Mechanical ventilation-induced neutrophil extracellular vesicles as mediators of postoperative pulmonary complications

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All authors were involved in the drafting of the article or revising it critically for important intellectual content, gave their final approval of the version to be published and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

At a Glance commentary:

Scientific Knowledge on the Subject:

Postoperative pulmonary complications (PPCs) remain common after major surgery, particularly esophagectomy. Mechanical ventilation is an important contributor to perioperative lung injury and PPCs, with this risk particularly relevant during esophagectomy, which frequently requires one-lung ventilation (OLV), exposing the ventilated lung to increased mechanical stress and alveolar stretch. However, the mechanisms linking this mechanical stress to early alveolar inflammation and subsequent PPCs remain incompletely understood. Extracellular vesicles (EVs) are important mediators of intercellular inflammatory signalling, but their contribution to ventilation-induced perioperative lung inflammation is unknown.

What This Study Adds to the Field:

Using serial bilateral bronchoalveolar lavage in patients undergoing esophagectomy, we identify a marked, lung-specific increase in neutrophil-derived EVs (NEVs) following OLV that is associated with alveolar inflammation and subsequent PPC development. Patient-derived NEVs are enriched in MMP-8 and MMP-9 and induce epithelial activation in vitro, which is attenuated by MMP inhibition. Mechanistically, alveolar stretch promotes NEV release through epithelial ATP signalling and P2X7 receptor activation. These findings identify a previously unrecognised mechanistic pathway linking mechanical ventilation to neutrophil-mediated

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alveolar inflammation and PPCs, and nominate NEVs as potential biomarkers and therapeutic targets.

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Short running title: Ventilation-induced NEVs as mediators of PPCs

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Impact of Research: This study elucidates a previously unrecognised mechanistic link between mechanical ventilation-induced alveolar stretch and the release of neutrophil-derived extracellular vesicles (NEVs), which act as mediators of alveolar inflammation and may predispose patients to postoperative pulmonary complications. By identifying matrix metalloproteinases (MMP-8 and MMP-9) as bioactive cargoes within these vesicles and

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demonstrating that their inhibition reduces NEV-induced inflammatory responses, we expand our understanding of the cellular communication contributing to perioperative lung injury and reveal a novel and potentially translationally relevant therapeutic target to mitigate postoperative morbidity.

This article has an online data supplement, which is accessible from this issue's table of contents online at **www.atsjournals.org**.

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Abstract

Rationale

Postoperative pulmonary complications (PPC) remain a leading cause of perioperative morbidity and mortality, yet the underlying mechanisms are poorly understood. While alveolar stretch from mechanical ventilation is a known driver of lung inflammation, the role of extracellular vesicles (EVs) is unknown.

Objectives

To characterise the biogenesis of alveolar stretch-induced EVs and determine their role in perioperative alveolar inflammation and PPC development.

Methods

Serial, bilateral bronchoalveolar lavage (BAL) samples were obtained from forty-two patients undergoing esophagectomy and requiring one- and two-lung ventilation. EV subtypes were identified using flow cytometry and correlated with inflammatory mediators and clinical data. Western blotting identified neutrophil-specific inflammatory matrix metalloproteinases (MMP-8/9) within patient-derived BAL neutrophil-EVs (NEV), and their bioactivity was assessed using a primary human alveolar epithelial cell-macrophage coculture. An in vitro alveolar stretch model, comprising epithelial cells and neutrophils, was used to explore mechanisms of NEV biogenesis.

Measurements and Main Results

Unphysiological one-lung ventilation, but not two-lung ventilation, induced a marked increase in NEVs (4.8-fold), which were associated with alveolar inflammation and a greater incidence

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of PPCs. BAL NEVs contained abundant MMP-8/9, generating pro-inflammatory responses in vitro, which were reduced by MMP inhibition. In vitro, NEV production was induced by injurious stretch of an epithelial-neutrophil co-culture through an ATP-dependent mechanism.

Conclusions

We identify a novel mechanistic pathway whereby mechanical ventilation induces NEV release, propagating alveolar inflammation via their biologically active cargo, which may be associated with PPCs. This suggests NEVs could become a potential biomarker and therapeutic target for reducing ventilator-induced lung inflammation and PPCs.

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Introduction

Postoperative pulmonary complications (PPCs) occur in up to 39% of major surgical cases and are independently associated with increased morbidity, mortality, and healthcare burden worldwide (1–3). These complications are especially common in high-risk patients and procedures such as esophagectomy, where the incidence may exceed 50% (4–6). Despite widespread adoption of lung-protective ventilation strategies, PPCs remain prevalent, underscoring a persistent gap in our understanding of their underlying pathophysiology.

Whilst mechanical ventilation is essential for intraoperative respiratory support or during critical illness, it can produce unphysiological alveolar stretch, triggering lung inflammation and predisposing the lungs to PPCs or ventilator-induced lung injury (VILI). In high-risk surgeries such as esophagectomy, prolonged one-lung ventilation (OLV), which is required for surgical access, exacerbates this mechanical stress and the ensuing ventilator-associated lung inflammation, which is largely mediated by a host of cytokines/chemokines/proteases (7–9). However, most mechanistic insights into stretch-induced lung injury have emerged from animal models, which may not accurately represent perioperative and/ or human conditions.

Extracellular vesicles (EV) are increasingly recognised as potent mediators of intercellular signalling in inflammatory diseases (10–12). Specifically, among EV subtypes, leukocyte-derived EVs have been implicated in both acute and chronic pulmonary pathologies (10, 13, 14). These membrane-circumscribed vesicles, ranging from 0.1 to 1μm, encapsulate a variety of cellular mediators, offering protected and targeted delivery of bioactive cargo. Under normal resting conditions, low levels of EVs are present within the bronchoalveolar lavage (BAL) fluid, however, immediately following injury e.g., pathological alveolar stretch, damage-associated molecular patterns (DAMPs) such as adenosine triphosphate (ATP) are rapidly released, which can stimulate alveolar immune cells to produce proinflammatory EVs

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(15, 16). Inflammatory cargo within these EVs can include lipids, nucleic acids, metabolites and proteins such as TNF, IL-6 and IL-8, key cytokines in the pathogenesis of lung inflammation (10, 17). Our prior work has shown that EV-associated mediators may be more bioactive than their soluble counterparts and more resistant to degradation or neutralisation, and therefore are an efficient mechanism for intercellular communication, both within the alveolus and in distant cell signalling (16, 18, 19).

We hypothesised that ventilator-associated alveolar stretch triggers the rapid release of proinflammatory EVs within the alveolar space, contributing to perioperative lung inflammation and predisposing patients to PPCs. In this study, we used patients undergoing esophagectomy as a human model of ventilator-associated alveolar stretch and inflammation. Multiple BAL samples were collected from both lungs of the same patient intraoperatively, allowing detailed characterisation of EV dynamics within the alveolar compartment in response to mechanical ventilation and determination of their pro-inflammatory impact. This was complemented with in vitro models to assess the bioactivity of EVs and their biogenesis mechanisms. Herein, we elucidate a novel EV-mediated pathway of alveolar inflammation and identify that specifically neutrophil-derived EVs (NEV) are potential biomarkers and therapeutic targets for the prevention of PPCs.

Some of the results of these studies have been previously reported in the form of abstracts (20, 21).

