydrated multilamellar bodies (Fig. S3F, right) but denser objects and less well-defined membranes can be detected (e.g. all Fig. S3G panel images). Moreover, several membrane forms and dense-core particles of < 100 nm in diameter appear in the lung tissue sample coming from dolphins with lung disease, which could correspond to packed inclusion bodies from alveolar type II cells. Have-Opbroek et al. (1990)

Lung pathology alters pulmonary membrane ultrastructure

To further understand the effect of disease severity and cardiolipin excess on the structural integrity of pulmonary membranes, we inspected membrane suspensions of lung exudates using small and wide-angle X-ray scattering -SAXS/WAXS (Fig. 5A-C). Fig. 5A-B presents SAXS/WAXS data as integrated intensity (arbitrary units) as a function of wave vector q (nm^{-1}) for lung extracts from dolphins with and without lung pathologies. Several peaks can be observed in the SAXS regime (Fig. 5A). As shown in Fig. 1, extracted pulmonary membranes can be reconstituted in water, self-assembling into several vesicular and multilamellar bodies (MLBs). SAXS is very sensitive to membrane periodicity at the nanoscale, hence allows for accurate probing of inter-bilayer distances (d) in MLBs. The observed Bragg peaks are consistent with a multilamellar phase of pulmonary lipids yielding peak q -position ratios of 1:2:3:4(...). This indicates that lung extracts indeed self-assemble into MLBs where bilayers are coherently correlated and separated by a water layer of conserved thickness. In addition to the 100 and 200 Bragg peaks, a scattering form factor envelope is also observed, indicating the presence of vesicular structures. Compared to healthy dolphins, the SAXS pattern is clearly altered for pathological samples. More peaks (up to 500) can be resolved that shift to higher q values, and the form factor envelope is less discernible. Similar results were obtained when adding cardiolipin to lung membrane model systems. Steer et al. (2018). In addition, our lab and others previously found that bovine-extracted pulmonary membranes exhibit a coexistence of two lamellar phases, de la Serna et al. (2013); Larsson et al. (2002); Steer et al. (2018) one rich in unsaturated lipids and one in saturated lipids (0.5-2 nm thicker) that merge at temperatures above 40°C. de la Serna et al. (2013). The SAXS data of lung exudates present a single lamellar phase even though lipid domains are clearly present, as shown in Fig. 3A and will be later demonstrated by cryogenic electron microscopy (cryo-EM). This is likely because the phase-separated domains are small and not fully aligned in the MLBs.

The average separation between bilayers in a multilayer stack (d -spacing) results from the sum of the lipid bilayer thickness d_b and the water layer thickness d_w and can be calculated from the n th diffraction peak as $d = n2\pi/q_n$. In our previous work, Steer et al. (2018), when cardiolipin was artificially introduced, we observed that phase separation was exacerbated and that the two multilamellar systems had very different d -spacings ($\Delta d > 5$ nm), which was attributed to different water layer thicknesses. Fig. 5C plots d obtained from membranes extracts calculated from q_{100} in Fig. 5A. The bilayer thickness d_b should not vary significantly with lipid composition and was previously estimated to range 5-8 nm Porras-Gómez et al. (2022b) (see also later Fig. 5D). Hence, drastic changes in d likely arise from alterations in the hydration layer d_w . SAXS data indicate that lung fluid from dolphins with pulmonary disease exhibits thin water layers ($d \approx 7$ nm indicates d_w of 2-6 nm). Conversely, samples from dolphins with no lung diseases exhibit very well-hydrated pulmonary membranes with water layers of $d_w \approx 40$ nm. These results reinforce that