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Methods

Study approval

This study was conducted under the 'Vesicles In PERioperative inflammation (VIPER) (REC:21/PR/1554) and the 'Multiorgan Failure in COVID-19' (REC:20/NE/0153) studies, with informed consent obtained from all participants

Patient sampling and study design

Patients undergoing transthoracic esophagectomy between April 2022 and June 2023 were recruited as a human model of ventilator-induced alveolar stretch. No change was made to the standardised perioperative care of these patients. Serial BAL was performed separately in each of the two lungs at three predefined intraoperative time points: after anaesthetic induction (baseline), post–two-lung ventilation (TLV), and post–OLV (Figure E1 in the online supplement). This unique study design enabled the detection of early inflammatory changes induced by excessive alveolar stretch, with the data of post-OLV compared to that of pre-OLV in the same ventilated lung in each patient. Changes in the non-ventilated lung during the same OLV period, and changes in the ventilated lungs during the TLV period served as 'internal controls' within each patient, while 'external controls' were patients undergoing gastrectomy and requiring TLV only. Ventilatory and physiological data were recorded continuously. Two independent Consultant intensivists (>20 years' experience) reviewed clinical notes to identify PPCs within 7 days post-surgery, using the 2015 European Perioperative Clinical Outcome definitions with minor esophagectomy-specific modifications (Table E1 in the online supplement).

BAL fluid analysis and EV quantification

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BAL fluid was processed within 30 minutes. EVs were enumerated and phenotyped using fluorescent-labelled antibodies and well-established flow cytometry protocols (Figure E2 and Tables E2A and E3) (13, 18, 22, 23). Cell-free supernatants were analysed for the soluble receptor for advanced glycation end products (sRAGE), interleukin (IL)-6 and IL-8, and BAL cell counts were determined using flow cytometry (Figure E3 and Table E2B).

BAL EV isolation and western blotting

EV subtypes were isolated from the BAL fluid of the ventilated lung following OLV using an immunomagnetic bead separation technique (MACS® MicroBeads). For western blotting, standardized EV numbers were lysed and the abundance of MMP-8 and MMP-9 was quantified using the Jess™ automated platform (Figure E4).

Bioactivity assay

To determine the proinflammatory activity of patient-derived NEVs, we used an in vitro alveolar model comprising primary human alveolar epithelial cells (HAECs, Cell Biologics, Chicago, USA) cultured with monocyte-derived macrophages (MDMs) differentiated using macrophage colony-stimulating factor and granulocyte-macrophage colony-stimulating factor. Co-cultures were incubated with NEVs (1×10^5/μl) isolated from BAL fluid of the ventilated lung after OLV. Controls included post-wash supernatant (PWSN), non-neutrophil EVs from the BAL fluid of the same patient, and platelet-derived EVs (PEVs) isolated from healthy volunteer blood. Involvement of MMP pathways was assessed by pre-incubation with doxycycline (20 μM) (Merck Life Science, #D9891), a pan-MMP inhibitor. After 4 hours, expression of intercellular adhesion molecule-1 (ICAM-1) and tissue factor (TF) on HAECs was quantified by flow cytometry.

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In vitro alveolar model of stretch-induced NEV production

To investigate mechanisms of stretch-induced NEV production, HAECs were seeded on BioFlex plates (Flexcell, Burlington, USA) and neutrophils, isolated from the blood of healthy volunteers, were added (1:2). After 30 minutes rest, the co-culture was subjected to high cyclical stretch (21%) using the Flexcell® system for a further 30 minutes, with EVs quantified over time. In some experiments, cells were pretreated with KN-62 (100nM) (Abcam, #ab120421), a specific P2X7 inhibitor. In separate experiments, individual monocultures of HAECs or neutrophils were subjected to the same stretch conditions and EVs were quantified over time. In the HAEC monoculture, ATP release over time was also assessed via bioluminescence.

Statistical analysis

Statistical analysis was performed using GraphPad Prism with figure legends detailing the specific statistical test. Data normality was assessed using the Shapiro-Wilk test, and paired statistical analyses, corrected for multiple comparisons, were used where appropriate. Flow cytometry and western blotting data were analysed using FlowJo™ and Compass for Simply Western, respectively.

Results

Effect of OLV on EV production

To determine whether unphysiological alveolar stretch induces EV production in patients, we obtained serial BAL samples from 42 patients undergoing esophagectomy and requiring OLV (Figure E5). The demographics of this cohort were in keeping with the esophagogastric cancer

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population of England and Wales, esophagogastric cancer being the indication for surgery for all but one patient who had achalasia (Table E4) (24). Seven patients undergoing gastrectomy were external controls. During OLV, one lung of the patient was exposed to proportionally greater tidal volumes (V_t) compared to TLV, with a significantly greater peak airway pressure (P_{peak}) and a proportionally greater surrogate mechanical power (Table 1). Patients undergoing esophagectomy received TLV for a mean (SD) of 372 +/- 107 minutes and OLV for 169 +/- 52 minutes, whilst patients undergoing gastrectomy received TLV for a mean (SD) of 357 +/- 107 minutes.

OLV caused significant increases, albeit small, in all EV subtypes in BAL fluid in the ventilated lung, yet NEVs demonstrated a profound increase, where a 4.8-fold increase was identified post-OLV compared to pre-OLV (Figures 1A+B and Table E5). In contrast, no significant changes in any EV subtype numbers were observed in the non-ventilated lungs during OLV (Figure 1C and Table E6), nor during protective TLV in patients undergoing both esophagectomy and gastrectomy (Figure 1C and Table E7 and E8), highlighting that the phenomenon of NEV release was unique to the period of unphysiological alveolar stretch caused by OLV. BAL recovery volumes showed little variability across lungs and time points, indicating stable sampling conditions throughout this study (Table E9). In all BAL samples sRAGE was consistently detected (Table E10), confirming that we have sampled the alveolar space.

OLV elicits alveolar inflammation

To characterise immune cell recruitment to the alveolus during the perioperative period, we identified BAL alveolar macrophages (AM) and neutrophils via flow cytometry and found a significant increase in both cells following unphysiological OLV only in the ventilated lung

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(Figure 2). Enzyme-linked immunosorbent assays on the cell-free BAL fluid for the proinflammatory cytokines IL-6 and IL-8 demonstrated a generalised inflammatory response in both lungs following OLV (Figure 3); TLV in either lung did not cause a significant change in cytokine concentrations (data not shown).

NEVs as a biomarker for PPCs

Within 7 days of surgery, 20 patients (48%) sustained a PPC, in keeping with previously reported studies (Table E11) (4–6). PPCs were more common in the lung ventilated during OLV than in the collapsed, non-ventilated lung (ventilated lung: 45% vs. non-ventilated lung: 15%, p=0.038) (Figure 4A). In the ventilated lung during OLV, BAL NEVs increased significantly more in patients who sustained a PPC compared to patients who did not (Figure 4A). As a result, patients who sustained a PPC had a significantly greater BAL NEV burden after OLV compared to patients who did not develop a PPC, demonstrating that NEVs could be a sensitive and effective biomarker of early alveolar inflammation and the development of PPCs (Figure 4B). Importantly, there was no relationship between PPC occurrence and NEV levels after OLV in the non-ventilated lung, or between PPC occurrence and any other measured BAL mediator in both the ventilated and non-ventilated lungs, including cell counts of neutrophils or AMs and levels of IL-6 or IL-8 (Figure E6). Additionally, ventilation parameters were not significantly different between those with and without PPCs (Table E12).