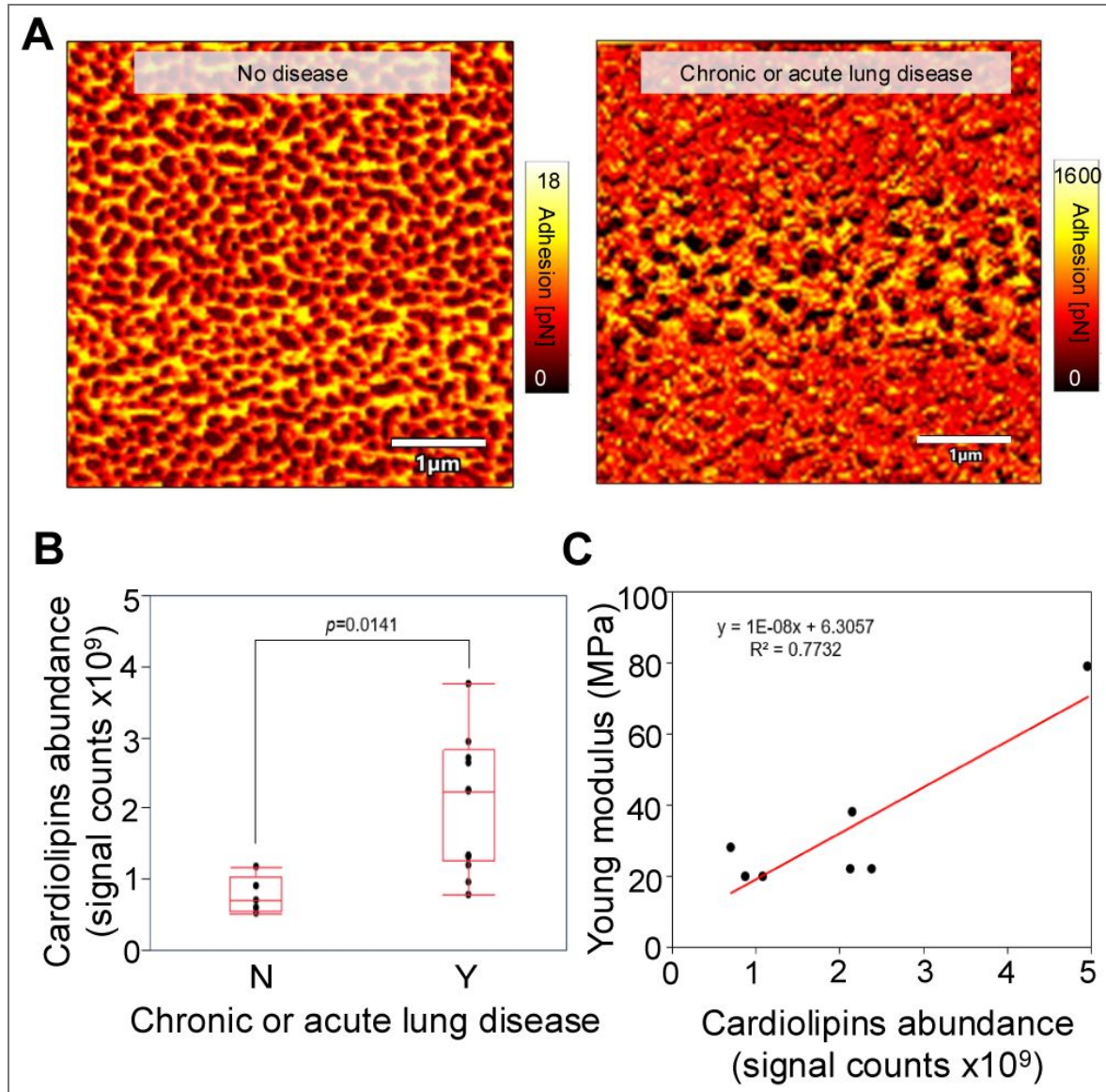


Figure 4. Microscopy analysis of lung extracts and lung tissue.

A) AFM adhesion maps of multilayered lung fluid extracts from dolphins with no disease and with lung disease. B) One-way ANOVA analysis of cardiolipin abundance by lung disease condition. Cardiolipins' abundance values correspond to the normalized signal counts to internal standards and sample weight. C) Bivariate fit of Young modulus by cardiolipin abundance. Linear fit and corresponding equation are shown. Elasticity means of lung extracts were calculated from fast force mapping.

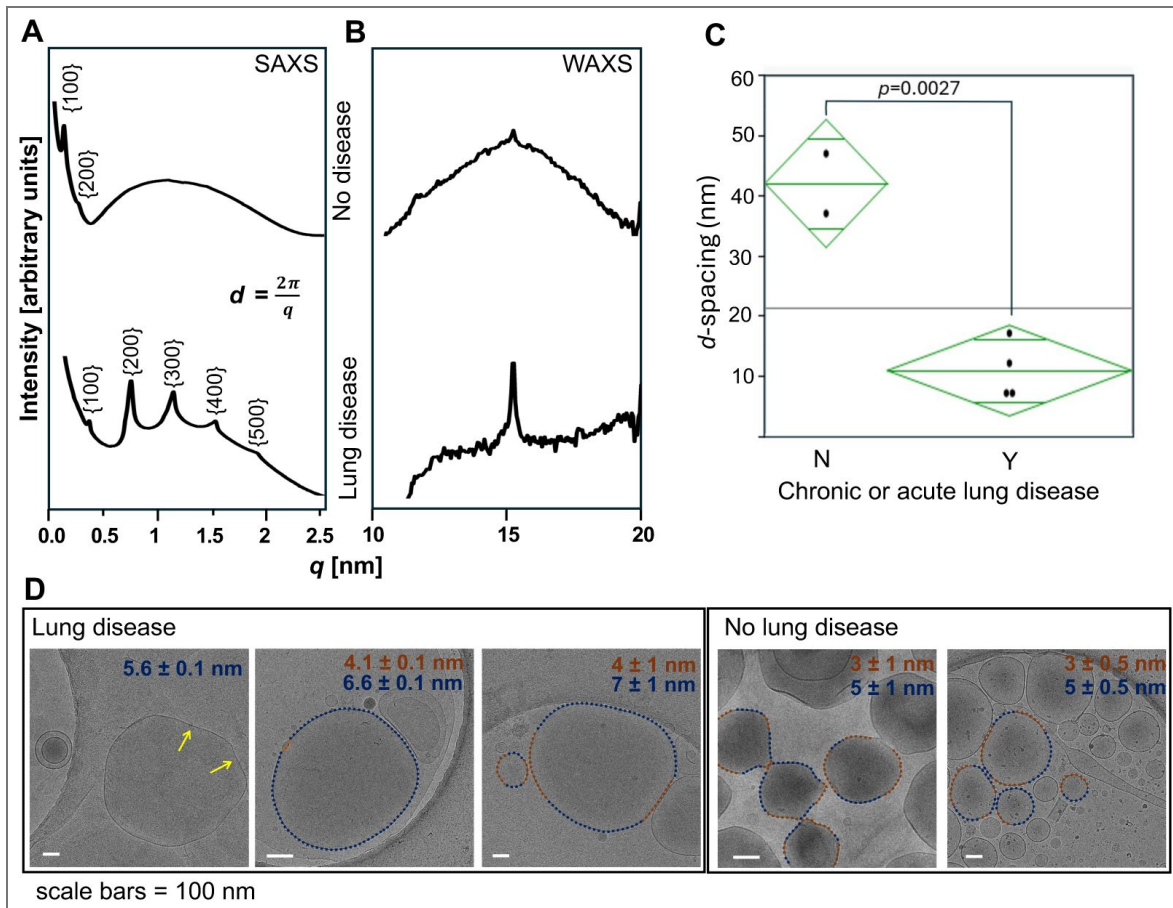


Figure 5. Structural characterization of pulmonary membrane complexes.