In vitro biological activity of NEVs released following OLV

EVs have the potential to provide a highly effective mechanism for cell-to-cell communication, partly due to their protected cargo. Using a capillary-based automated western blotting system (Jess, Bio-Techne), which enables precise quantification of proteins in a standardized and reproducible manner, we measured the relative abundance of MMP-8 and MMP-9, associated

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with NEVs and non-neutrophil EVs within the BAL fluid after OLV. We targeted these neutrophil-specific inflammatory mediators as they have previously been implicated in the pathophysiology of VILI and ARDS and have been found to have significant bioactivity when associated with NEVs (8, 9, 19, 25). We detected abundant MMP-8 and MMP-9 in all NEV samples, significantly more than the non-neutrophil EV controls (Figure 5A).

To determine the bioactivity of NEVs released following OLV, we used a co-culture of primary HAECs and an M1/M2 mix of MDMs (2:1) as an in vitro alveolar model. Macrophages were utilised as the immune component given the sentinel role they play in the recognition of invading alveolar pathogens. When applied to the epithelial–macrophage co-culture, NEVs, but not non-neutrophil EVs or PEVs, induced significant upregulation of ICAM-1 and TF on epithelial cells (Figure 5B). This inflammatory effect was abolished by doxycycline, a pan-MMP inhibitor, implicating NEV-associated MMPs in the observed bioactivity.

Mechanisms of stretch-induced NEV production in vitro

To elucidate the mechanism underlying NEV generation observed in patients during OLV, we conducted an in vitro cell stretch experiment, in which we mechanically stretched HAECs in co-culture with neutrophils to simulate overstretched alveoli with infiltrating neutrophils. We found that cyclical high stretch (21%) induced a significant release of NEVs (Figure 6A) but not HAEC-EVs (Figure E7A), in keeping with our in vivo findings. In addition, no EV increase was observed when monocultures of either HAECs or neutrophils were subjected to the same stretch conditions (Figures E7B+C), indicating that cell–cell interaction and/or epithelial-derived mediators are essential for NEV biogenesis. Notably, stretch did not result in significant cell death, confirming that vesicle release was an active process rather than simply a consequence of necrosis (data not shown).

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Next, we found that cyclical stretch of an HAEC monoculture triggered a rapid release of ATP from epithelial cells into the extracellular environment (Figure 6B). Consistent with this, pre-treatment of the HAEC-neutrophil co-culture with KN-62, a selective P2X7 receptor antagonist, completely abrogated stretch-induced NEV production (Figure 6C), indicating that NEV release was mediated through an ATP-P2X7-dependent signalling axis.

Discussion

Our study identifies several novel findings describing how mechanical ventilation may contribute to alveolar inflammation and potentially PPCs through the release and activity of NEVs. Firstly, using patients undergoing esophagectomy as a controlled human model of stretch-induced lung inflammation, we have profiled clinically relevant compartmental EV dynamics in response to unphysiological alveolar stretch. By conducting serial BAL sampling from both lungs at defined intraoperative time points, we identified that alveolar NEVs are significantly released in the lung exposed to OLV, which are associated with increased markers of alveolar inflammation and, most importantly, may be associated with the development of PPCs. Next, by utilising innovative quantitative western blotting methodologies, we showed that these stretched-induced NEVs are enriched with proinflammatory MMP-8 and MMP-9, which inflamed epithelial cells in vitro, implicating NEV-associated MMPs in the propagation of alveolar inflammation following unphysiological stretch. Finally, we established that NEV release following alveolar stretch is largely driven by epithelial–neutrophil interactions, specifically by stretch-induced ATP release from mechanosensitive epithelial cells that stimulate neutrophils within the alveolar space to produce EVs via an ATP–P2X7–dependent pathway. These findings provide a direct mechanistic link between alveolar stretch, purinergic

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signalling, and the release of bioactive neutrophil EVs (Figure 7), suggesting a potential role for NEVs as diagnostic and therapeutic targets for lung inflammation with injurious mechanical ventilation.

Despite widespread adoption of lung-protective ventilation strategies, PPCs continue to affect up to half of patients following high-risk surgery, including esophagectomy (1–5, 26–28). The cause of PPCs is multifactorial but mechanical ventilation used during the perioperative period is likely to play a crucial role. In our cohort, nearly 50% of patients undergoing esophagectomy developed a PPC, and unilateral complications were significantly more likely to affect the lung that was ventilated during OLV. This supports the concept that alveolar overstretch associated with OLV, rather than collapse/atelectasis, is an important contributor to PPCs in this setting.

Although it is well appreciated that unphysiological ventilation outside normal physiological boundaries is associated with inferior outcomes (29, 30), the optimal strategy for delivering perioperative mechanical ventilation is yet to be determined and there remains a lack of high-quality trial data, meaning that expert opinion provides the mainstay of evidence (2, 3, 31, 32). Presently, guidelines recommend perioperative tidal volumes to be 6 - 8 ml/kg predicted body weight. However, in our cohort during OLV, the single lung was exposed to 5.1ml/kg, a tidal volume equivalent to ~10.2ml/kg for two lungs, which is considered unphysiological and as an exposure to excess stretch (32, 33). Although our data found no relationship between ventilator parameters and PPC occurrence, our unique methodology of serial BAL procedures in each of the ventilated and non-ventilated lungs during the period of OLV, clearly demonstrated that lung overstretch triggered significant alveolar inflammation and neutrophil activation. Hence, simply observing overall ventilator parameters/metrics is not enough; careful mechanism-based

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evaluation of stretch-induced inflammation is crucial to predict and subsequently prevent PPC evolution.

Esophagectomy has long been considered a well-controlled and ethically acceptable human model of VILI (34–36). Yet recent advances in perioperative care have led to patients undergoing esophagectomy showing fewer clinical manifestations of post-operative ARDS, and traditional BAL procedures demonstrating less obvious indicators of intra-alveolar inflammation (37). However, if we precisely evaluate local changes in the affected, over-ventilated lung during OLV, as we have done with our novel experimental design, we are able to detect early stretch-induced inflammatory changes within the alveoli, providing strong evidence that esophagectomy remains an excellent and reproducible model of VILI in humans. Whilst the incidence of postoperative ARDS in our cohort was low (4.8%), we determined that unphysiological overstretch of the ventilated lung during OLV leads to alveolar inflammation, with significant immune cell recruitment, an increase in a range of EVs (which reflects host cell activation) and raised BAL cytokines; these changes do not occur following more physiological TLV. Furthermore, our identification of NEVs as both mediators and predictors of PPC risk represents a key advance toward more personalised and biologically informed strategies for perioperative lung protection.

Our study identified that pathological stretch of alveolar cells, both in patients and in vitro, induces the release of EVs, particularly NEVs. EVs have been increasingly recognised as potential biomarkers in acute and chronic lung pathologies. Mahida et al proposed that $CD14^{+ve}$ BAL EVs can be a biomarker of ARDS, although they almost exclusively focused on smaller EVs (i.e., less than 100nm), previously termed exosomes (11). Our previous work identified elevated concentrations of NEVs in COPD patients, with a direct correlation between NEV

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burden and symptom severity (13). In this current study, we show that patients who develop PPCs following esophagectomy have significantly higher NEV concentrations in the ventilated lung compared to patients without a complication, although whether NEVs can independently predict PPC occurrence requires future multicentre studies. Yet, importantly, this association was not observed for BAL neutrophil numbers per se, indicating that it is likely neutrophil activation, rather than just their presence, that underpins NEV release and PPC risk, a concept in keeping with our previous COPD study (13).