SAXS (A) and WAXS (B) Intensity vs q data. C) One-way ANOVA analysis of lattice parameter d response to lung disease condition. d -spacing was calculated from SAXS peak positions in A. D) Cryo-EM data where scale bars correspond to 100 nm. The Cryo-EM images depict lung extracts from dolphins with chronic or acute lung disease (left) and dolphins with no lung disease (right). Nanodomain size is highlighted in blue (> 5 nm) and in orange (< 5 nm), and was estimated with rim analysis.

pneumonia-associated membrane alteration leads to aggregation of lipids and dehydration of pulmonary multilamellar bodies. As expected, pulmonary extracts from dolphins with no lung disease exhibit large d -spacing, indicating that lung membranes in the alveolar hypophase are fully hydrated, which is imperative for membrane protein function and general lung membrane homeostasis.

WAXS probes membrane organization at the molecular level, i.e., the configurations of lipid fatty acid chains, related to membrane fluidity and acyl chain order. The shape and width of the characteristic WAXS peak at $q \approx 15 \text{ nm}^{-1}$ for lipid bilayers enable the identification of fluid/gel phases (L_α/L_β) (Fig. 5B). For instance, lung extracts from dolphins with no lung disease diagnosis exhibit broader peaks indicative of the L_α phase, fluid nature of these membranes. On the contrary, lung extracts from dolphins with chronic/acute lung disease display a coexistence of sharper peaks, which are indicative of a L_β phase, containing closed-packed fatty acid chains. This could be attributed to alterations in acyl chain saturation and/or clustering induced by disease both consistent with the observed increased membrane stiffness (Fig. 4). Porras-Gómez et al. (2022b).

With cryogenic electron microscopy, we observed vesicles (Fig. 5D) in extracts from both diseased (left group) and healthy (right group) lungs displaying high-contrast phospholipid bilayers. The images were analyzed with respect to bilayer thickness and, on average, bilayers from lungs with disease are 1-2 nm thicker than from healthy lung membranes. In line with the presence of L_α/L_β domains Heberle et al. (2020); Cornell et al. (2020) we detect two distinct bilayer thicknesses in most vesicles, the L_β represented in blue and the L_α represented in orange, with the L_β domains being roughly 2-3 nm thicker. The main difference between the vesicles from diseased lungs compared to healthy lungs is the size of the vesicle and the fact that larger domains of thicker and stiffer L_β (blue) membranes are detected. This is clear by the fact that membranes persist in a straight form (yellow arrows on the left group). This, together with thicker L_β domains and a prominent WAXS peak at 15 nm^{-1} , is consistent with exacerbated phase separation and membrane stiffening caused by cardiolipin enhancement. Healthy membranes appear more fluid, and a few membrane contacts are apparent between the L_α domains of apposed vesicles, which are attributed to the natural process of surfactant protein-driven membrane contacts. Parra et al. (2013). Indeed, multilamellar polygonal vesicles have been observed by cryo-EM with surfactant protein preferentially located at the membrane bends. de la Serna et al. (2013). We observe that healthy discrete multilamellar vesicles display an average bilayer separation of $d \approx 6.6 \text{ nm}$ as well as membrane bends where protein is putatively enriched (Fig. S4). It is also possible that cholesterol facilitates the coexistence of untilted and tilted microdomains that would change the bilayer angle. Indeed, protein-free binary mixtures of DPPC and cholesterol also form polygonal vesicles (Fig. S5). We propose that both cholesterol and surfactant proteins are enriched at the bends of lung membranes.

Discussion

Supraphysiological amounts of cardiolipins have been detected in lung fluid in terrestrial mammals with pneumonia Ray et al. (2010) and have been shown to disrupt the structure, mechanical properties, and oxygen permeation of the lipid membrane. Steer et al. (2018); Porras-Gómez et al. (2022b); Kim et al. (2020). In this work, we found analogous alterations in the lipidome, phase behavior, and mechanical and structural properties of lung membranes extracted from clinically affected Navy bottlenose dolphins. This knowledge has the potential to impact future developments of lung disease diagnosis and therapies targeting lung membranes in mammals.

Lipidome analysis shows that both cardiolipins and plasmalogen phosphocholines are more abundant in diseased lungs compared to healthy marine mammal lungs. Plasmalogens, phospholipids associated with inflammatory responses, Bozelli Jr et al. (2021); Braverman and Moser (2012) are more abundant in lungs with a recent history of disease compared to chronic disease, which positions plasmalogen activity in the early stages of disease. The fact that lung extracts from dolphins with no lung diseases present reduced levels of plasmalogen

phosphocholines could be related to the absence of inflammatory triggers. However, further research is required to identify a relation between lung pathology and plasmalogen abundance, and in this paper, we focus on the effects of cardiolipin. While lipidomics clearly suggests that the presence of lung disease significantly alters lipid signatures, our analysis is restricted by the low number of disease-free samples. Lung exudates are collected as part of BAL retrieval, which is not routinely performed in healthy dolphins. Our study mitigates this by employing a multi-prong, robust, and quantitative approach where lipidomics data is backed by several scattering and imaging tools done in multiple replicates per sample, as well as robust statistical analyses.