Our data also provide evidence that NEVs are not merely bystanders of a disease process, but in fact, they may play an important mechanistic role in enhancing stretch-induced lung inflammation. NEVs were associated with alveolar inflammation and may contribute to the observed response. We also showed that BAL NEVs by themselves have substantial bioactivity when applied to a realistic alveolar model consisting of not only HAECs but also accompanying macrophages. These findings are consistent with, but do not prove, an EV-mediated pathway. Previous studies have suggested that the proinflammatory activities of inflammatory mediators during VILI may not be mediated by their simple extracellular 'soluble' form (16). Our interrogation of BAL NEVs identified significant quantities of MMP-8/9, zinc-dependent metalloproteinases most highly expressed in neutrophils that are implicated in the modulation of extracellular matrix proteins as well as the processing of cytokines and cell surface molecules (38–40). Recent animal VILI studies have associated MMP-8/9 with worsening lung injury (8, 9). Unlike other MMPs, MMP-8/9 are transcribed within neutrophil precursors in the bone marrow and stored within secretory granules as inactive proMMPs, facilitating rapid degranulation following stimulation (41–43). MMP-8/9 are also associated with the surface membrane of neutrophils, in which context they exhibit significant bioactivity and ability to evade endogenous regulatory proteins such as tissue-

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inhibitor of MMP (TIMP-1) (40). Given that EVs are formed from the blebbing of the host cell membrane 'right-side-out', it is plausible that NEVs produced following alveolar stretch incorporate MMP-8/9 located on the activated neutrophil membrane, thus transferring this proinflammatory cargo. Indeed, in activated breast cancer cells, the membrane distribution of proteins such as MMP-9 is non-random, with a greater concentration of proteins at the site of vesicle shedding, leading to an enrichment of cargo associated with EVs (44).

By developing a novel methodology to quantify cargo within distinct EV populations, we identified a high abundance of MMP-8/9 specifically in NEVs, confirming the selective transfer of MMPs from parental neutrophils to NEVs in patients. We also probed BAL NEVs for other proinflammatory proteins associated with neutrophils, namely neutrophil elastase, Cathepsin G, IL-6 and IL-8, but interestingly, these mediators were not routinely identified. Our adoptive transfer experiments highlighted the bioactivity of these stretch-induced BAL NEVs, which was significantly inhibited by doxycycline, an MMP inhibitor, thus implicating NEV-associated MMPs as drivers in the pathophysiology of alveolar inflammation. Our previous work identified that MMPs associated with circulating NEVs in critical illness exert their bioactivity on endothelial cells in a monocyte-dependent fashion (19); we speculate that within the alveolus, MMP-mediated macrophage activation similarly plays a role in activating epithelial cells.

The cellular mechanisms governing EV biogenesis remain incompletely understood, but DAMPs such as ATP have emerged as key regulators. We have previously identified ATP as a potent stimulator of proinflammatory alveolar macrophage-derived EV production (16). Under homeostatic conditions, ATP is maintained intracellularly, but in response to cellular stress, including mechanical stretch, it is actively released via exocytosis or through mechanosensitive

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channels such as Piezo-1 (15, 45). Extracellular ATP then signals through purinergic P2X7 receptors on immune cells, increasing membrane calcium permeability, which activates downstream enzymes, including acid sphingomyelinase, driving cytoskeletal disruption and subsequent EV production (46–51). By exposing a monoculture of primary HAEC to high cyclical stretch, we confirmed ATP efflux in response to stretch but not HAEC-EV release. However, NEVs were released when the HAECs were in co-culture with neutrophils, and this was abolished by P2X7 receptor blockade, indicating that both epithelial–immune cell interactions and ATP signalling are required for EV production. Summarily, our findings highlight a specific, mechanosensitive, and ATP-dependent mechanism of NEV generation.

Our study has limitations. First, serial sampling of the same lung lobe by saline instillation may lead to a theoretical dilution of BAL mediators with subsequent BAL sample retrieval, potentially explaining the significant decrease seen in EVs between sampling points 1 and 2 (period of TLV for patients undergoing esophagectomy). However, we feel the benefit of sampling the same lung lobe at multiple time points to provide well-controlled data outweighs the theoretical risk of sample dilution. Furthermore, the BAL instillation volume was purposefully kept low to reduce the risk of iatrogenic pulmonary complications or inflammation in this high-risk group. Despite our confidence in sampling the alveolar space, as evidenced by our consistent sRAGE data, we acknowledge that our sample may include some bronchial wash (Table E10). Second, the in vivo alveolar environment is complex with multiple cell-cell interactions (from within the alveolus and the systemic circulation) and an air-liquid interface, and we accept that the role of other cells is not being fully appreciated in our current in vitro models. However, by co-culturing human epithelial and appropriate immune cells, our models were designed to generate focused mechanistic data for specific cell types/ interactions instead of examining the alveolus as a whole. Furthermore, our human model replicated our in vitro

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findings regarding EV production, providing confidence in the veracity of our in vitro data. In addition, the human alveolus contains predominantly type 1 pneumocytes, whereas most cultured cells are type 2 pneumocytes; our use of primary cells at early passage (within 7) was designed to maximise the presence of type 1 pneumocytes (52).

In conclusion, we have identified a novel pathway linking mechanical ventilation-induced alveolar overstretch to the rapid release of proinflammatory NEVs, mediated through an ATP–P2X7–dependent mechanism. These vesicles carry active MMPs capable of inducing alveolar inflammation and are associated with the development of PPCs. NEVs thus represent both a potential biomarker of risk and a therapeutic target in perioperative lung injury.

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References

1. Fernandez-Bustamante A, Frendl G, Sprung J, Kor DJ, Subramaniam B, Martinez Ruiz R, *et al.* Postoperative Pulmonary Complications, Early Mortality, and Hospital Stay Following Noncardiothoracic Surgery. *JAMA Surg* 2017;152: 157-162.
2. Karalapillai D, Weinberg L, Peyton P, Ellard L, Hu R, Pearce B, *et al.* Effect of Intraoperative Low Tidal Volume vs Conventional Tidal Volume on Postoperative Pulmonary Complications in Patients Undergoing Major Surgery. *JAMA* 2020;324:848-858.
3. Odor PM, Bampoe S, Gilhooly D, Creagh-Brown B, Moonesinghe SR. Perioperative interventions for prevention of postoperative pulmonary complications: systematic review and meta-analysis. *BMJ* 2020; 368: m540.
4. Hulscher JBF, van Sandick JW, de Boer AGEM, Wijnhoven BPL, Tijssen JGP, Fockens P, *et al.* Extended Transthoracic Resection Compared with Limited Transhiatal Resection for Adenocarcinoma of the Esophagus. *New Eng J Med* 2002;347:1662–1669.
5. van der Werf LR, Busweiler LAD, van Sandick JW, van Berge Henegouwen MI, Wijnhoven BPL. Reporting National Outcomes After Esophagectomy and Gastrectomy According to the Esophageal Complications Consensus Group (ECCG). *Ann Surg* 2020;271:1095–1101.
6. Lindner K, Fritz M, Haane C, Senninger N, Palmes D, Hummel R. Postoperative Complications Do Not Affect Long-Term Outcome in Esophageal Cancer Patients. *World J Surg* 2014;38:2652–2661.
7. Vlahakis NE, Schroeder MA, Limper AH, Hubmayr RD. Stretch induces cytokine release by alveolar epithelial cells in vitro. *Am J of Physiol Lung Cell Mol Physiol* 1999;277:L167–L173.