Alterations in saturation and lipid profiles are expected to affect the phase behavior of membranes. Unsaturated phospholipids tend to form fluid films that are not as efficient at lowering surface tension but present better respreading relative to fully saturated phospholipids. [Notter \(2000\)](#)  Here, we observe that membranes extracted from diseased lungs have less saturation of acyl chains and stronger L_{β} phases, resulting in bigger yet less circular domains. In other words, diseased membranes have less ability to sustain the height mismatch and line tension between the L_{β} and the L_{α} phases. [García-Sáez et al. \(2007\)](#)  Loss of membrane homeostasis has been associated with the enhancement of gel L_{β} phases in senescing cells, [Ayres \(1984\)](#) ; [Thompson et al. \(1987\)](#)  during irreversible ischemic cell injury, [Farber et al. \(1981\)](#)  and in erythrocyte cells in cognitive impairment patients. [Vignini et al. \(2019\)](#)  Also, cold-shock injury in cells directly results from $\alpha L_{\alpha}/L_{\beta}$ phase transition in plasma membranes. [Drobnis et al. \(1993\)](#) 

Native pulmonary membranes, as the ones used in this study, comprise multiple lipid species, hydrophobic surfactant proteins, and the molecular organization in these domains remains unclear. [Sear and Cuesta \(2003\)](#) ; [Jacobs and Frenkel \(2017\)](#)  The L_{β} - L_{α} domains have been reported to align across lipid-stacked multilayers. [Mills et al. \(2008a,b\)](#)  In fact, a reversible monolayer-to-multilayer transition has been reported to occur in pulmonary membranes after further compression. [Zuo et al. \(2008\)](#) ; [Zhang et al. \(2011a\)](#) ; [Porras-Gómez et al. \(2022a\)](#)  Such transition seems to initiate at the edge of the L_{β} domains where there is an acyl chain mismatch [Keating et al. \(2012\)](#)  and altered elastic and frictional properties. [Zuo et al. \(2008\)](#)  The resultant multilayer structures could provide additional stability, thereby allowing very low surface tensions. [Zuo et al. \(2008\)](#)  This process is reversible, indicating that after squeeze-out, the excluded multilayers remain closely associated with the interfacial monolayer rather than escaping into the hypophase. The excess lipid stored in the multilayer assemblies readily returns into the interface to decrease surface tension. Hence, multilayer structures are required for pulmonary film stability, facilitating low compressibility and low surface tension, and serving as a lipid reservoir for film replenishment. [Zuo et al. \(2008\)](#)  Here, we demonstrate a direct link between lateral membrane heterogeneity and the physiological function of native pulmonary membranes under pathological conditions.

Finally, as expected, lung pathology impacts the mechanical integrity of lung tissue. Pulmonary membranes and tissue from dolphins with no lung disease appear softer because they are composed mostly of discrete membranes separated and cushioned by water layers. In contrast, lung extract membranes from dolphins with chronic/acute lung diseases are dehydrated [Steer et al. \(2018\)](#)  and highly interconnected by hydrophobic cardiolipin-rich stacks, leading to membranes with higher Young modulus [Porras-Gómez et al. \(2022b\)](#)  and stronger adhesion forces. Similar phenomena were observed in porcine pulmonary extracts, where membranes exposed to chemicals known to show acute inhalation toxicity were not able to reach low surface tensions upon compression compared to the control. [Da Silva et al. \(2021\)](#)  The latter correlates with the fact that these same samples exhibit smaller d -spacing related to their state of aggregation and dehydration.

Overall, this study advances understanding of how specific lipid alterations fundamentally impact the structure, composition and mechanics of diseased marine mammal lung membranes. Findings from Navy bottlenose dolphins highlight the role of membrane heterogeneity in disease progression and lay the groundwork for improved diagnostic and therapeutic strategies targeting pulmonary membranes in mammals.

Materials and methods

Animals

A total of 7 bronchoalveolar lavage (BAL) samples opportunistically collected from U.S. Navy Marine Mammal Program bottlenose dolphins, *Tursiops truncatus*, between February 2017 and April 2022, were analyzed in this study. Samples from Navy animals were collected during their routine care and under the authority codified in U.S. Code, Title 10, Section 7524. Secretary of the Navy Instruction 3900.41H directs that Navy marine mammals be provided the highest quality of care. The U.S. Navy Marine Mammal Program (MMP), Naval Information Warfare Center (NIWC) Pacific, houses and cares for a population of bottlenose dolphins and California sea lions in San Diego Bay (CA, USA). NIWC Pacific is accredited by AAALAC International and adheres to the national standards of the U.S. Public Health Service policy on the Humane Care and Use of Laboratory Animals and the Animal Welfare Act. Five BAL samples were collected opportunistically from animals with naturally occurring respiratory diseases of variable severity. Two BAL samples were also collected opportunistically from the same species without clinical evidence of lung pathology. Based on bacterial and fungal cultures, gross and histopathologic diagnoses, the study included one case of active pneumonia, one case of chronic pulmonary disease of unknown etiology, one case of chronic pulmonary aspergillosis and methicillin-resistant *Staphylococcus aureus* (MRSA), one case of pulmonary aspergillosis with secondary stenotic airways, one case of suspected *Aspergillus* pneumonia at the time of collection, one case with no prior history of pulmonary disease but with recent ultrasound evidence of alveolar interstitial syndrome, and one case with clinically normal lungs where lung extracts were collected post-mortem. In addition, post-mortem lung tissue samples from 3 dolphins collected during necropsies were included – one sample from a naturally-aborted second-trimester fetus, one sample from a geriatric dolphin with multifocal mild mineralization in the epithelium of the terminal bronchioles and alveolar septa, and one sample from a dolphin with histopathologic evidence of interstitial fibrosis with acute bacterial bronchopneumonia.

Bronchoalveolar lavage protocol

The raw marine mammal lung exudate was collected via standard bronchoalveolar lavage (BAL) aspiration techniques using a sterilized video endoscope (Karl Storz Slim Gastroscope, outer diameter 5.9 mm, working length 1,100 mm, biopsy channel 2 mm). Briefly, the tip of the bronchoscope was wedged gently in a lung bronchus. A divided, weight-adjusted volume of sterile saline solution (NaCl) was infused, followed by syringe aspiration of the combined fluid and lung exudate. A portion of the recovered fluid and exudate was aliquoted into a large sterile vial and immediately placed into a -80 °C freezer for pulmonary membrane analysis.

Lipid extraction

Lipid extraction was performed according to a modified Bligh and Dyer procedure, [Bligh and Dyer \(1959\)](#) [\[1\]](#) keeping a ratio of 1:1:0.9 (v/v/v) between chloroform/methanol (HPLC grade) and the corresponding aqueous BAL sample. A volume of 200 µL of BAL fluid was placed in a microcentrifuge tube. Volumes of 500 µL of methanol and 250 µL chloroform (chilled to -20 °C) respectively were added. Tubes were mixed using a vortex for 10 sec. The 1-phase solution was left still at 4 °C for 15-30 minutes. Afterward, 250 µL of ultrapure water and 250 µL of chloroform were added, respectively. The solution, which becomes biphasic at this point, was centrifuged for 10 min at 16000 xg. Finally, the upper polar phase of the solution was discarded, while the bottom organic phase containing the lipid material was transferred to a clean and previously weighed vial. The organic solvent was evaporated in a vacuum desiccator overnight. Weighted dried lipid extracts were stored at -20 °C. Extracts contain all lipid species and hydrophobic surfactant proteins SP-B and SP-C.

Lipidomics

Dry lipid extracts from BALs were redissolved in a solution of 100 μ L of methanol:chloroform (1:2 v/v) and subsequently analyzed by using the Thermo Q-Exactive MS system in the Metabolomics Laboratory of the Roy J. Carver Biotechnology Center, University of Illinois Urbana-Champaign. The mass spectra acquisition protocol was previously reported. Zhang et al. (2018) [\[1\]](#) The lipid signal responses (peak area counts) were normalized to the corresponding internal standard signals and sample weight. Lipid species were identified by lipid headgroup and fatty acid chain lengths resulting from the generated ions via electrospray ionization (ESI). Since this is based on lipid species affinity to ESI, data are presented as relative abundance, allowing comparison of lipid species between samples. Lipidomics was performed on duplicates and triplicates depending on the sample.

Lipidomic data analysis

For the lipid analysis, the full data set contained quantitative information from ≈ 1060 lipid species. Lipids with $>80\%$ missing values were filtered out from the analysis (below the detection limit), and the remaining ≈ 490 lipid species served as the input to subsequent multivariate analyses. Missing values were imputed using the median value of each variable. Data were log-transformed and auto-scaled with mean-centering and divided by the standard deviation of each variable. The top 30 lipids with the highest variable importance in projection (VIP) scores were selected from component 1. Lipid enrichment analysis was performed in LION using 1-way ANOVA F-test P-values or Log_2 fold differences (lung pathology vs. no lung pathology). Distributions of all LION-terms over the ranked list and compared to uniform distributions by using 1-tailed Kolmogorov-Smirnov as previously described. Molenaar et al. (2019) [\[2\]](#) Enriched LION terms are significant when associated lipids are higher ranked than expected by chance. Z-scores are averages $\pm$ SEM and analyzed with Kruskal-Wallis test ($*p < 0.05$). Lipid species-level analysis of cardiolipins was performed by using Log_2 fold differences and FDR q -values (chronic/acute lung disease vs. no lung disease).

Atomic Force Microscopy

BAL extracts were dissolved in chloroform and afterward spin-coated onto 1 cm^2 thermal oxide SiO_2 substrates (University Wafer Inc., MA, USA) at 4000 RPM for 30 s using a spin coater (VTC-100A, MTI Corporation, California, USA). The samples were vacuum-dried for at least 2 hours and scanned in air afterward using a high-speed Cypher AFM (Asylum Research-Oxford Instruments, California, USA). Contact mode AFM probes (ContE-G, $K = 0.2$ N/m) with platinum overall coating were used (NanoAndMore U.S.A. Corporation, California, USA). Lung tissue from dolphins kept at -80°C was snap-frozen after submerging directly in isopentane in a liquid nitrogen bath for about 15 s. Afterward, the frozen tissue was embedded in an optimal cutting temperature (OCT) compound. The tissue was then cryo-cut into 5 μm sections onto poly-L-lysine-coated Wenderott et al. (2020) [\[3\]](#) thermal oxide SiO_2 substrates (2.5 cm^2). To ensure the AFM tip scanned the tissue and not the OCT, only one slide was deposited in the center of a substrate, making it visually easier to locate the tissue sample. Phosphate-buffered saline (PBS) was added on top of the sample and on the AFM tip before bringing them into contact. We used gold-coated (on both sides) silicon nitride cantilevers (HYDRA6R-200NGG, AppNano, Mountain View, CA) with a nominal spring constant of 0.035 N/m. Gold coating on both sides decreases adhesion and improves tip-tissue interaction. For both BAL extracts and tissue samples, the cantilever spring constant and the resonance frequency were calibrated using the thermal spectrum before each experiment. The amplitude sensitivity was calibrated in air and in fluid (for tissue). The force applied to the samples was fixed to the lowest possible value, allowing reproducible mapping (1 V corresponding to a force range of 1–2 nN). The scan rate was set to 100 Hz on an area of 5 $\times$ 5 μm . The total Z-piezo (vertical) displacement was set to up to 2 μm . Force maps comprised 128 points $\times$ 128 lines, resulting in 16,384 data points per map. Force maps were processed using the accompanying Asylum Research