ACCEPTED MANUSCRIPT

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8. Kim JH, Suk MH, Yoon DW, Lee SH, Hur GY, Jung KH, *et al.* Inhibition of matrix metalloproteinase-9 prevents neutrophilic inflammation in ventilator-induced lung injury. *Am J of Physiol Lung Cell Mol Physiol* 2006;291:L580–L587.

9. Albaiceta GM, Gutierrez-Fernández A, García-Prieto E, Puente XS, Parra D, Astudillo A, *et al.* Absence or Inhibition of Matrix Metalloproteinase–8 Decreases Ventilator-Induced Lung Injury. *Am J Respir Cell Mol Biol* 2010;43:555–563.

10. Soni S, Wilson MR, O'Dea KP, Yoshida M, Katbeh U, Woods SJ, *et al.* Alveolar macrophage-derived microvesicles mediate acute lung injury. *Thorax* 2016;71:.1020-1029.

11. Mahida RY, Price J, Lugg ST, Li H, Parekh D, Scott A, *et al.* CD14-positive extracellular vesicles in bronchoalveolar lavage fluid as a new biomarker of acute respiratory distress syndrome. *Am J of Physiol Lung Cell Mol Physiol* 2022;322:L617–L624.

12. Raeven P, Zipperle J, Drechsler S. Extracellular Vesicles as Markers and Mediators in Sepsis. *Theranostics* 2018;8:3348–3365.

13. Soni S, Garner JL, O'Dea KP, Koh M, Finney L, Tirlapur N, *et al.* Intra-alveolar neutrophil-derived microvesicles are associated with disease severity in COPD. *Am J of Physiol Lung Cell Mol Physiol* 2021;320:L73–L83.

14. Guervilly C, Lacroix R, Forel J-M, Roch A, Camoin-Jau L, Papazian L, *et al.* High levels of circulating leukocyte microparticles are associated with better outcome in acute respiratory distress syndrome. *Crit Care* 2011;15:R31.

15. Diem K, Fauler M, Fois G, Hellmann A, Winokurow N, Schumacher S, *et al.* Mechanical stretch activates piezo1 in caveolae of alveolar type I cells to trigger ATP release and paracrine stimulation of surfactant secretion from alveolar type II cells. *The FASEB J* 2020;34:12785–12804.

ACCEPTED MANUSCRIPT

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16. Soni S, O'Dea KP, Tan YY, Cho K, Abe E, Romano R, *et al.* ATP redirects cytokine trafficking and promotes novel membrane TNF signaling *via* microvesicles. *The FASEB J* 2019;33:6442–6455.

17. Parsons PE, Eisner MD, Thompson BT, Matthay MA, Ancukiewicz M, Bernard GR, *et al.* Lower tidal volume ventilation and plasma cytokine markers of inflammation in patients with acute lung injury. *Crit Care Med* 2005;33: 1-6.

18. Tan YY, O'Dea KP, Tsiridou DM, Pac Soo A, Koh MW, Beckett F, *et al.* Circulating Myeloid Cell–derived Extracellular Vesicles as Mediators of Indirect Acute Lung Injury. *Am J Respir Cell Mol Biol* 2023;68:140–149.

19. Stephens JR, Iki Y, Yasuda T, Brown E, Tsiridou D, Green A, *et al.* Human neutrophil-derived extracellular vesicles induce renal endothelial inflammation in critical illness: an ex vivo investigation. *Br J Anaesth* 2025;135:920–929.

20. Stephens J, Soni S, Boshier P, Iki Y, Cheng X, Wilson MR, *et al.* Intra-alveolar Neutrophil-derived Microvesicles After One-lung Ventilation Are Associated With Post-operative Pulmonary Complications in Patients Undergoing Esophagectomy. *B67 ALL THE LUNG'S A STAGE: IMMUNE CELLS ARE THE PLAYERS* American Thoracic Society; 2023. p.A3994–A3994.

21. Stephens Jonny, CAna, WRebeca, IYoichi, CXingzhi, BPiers, WMichael, TMasao, SS. The role of neutrophil-derived microvesicles during the pathophysiology of postoperative pulmonary complications after major complex surgery. *Intensive Care Med Exp* 2024;12:1–858.

22. O'Dea KP, Tan YY, Shah S, V Patel B, C Tatham K, Wilson MR, *et al.* Monocytes mediate homing of circulating microvesicles to the pulmonary vasculature during low-grade systemic inflammation. *J Extracell Vesicles* 2020;9:.1706708.

ACCEPTED MANUSCRIPT

Downloaded from academic.oup.com/ajrccm/advance-article/doi/10.1093/ajrccm/aamag457/8796056 by guest on 16 September 2026

23. O'Dea KP, Porter JR, Tirlapur N, Katbeh U, Singh S, Handy JM, *et al.* Circulating Microvesicles Are Elevated Acutely following Major Burns Injury and Associated with Clinical Severity. *PLoS One* 2016;11:e0167801.

24. National Cancer Audit Collaborating Centre. *National Oesophago-Gastric Cancer Audit (NOGCA) State of the Nation Report*. London: 2025.

25. Davey A, McAuley DF, O'Kane CM. Matrix metalloproteinases in acute lung injury: mediators of injury and drivers of repair. *Eur Respir J* 2011;38:959–970.

26. Miskovic A, Lumb AB. Postoperative pulmonary complications. *Br J Anaesth* 2017;118:317–334.

27. Neto AS, da Costa LG V., Hemmes SNT, Canet J, Hedenstierna G, Jaber S, *et al.* The LAS VEGAS risk score for prediction of postoperative pulmonary complications. *Eur J Anaesthesiol* 2018;35:691–701.

28. Zingg U, Smithers BM, Gotley DC, Smith G, Aly A, Clough A, *et al.* Factors Associated with Postoperative Pulmonary Morbidity After Esophagectomy for Cancer. *Ann Surg Oncol* 2011;18:1460–1468.

29. Ventilation with Lower Tidal Volumes as Compared with Traditional Tidal Volumes for Acute Lung Injury and the Acute Respiratory Distress Syndrome. *N Eng J Med* 2000;342:1301-1308.

30. Futier E, Constantin J-M, Paugam-Burtz C, Pascal J, Eurin M, Neuschwander A, *et al.* A Trial of Intraoperative Low-Tidal-Volume Ventilation in Abdominal Surgery. *N Eng J Med* 2013;369:428–437.

31. Beck-Schimmer B, Schimmer RC. Perioperative tidal volume and intra-operative open lung strategy in healthy lungs: where are we going? *Best Pract Res Clin Anaesthesiol* 2010;24:199–210.

ACCEPTED MANUSCRIPT

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32. Young CC, Harris EM, Vacchiano C, Bodnar S, Bukowy B, Elliott RRD, *et al.* Lung-protective ventilation for the surgical patient: international expert panel-based consensus recommendations. *Br J Anaesth* 2019;123:898–913.

33. Schaefer MS, Tenge T, Futier E. How to set the ventilator during surgery? Key principles to guide intraoperative mechanical ventilation. *Intensive Care Med* 2026;doi:10.1007/s00134-026-08301-z.

34. Rocker GM, Wiseman MS, Pearson D, Shale DJ. Neutrophil degranulation and increased pulmonary capillary permeability following oesophagectomy: A model of early lung injury in man. *British Journal of Surgery* 2005;75:883–886.

35. Proudfoot AG, McAuley DF, Griffiths MJD, Hind M. Human models of acute lung injury. *Dis Model Mech* 2011;4:145–153.

36. Glass A, O'Kane CM, Dhrampal A, Fontaine E, Ryan J, Loubani M, *et al.* Effect of simvastatin on postoperative complications in patients undergoing one-lung ventilation during surgery: the Prevention HARP-2 randomised controlled trial. *Thorax* 2025; 223072.

37. Ryan J, Bayliffe AI, McAuley DF, Yeung J, Thickett DR, Howells PA, *et al.* A nebulised antitumour necrosis factor receptor-1 domain antibody in patients at risk of postoperative lung injury: A randomised, placebo-controlled pilot study. *Eur J Anaesthesiol* 2020;37:1014–1024.

38. García-Prieto E, González-López A, Cabrera S, Astudillo A, Gutiérrez-Fernández A, Fanjul-Fernandez M, *et al.* Resistance to Bleomycin-Induced Lung Fibrosis in MMP-8 Deficient Mice Is Mediated by Interleukin-10. *PLoS One* 2010;5:e13242.

39. Craig VJ, Quintero PA, Fyfe SE, Patel AS, Knolle MD, Kobzik L, *et al.* Profibrotic Activities for Matrix Metalloproteinase-8 during Bleomycin-Mediated Lung Injury. *The Journal of Immunology* 2013;190:4283–4296.

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40. Owen CA, Hu Z, Lopez-Otin C, Shapiro SD. Membrane-Bound Matrix Metalloproteinase-8 on Activated Polymorphonuclear Cells Is a Potent, Tissue Inhibitor of Metalloproteinase-Resistant Collagenase and Serpinase. *The Journal of Immunology* 2004;172:7791–7803.

41. Cowland JB, Borregaard N. The individual regulation of granule protein mRNA levels during neutrophil maturation explains the heterogeneity of neutrophil granules. *J Leukoc Biol* 1999;66:989–995.

42. Murphy G, Reynolds JJ, Bretz U, Baggiolini M. Collagenase is a component of the specific granules of human neutrophil leucocytes. *Biochem J* 1977;162:195–7.

43. Owen C. Leukocyte cell surface proteinases: Regulation of expression, functions, and mechanisms of surface localization. *Int J Biochem Cell Biol* 2008;40:1246–1272.

44. Dolo V, Ginestra A, Cassarà D, Violini S, Lucania G, Torrisi MR, *et al.* Selective localization of matrix metalloproteinase 9, beta1 integrins, and human lymphocyte antigen class I molecules on membrane vesicles shed by 8701-BC breast carcinoma cells. *Cancer Res* 1998;58:4468–74.

45. Dosch M, Gerber J, Jebbawi F, Beldi G. Mechanisms of ATP Release by Inflammatory Cells. *Int J Mol Sci* 2018;19:1222.

46. di Virgilio F, Dal Ben D, Sarti AC, Giuliani AL, Falzoni S. The P2X7 Receptor in Infection and Inflammation. *Immunity* 2017;47:15–31.

47. Pizzirani C, Ferrari D, Chiozzi P, Adinolfi E, Sandonà D, Savaglio E, *et al.* Stimulation of P2 receptors causes release of IL-1β–loaded microvesicles from human dendritic cells. *Blood* 2007;109:3856–3864.

48. Thomas LM, Salter RD. Activation of Macrophages by P2X7-Induced Microvesicles from Myeloid Cells Is Mediated by Phospholipids and Is Partially Dependent on TLR4. *The Journal of Immunology* 2010;185:3740–3749.

ACCEPTED MANUSCRIPT

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49. Bianco F, Perrotta C, Novellino L, Francolini M, Riganti L, Menna E, *et al.* Acid sphingomyelinase activity triggers microparticle release from glial cells. *EMBO J* 2009;28:1043–1054.

50. Soni S, Tirlapur N, O'Dea KP, Takata M, Wilson MR. Microvesicles as new therapeutic targets for the treatment of the acute respiratory distress syndrome (ARDS). *Expert Opin Ther Targets* 2019;23:931–941.

51. Baldi RF, Koh MW, Thomas C, Sabbat T, Wang B, Tsatsari S, *et al.* Ventilator-induced Lung Injury Promotes Inflammation within the Pleural Cavity. *Am J Respir Cell Mol Biol* 2024;71:43–52.

52. Herbst CJ, Lopez-Rodriguez E, Gluhovic V, Schulz S, Brandt R, Timm S, *et al.* Characterization of Commercially Available Human Primary Alveolar Epithelial Cells. *Am J Respir Cell Mol Biol* 2024;70:339–350.

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Figure legends

Fig 1. BAL NEVs in response to OLV and TLV

Serial bronchoalveolar lavage (BAL) samples were obtained from both lungs of patients undergoing esophagectomy, before and after one- and two-lung ventilation. Extracellular vesicle (EV) subtypes were profiled using flow cytometry. A) An example of $CD66b^{+ve}$ neutrophil EVs (NEVs) from a single patient, highlighting a profound release of NEVs into the ventilated lung only following one-lung ventilation (OLV). B) In all patients, there was a significant increase in BAL NEVs in the ventilated lung following OLV. C) However, in the non-ventilated lung, there was no significant increase in BAL NEVs following OLV, nor following protective two-lung ventilation (TLV) in the same patients or patients undergoing gastrectomy. Data were analysed by Wilcoxon matched-pairs signed rank test, *** p <0.001.

Fig 2. Alveolar cell recruitment in response to one- and two-lung ventilation

Cells within the bronchoalveolar lavage (BAL) fluid of both lungs pre- and post-one-lung ventilation (OLV) and two-lung ventilation (TLV) were profiled using flow cytometry. A) OLV induced a significant recruitment of both neutrophils and macrophages in the ventilated lung. However, there was no increase in BAL cell numbers in the B) non-ventilated lung following OLV, or in the C) same patients undergoing protective TLV (internal controls) or D) patients undergoing gastrectomy who experience protective TLV (external controls). Data were analysed by Wilcoxon matched-pairs signed rank test. * p <0.05, ** p <0.01.

Fig 3. Soluble IL-6 and IL-8 before and after OLV.

Enzyme-linked immunosorbent assays were performed to quantify the proinflammatory cytokines interleukin (IL)-6 and IL-8 within cell-free bronchoalveolar lavage fluid of both

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lungs, pre- and post-one-lung ventilation (OLV). OLV led to an increase in IL-6 and IL-8 in both lungs, although the increase was greatest in the ventilated lung. Data were analysed by Wilcoxon matched-pairs signed rank test. ** p <0.01, *** p <0.001.