software v.16. All reported Young modulus E values resulted from the peak of a Gaussian fit of E distribution obtained from the respective force maps. AFM force mapping was performed in a high-speed Cypher AFM (Asylum Research Oxford Instruments, California, USA).

Circularity analysis

To study the effect of phase separation in pulmonary extracts, the domains were characterized by their area and circularity. Open-source ImageJ software (National Institute of Health, USA) was used to obtain quantitative circularity and domain area. Circularity was calculated as $4\pi \times \text{area} / (\text{perimeter})^2$ for every measured domain. Statistical analyses were performed using JMP software.

Confocal microscopy

For confocal microscopy, lipid extracts were fluorescently labeled by incorporating Rhodamine lipids (16:0 and 18:1 Liss Rhod PE) in a total concentration of 0.1 mol% and imaged in an LSM 800 confocal microscope (Carl Zeiss 407 Microimaging, Jena, Germany). For TEM imaging, BAL lipid extracts were rehydrated at 2 mg/mL and incubated at 55 °C for 24 h. After negative staining, Baxa (2018) [5](#) μL were deposited on Holey Carbon supported Copper grids, size 200 mesh, size 100 nm (Sigma Aldrich) and imaged in a JEM-1400 transmission electron microscope (JEOL USA Inc).

X-ray scattering

X-ray analysis detailed protocol can be found in previous work. Rueben et al. (2022) [5](#) Briefly, dried lipid extracts were rehydrated into quartz capillaries to a final concentration of 45 mg/mL. Samples were analyzed using Synchrotron SAXS and WAXS at beamline 12-ID-B of the Advanced Photon Source at Argonne National Laboratory. For peak area comparisons, peaks were analyzed using the Peak Analyzer tool included in OriginPro 2019. Scattering data from water in the capillary was subtracted from all WAXS sample data to enhance WAXS peak intensity.

Transmission electron microscopy

BAL lipid extracts were rehydrated at 2 mg/mL and incubated at 55 °C for 24 h. After negative staining, Baxa (2018) [5](#) μL were deposited on Holey Carbon supported Copper grids, size 200 mesh, size 100 nm (Sigma Aldrich) and imaged in a JEM-1400 transmission electron microscope (JEOL USA Inc). Tissue samples were fixed overnight at 4°C in 2.5% (w/v) paraformaldehyde and 2% (w/v) glutaraldehyde dissolved in 0.2 M phosphate buffer, pH 7.2. They were then incubated in 2% (w/v) osmium tetroxide (EMS, 19170) for 1 hour at ambient temperature. A volume of 5% (w/v) potassium ferrocyanide (EMS, 25154-5) was then added to the samples and incubated for an additional 15 minutes. After extensive washes in water, samples were *en bloc* stained with saturated uranyl acetate (JT Baker Chemicals, 4192) in water and dehydrated in ascending concentrations of ethanol until the tissues were in 100% ethanol. Ethanol was replaced with pure acetonitrile, and samples were incubated for 10 minutes. Samples were then infiltrated with ascending concentrations of LX112 resin (LADD, 21210) in acetonitrile mixtures. Finally, samples were embedded in 100% LX112 resin and polymerized at 60°C overnight. Embedded samples were then cut into 70 nm sections using a diamond knife mounted on a Leica UC6 Ultramicrotome. Sections were collected on 200 mesh holey carbon copper grids (SPI Supplies, 3620C-MB) and imaged using a JEOL JEM-1400 transmission electron microscope.

Cryogenic electron microscopy

BAL organic extracts rehydrated in water and whole BAL aqueous samples for cryogenic BAL organic extracts rehydrated in water and whole BAL aqueous samples for cryogenic transmission electron microscopy were prepared on a QUANTIFOIL R 2/1 copper mesh grid (Electron Microscopy Sciences) using a Vitrobot Mark IV (Thermo Fisher Scientific). A volume of 3.5 μL of sample was applied to the glow-discharged surface of the grid at a chamber temperature of 6 °C with a relative humidity level of 100%, and then vitrified in liquid ethane. Before vitrification, samples were blotted for 6 seconds with a blot force of 6. All Cryo-EM grids were imaged using a

200 keV Glacios (Thermo Fisher Scientific) TEM equipped with a Falcon 4 direct electron detector. Images were collected using EPU automated acquisition software (Thermo Fisher). Additional qualitative analysis was conducted using FIJI software Schindelin et al. (2012) [to perform](#) thickness measurements on a single bilayer and to evaluate the distance between adjacent lamellae. The thickness measurements were taken by evaluating the distance between the outer boundaries of the high-contrast bilayer regions present in the images. The average interlamellar spacing values were obtained using the inverted FFT method, usually implemented to calculate d-spacing values from the crystalline structure of individual nanoparticles. By implementing this analysis on cryo-EM images containing clustered bilayers, the electron intensity profiles were generated. Subsequently, peak-to-peak measurements were extracted from all electron profiles to obtain the average interlamellar spacings.