Fig 4. BAL NEVs as a biomarker for PPCs

A) Following esophagectomy, 48% of patients experienced a postoperative pulmonary complication (PPC), most commonly pulmonary infection. If a complication did occur, it was significantly more likely to affect the lung exposed to alveolar stretch during one-lung ventilation (OLV) (45% v 15%, p=0.038). Furthermore, in the ventilated lung following OLV, neutrophil-derived extracellular vesicles (NEV) increased significantly more in patients who experienced a PPC than those who did not (change in NEVs: PPC 2471 v no PPC 211 NEV/µl, p=0.044). B) Patients who sustained a PPC had significantly more bronchoalveolar lavage (BAL) NEVs within the ventilated lung after OLV compared to patients who didn't experience a PPC, which suggests that NEVs may be a clinically relevant biomarker for PPCs. Data were analysed by Chi-square test or Mann-Whitney test. * p <0.05.

Fig 5. The bioactivity of MMP-8 and MMP-9 associated with stretch-induced BAL NEVs

A) Neutrophil extracellular vesicles (NEV) were isolated from the bronchoalveolar lavage fluid of the ventilated lung following one-lung ventilation. A standardised number of NEVs were lysed and probed for proinflammatory MMP-8 and MMP-9 using the Jess automated Western Blot system, which quantifies chemiluminescence allowing a relative comparison of protein abundance. The CD66b^{-ve} negative elute from NEV isolation i.e., non-neutrophil EVs, acted as controls. NEVs contained significantly more MMP-8 and MMP-9 than non-neutrophil EVs.

B) To determine the bioactivity of NEV-associated MMP-8 and MMP-9, an in vitro alveolar model was developed by coculturing primary human alveolar epithelial cells (HAEC) and

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monocyte-derived macrophages (2:1). Isolated NEVs were incubated with the coculture for 4 hours, following which HAEC activation was measured by quantifying HAEC adhesion molecules, intercellular adhesion molecule-1 (ICAM-1) and tissue factor (TF) by flow cytometry. To assess NEV bioactivity specifically, non-NEVs ($CD66b^{-ve}$) from the same patient were also assessed, alongside isolated platelet EVs and cells incubated with media alone (control). In an additional well, the coculture was preincubated with doxycycline, a pan-MMP inhibitor, before NEV stimulation to determine the specific bioactivity of NEV-associated MMPs. NEVs caused significant ICAM-1 and TF upregulation. This bioactivity was ablated by MMP inhibition, implicating MMPs in inducing inflammation. Data were analysed by Wilcoxon matched-pairs signed rank test or ANOVA with Dunnett correction for multiple comparisons. * p <0.05, *** p <0.001. n=6.

Fig 6. EV production and ATP release following cell stretch of an in vitro alveolar model

A) To establish mechanisms of stretch-induced NEV biogenesis, primary human alveolar epithelial cells (HAEC) were cultured with neutrophils isolated from the blood of healthy volunteers. This coculture was exposed to high cyclical stretch (21%) for 30 minutes. Neutrophil and epithelial cell extracellular vesicles (EV) were quantified using flow cytometry. Cell stretch induced the production of neutrophil-derived EVs (NEV). B) Next, the cyclical stretch of an HAEC monoculture led to a rapid release of adenosine triphosphate (ATP), a damage-associated molecular pattern known to stimulate the release of leukocyte EVs. C) Finally, to test the functional importance of ATP in the production of NEVs, separate HAEC-neutrophil cocultures were exposed to cyclical stretch, with or without prior incubation with KN-62, a P2X7 inhibitor. The inhibition of ATP acting at P2X7 receptors led to the ablation of the stretch-induced NEV production after 30 minutes, highlighting the crucial role of ATP in NEV biogenesis. Data were analysed by Wilcoxon matched-pairs signed rank test, t-test or non-

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parametric ANOVA (Friedman) with Dunn's correction for multiple comparisons. * p <0.05, ** p <0.01. n=5-10.

Fig 7. Proposed pathway detailing how mechanical ventilation-induced NEVs render patients prone to PPCs

Following alveolar stretch induced by mechanical ventilation, adenosine triphosphate (ATP) is released from alveolar epithelial cells, specifically. ATP interacts with resident and recruited leukocytes such as neutrophils to instigate the production of extracellular vesicles (EV) which are laden with proinflammatory cargo e.g., matrix metalloproteinase (MMP)-8 and MMP-9. Cargo associated with EVs can then induce alveolar inflammation, rendering patients prone to postoperative pulmonary complications. DAMP: damage-associated molecular pattern, ICAM-1: intercellular adhesion molecule-1.

Table 1. Mechanical ventilation and patient observation data of patients undergoing esophagectomy during the period of OLV and TLV

During the perioperative period, mechanical ventilator and patient observation data were recorded minutely by the computer software Cerner Anaesthesia. Data was subsequently extracted, separated into the periods of one- and two-lung ventilation, and then averaged. Please note that tidal volume and surrogate mechanical power values are provided 'per lung' because throughout one-lung ventilation, the entirety of the ventilator's energy is delivered to an individual lung instead of being distributed across both lungs. Therefore, the lung ventilated during one-lung ventilation receives 'proportionally' greater tidal volume and mechanical power than during two-lung ventilation. Data were analysed by Wilcoxon matched-pairs signed rank test. n=40. IQR = interquartile range, ml = milliliter, kg = kilogram, PBW = predicted

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body weight, J = joule, min = minute, cmH_20 = centimetre of water, kPa = kilopascals, mmHg = millimetre of mercury.

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Ventilatory parameter or patient observation	One-lung ventilation (median, (IQR))	Two-lung ventilation (median, (IQR))	p-value
Tidal volume (ml) (per lung)	351 (314 – 371)	239 (226 – 268)	-
Tidal volume per predicted body weight (ml/kg PBW) (per lung)	5.1 (4.7 – 5.6)	3.7 (3.3– 3.9)	-
Mechanical Power $_{Surrogate}$ (J/min) (per lung)	8.3 (7.3 – 11.0)	5.5 (4.6 – 6.6)	-
Positive End Expiratory Pressure (cmH_20)	5.9 (3.9 – 7.0)	6.0 (5.0 – 7.9)	**0.006**
Peak Pressure (cmH_20)	25.0 (20.0 – 26.0)	19.0 (18.0 – 20.0)	**<0.001**
End-tidal carbon dioxide (kPa)	5.5 (4.9 – 6.2)	4.9 (4.6 – 5.3)	**<0.001**
Respiratory rate (per minute)	18 (16 – 20)	16 (15 – 16)	**<0.001**
Heart rate (beats per minute)	77 (70 – 94)	74 (65 – 80)	**<0.001**
Systolic blood pressure (mmHg)	106 (99 – 112)	108 (102 – 108)	0.321
Diastolic blood pressure (mmHg)	60 (56 – 65)	60 (55 – 63)	0.307
Mean arterial pressure (mmHg)	76 (74 – 80)	77 (73 – 80)	0.748
Fraction of inspired oxygen (%)	77 (66 – 92)	42 (35 – 48)	**<0.001**
Peripheral oxygen saturations (%)	95 (92 – 97)	100 (99 – 100)	**<0.001**