Data availability

No data have been generated for this manuscript.

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Additional files

[Supplementary file 1](#) 

Additional information

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Peer reviews

Reviewer #1 (Public review):

Summary:

This paper describes a number of alterations in pulmonary surfactant recovered from bottlenosed dolphins. Although the sample consists of only seven diseased and two control animals, due to the difficulty in obtaining these animals, this is considered adequate. However, conclusions must be considered in view of this small sample size. The authors employ a number of sophisticated techniques to show differences in the composition and in the structure of bilayers formed by these two surfactant samples

Strengths:

The availability of these samples makes this study quite original. The authors apply mass spectroscopy to observe an increase of an acidic phospholipid and in the level of plasmalogens in the diseased (i.e. pneumonia) aquatic animals. They suggest these increases contribute to hampered function in vivo. They show alterations in lipid bilayers formed from lipid extracts of these surfactants by electron microscopy, by Atomic Force Microscopy and by small and wide-angle X-ray scattering -SAXS/WAXS. They have previously shown that adding small amounts of cardiolin to the clinical surfactant BLES results in altered bilayer structure, consistent with the current study.

Weaknesses:

It seems surprising to me that the small changes in cardiolipin can alter surfactant function i.e., reducing surface tension to near zero. As it happens, no surfactant function tests monitoring the reduction in surface tension were conducted. This would add a great deal to the paper. Further, the paper would benefit greatly from the inclusion of a table listing the lipid composition of surfactant recovered from diseased and normal animals and comparing this to the composition of BLES, a clinical surfactant. Finally, there is a possibility that the minor lipid identified by mass spec is the lysosomal marker, bis-(monoacylglycerol)phosphate rather than the metachronal marker, cardiolipin.

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Reviewer #2 (Public review):

Summary:

In this manuscript, Porras-Gómez et al. analyse the lipid composition and biophysical properties of pulmonary surfactant obtained by bronchoalveolar lavage (BAL) from a group

of bottlenose dolphins (*Tursiops truncatus*), including two healthy individuals and five affected by pneumonia. Through lipidomic analysis, the authors report an exacerbated presence of cardiolipin species in the BAL lipid extracts from diseased dolphins compared to healthy ones. Structural analyses using electron microscopy, atomic force microscopy, and X-ray scattering on rehydrated membrane samples reveal that lipids from diseased animals form membranes with a more pronounced L β phase and reduced fluidity. Moreover, the membranes from affected lungs appear more interconnected and less hydrated, as indicated by the X-ray scattering data. These findings provide valuable and convincing insights into how pulmonary disease alters the lipid composition and structural properties of surfactant in diving mammals, and may have broader implications for understanding surfactant dysfunction in marine mammals.

Strengths:

The study is well designed, and the experimental techniques were applied in a logical and coherent manner. The results are thoroughly analysed and discussed, and the manuscript is clearly written and well organized, making it both easy to follow and scientifically robust. Although the number of samples is limited, the rarity and logistical challenges of obtaining bronchoalveolar lavage material, particularly from animals affected by respiratory disease, make this study especially valuable and relevant.

Weaknesses:

In my opinion, the main issue lies in the treatment of the samples. Pulmonary surfactant is a lipoprotein complex produced by type II pneumocytes of the alveolar epithelium in the form of compact and highly dehydrated structures known as tubular myelin. Once secreted, these structures unfold and, upon contact with the air-liquid interface, form an interfacial monolayer connected to surfactant membranes in the subphase, thereby facilitating respiratory dynamics throughout the breathing cycle.

When bronchoalveolar lavages are treated using the Bligh and Dyer method to extract the hydrophobic fraction of these samples, the structural complexity of the surfactant is disrupted, and this organization cannot be completely restored once the lipids are rehydrated. Although these extracts contain the hydrophobic proteins SP-B and SP-C, the hydrophilic protein SP-A may play an essential role in the formation of pulmonary surfactant structures. It is well established that SP-A is crucial for the formation of tubular myelin, an intermediate structure between the lamellar bodies newly secreted by type II cells and the interfacial surfactant layers.

Moreover, and more importantly, bronchoalveolar lavage fluid may contain cells, tissue debris, and even bacteria that can alter the lipid composition of the samples used in the study after extraction by the Bligh and Dyer method. For this reason, most studies include a density gradient centrifugation step to isolate the surfactant membranes. Consequently, the samples used may be contaminated with phospholipids originating from other cells, such as macrophages, pneumocytes, or bacterial cells, particularly in lavages obtained from diseased animals.

Although the techniques employed provide valuable information about the behaviour of surfactant membranes and allow certain inferences regarding their functionality, no functional studies of these samples have been conducted using methods such as the constrained drop surfactometer or the captive bubble surfactometer. The observed alterations do not necessarily demonstrate that surfactant modulates its properties, as claimed by the authors, but rather indicate that it is altered by the presence of other lipids.

The spin-coating technique used to form lipid films for analysis by atomic force microscopy is not the most suitable approach to reproduce the structures generated by pulmonary surfactant. However, the results obtained may still provide valuable insights into the

biophysical behaviour of its components. The analysis of lung tissue shown in Supplementary Figure S3 presents the same limitation, as the samples were embedded in a cutting compound, and the measurements may have been taken from different regions of the tissue. Therefore, it cannot be ensured that the analysed structures correspond to those generated by pulmonary surfactant.

The finding that the structures formed in samples obtained from diseased animals are more tightly packed and dehydrated than those derived from the surfactant of healthy animals contrasts with the notion that the high efficiency of lamellar bodies in generating interfacial structures is related to their high degree of packing and dehydration. The formation of these structures involves the participation of the ABCA3 protein, which pumps phospholipids into the interior of lamellar bodies, and SP-B, which facilitates the formation of close membrane contacts.

While the results are interesting from a comparative perspective, the implications for surfactant performance and respiratory dynamics should be interpreted with caution.

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Reviewer #3 (Public review):

In this manuscript, the authors present data on the supposed composition of pulmonary surfactant obtained from bronchoalveolar lavages (BALs) of a small cohort of dolphins, a group of them suffering from pneumonia. The lipid compositional differences of the sample group are consistent with the different pathological situations of the specimens, suggesting that differences in surfactant composition are somehow associated (as a cause or as a consequence) with the particular pathophysiological contexts. It is particularly remarkable that an increase in cardiolipins and plasmalogens appears as an abnormal composition in pathological surfactants. The study is completed by analyzing the differences in membrane properties (order, packing, phase) of abnormal versus "control" membranes, concluding that pneumonia in dolphins is associated with a significant alteration of surfactant membranes that become more rigid, packed and thicker than those in surfactant from animals with no lung disease.

In general terms, the data provided are of interest as they somehow offer a framework of effects that may extend what is known about alterations of composition, biophysical properties and functional performance of pulmonary surfactant as a consequence of respiratory pathologies. A collection of pertinent biophysical methodologies (fluorescence, X-ray scattering, AFM) have been applied to complete a full characterization of membrane properties in the different samples.

However, the way the samples have been processed, i.e. by making organic extracts of hydrophobic (lipid and protein) components before surfactant membranes have been purified or at least, separated from bulk lavage, open the question of how much of the altered composition is actually occurring in surfactant or comes from other membranes (from cells, bacteria) that have been completely intermixed as a consequence of the organic extraction. Without an appropriate surfactant membrane obtention, the results of the study should be taken with caution and await confirmation. Specific questions that need to be considered include:

(1) As said, the direct organic extract of BAL samples ends in a full mix of lipid and protein components that in origin could be part of different membranes, either from different surfactant assemblies, or even from pulmonary cells or membrane debris, or microorganisms, collected within the lavage. Obtaining conclusions about the structure and properties of membranes artefactually reconstituted from such lipid and protein mixtures is far from correct.

It is mentioned that "subsequentially" to the organic extraction, the samples were subjected to ultracentrifugation to separate debris and membrane cells. I do not see what the ultracentrifugation is going to change if it is done after the organic extraction. It should have been done before the extraction, for the organic solvents to solubilize exclusively the large, and relatively light, surfactant membrane complexes.

On the other hand, the ulterior reconstitution of the obtained full lipid mixture surely ends in membrane assemblies whose compositional distribution and organization may differ significantly from those in the original membranes.

Taking all this into account, statements such as "These aggregate forms reproduce the expected membrane microstructures observed in native alveolar hypophase" or "pulmonary membranes can be successfully extracted and reconstituted from BALs of Navy dolphins" are simply not true and should be rephrased.

One can understand that the limitation of material may make it difficult to obtain first the purified surfactant membranes and then their organic extract. However, the limitation should be acknowledged to make the readers clear that the actual compositional effects caused in surfactant by pneumonia need confirmation.

(2) In some of the experiments, i.e. in the AFM characterization, supported membranes were prepared by the spray-dry method applied to organic solutions. Again, the spray-dry of organic lipid solutions ends in a lipid dispersion that may be very far from the real organization of the lipids in actual surfactant membranes.

(3) When stated that phospholipid concentrations are greater in BAL from pinnipeds than in humans, how has the actual concentration been determined? BAL volumes are typically subjected to large variations depending on the conditions used to obtain the lavage (including volume of saline instilled, level of atelectasia in the lung tissue, presence of inflammation and edema, etc). If total amounts of phospholipids in BAL are to be compared, certain normalization procedures should be applied, such as for instance, with respect to the urea concentration in serum.

(4) All the differences regarding membrane phase and lipid order/packing have been interpreted in terms of the potential coexistence of L β (gel)/L α (liquid crystalline) phases. However, it has been well established that in lipid systems containing cholesterol, such as pulmonary surfactant, phase coexistence can actually be of the type liquid-ordered (Lo)/liquid-disordered (Ld), very different in terms of mobility and true molecular order. Why do the authors consider that L β is the phase observed in the surfactant membranes they have reconstituted? The presence of round-shaped domains seems to indicate that a liquid/liquid phase segregation is actually occurring.

(5) In the same line as the previous comment, the authors state that SAXS shows that bovine-extracted pulmonary membranes exhibit a coexistence of two lamellar phases, one rich in unsaturated lipids and one in saturated lipids. SAXS and WAXS cannot provide compositional information, but structural parameters such as membrane thickness, or molecular order. This should be clarified.

(6) It is mentioned that the surfactant monolayer at the air-liquid interface is interconnected to tubular membranous structures (tubular myelin, TM). It is true that TM, when present, appears interconnected with the interface. However, it is widely recognized that there are many other structures connected with the interfacial film, including multilamellar membrane arrays or reservoirs that have not been mentioned here. Furthermore, TM is not required for surfactant function, because it is absent, for instance, in mice lacking expression of surfactant protein SP-A, which can breathe perfectly.

(7) In the Discussion, the authors mention that "...after squeeze-out, the excluded multilayers remain closely associated with the interfacial monolayer rather than escaping into the subphase". The authors may like to complete this discussion by specifying that the stable association of excluded assemblies with the interfacial film is actually possible thanks to the surfactant proteins.

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