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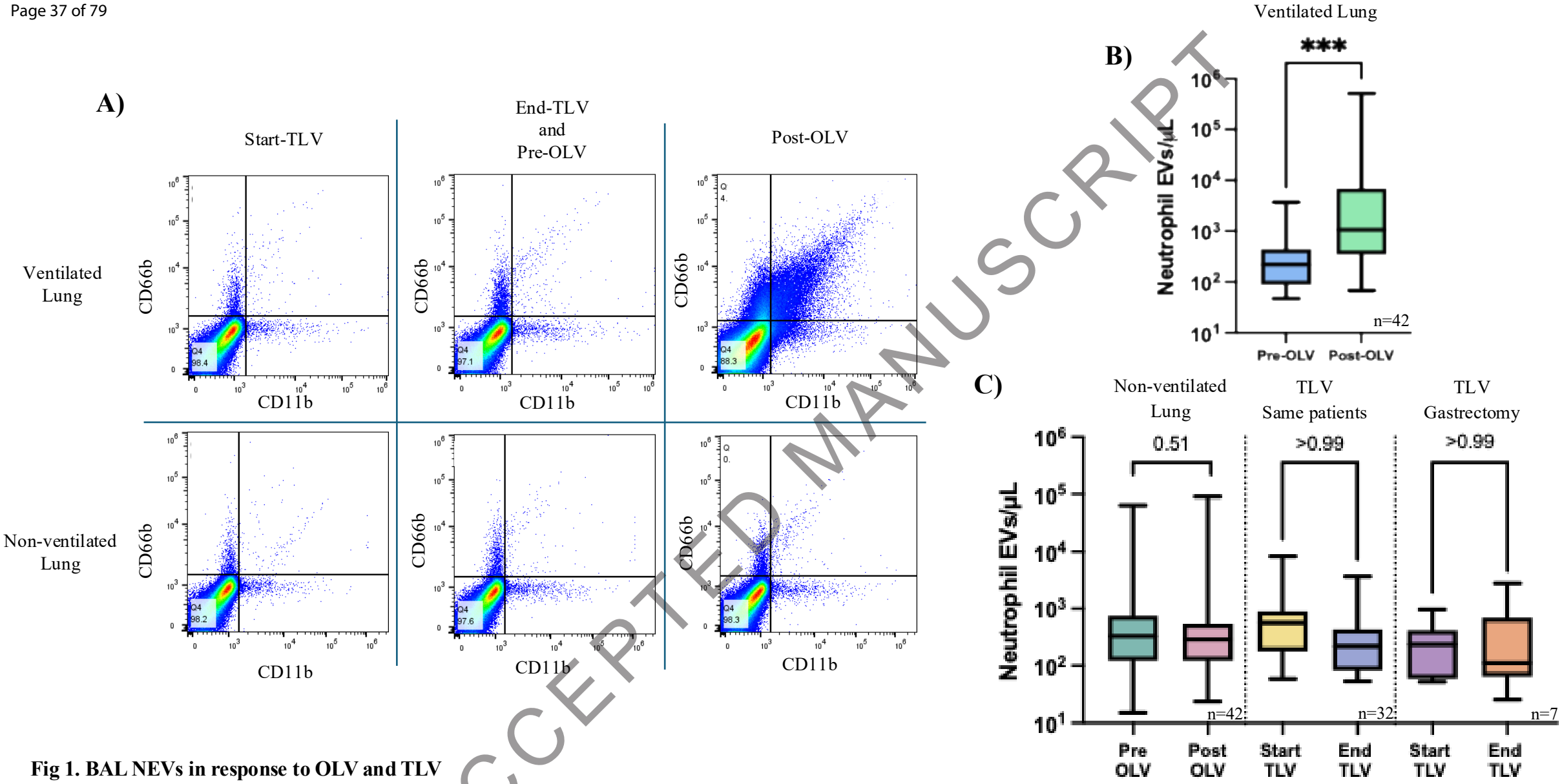


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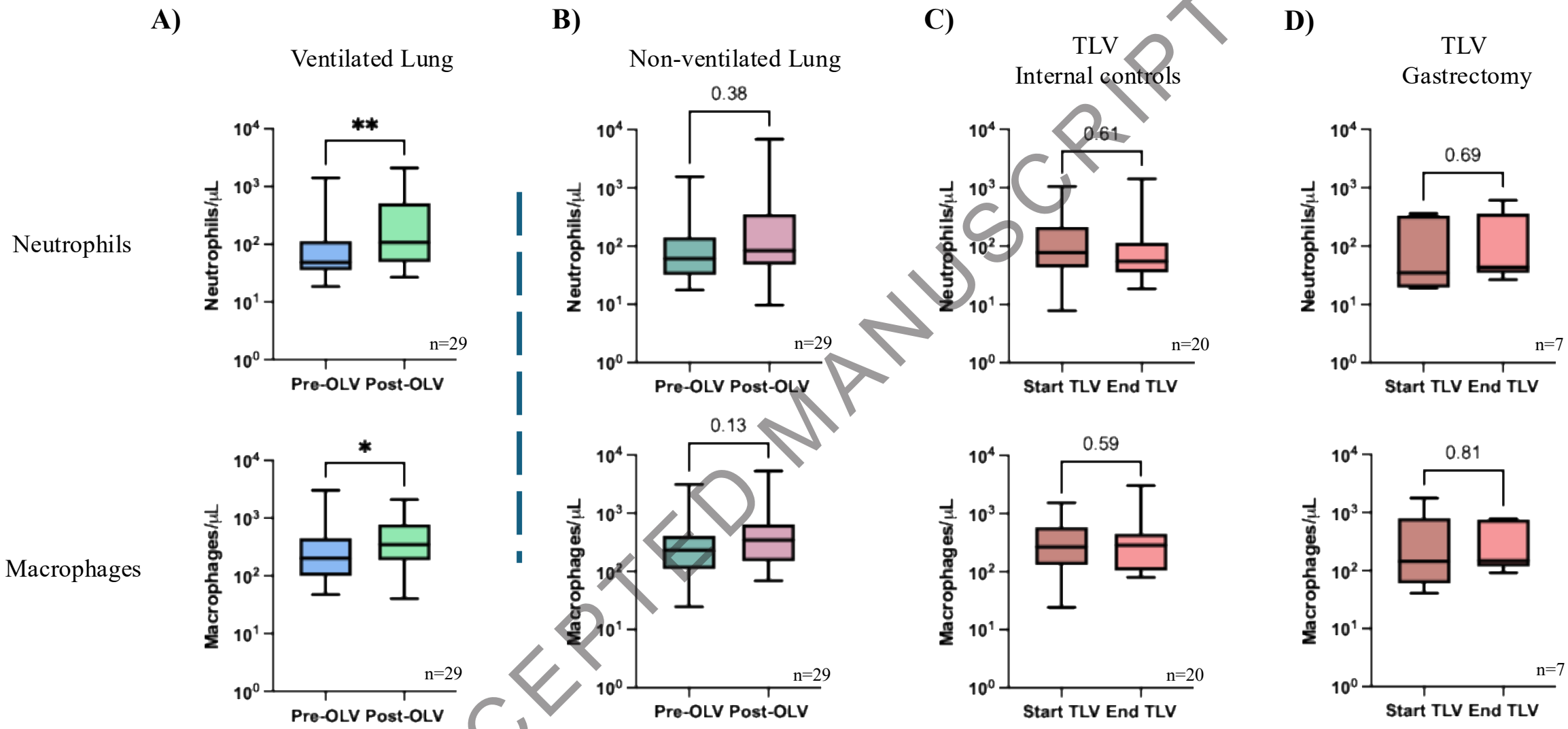


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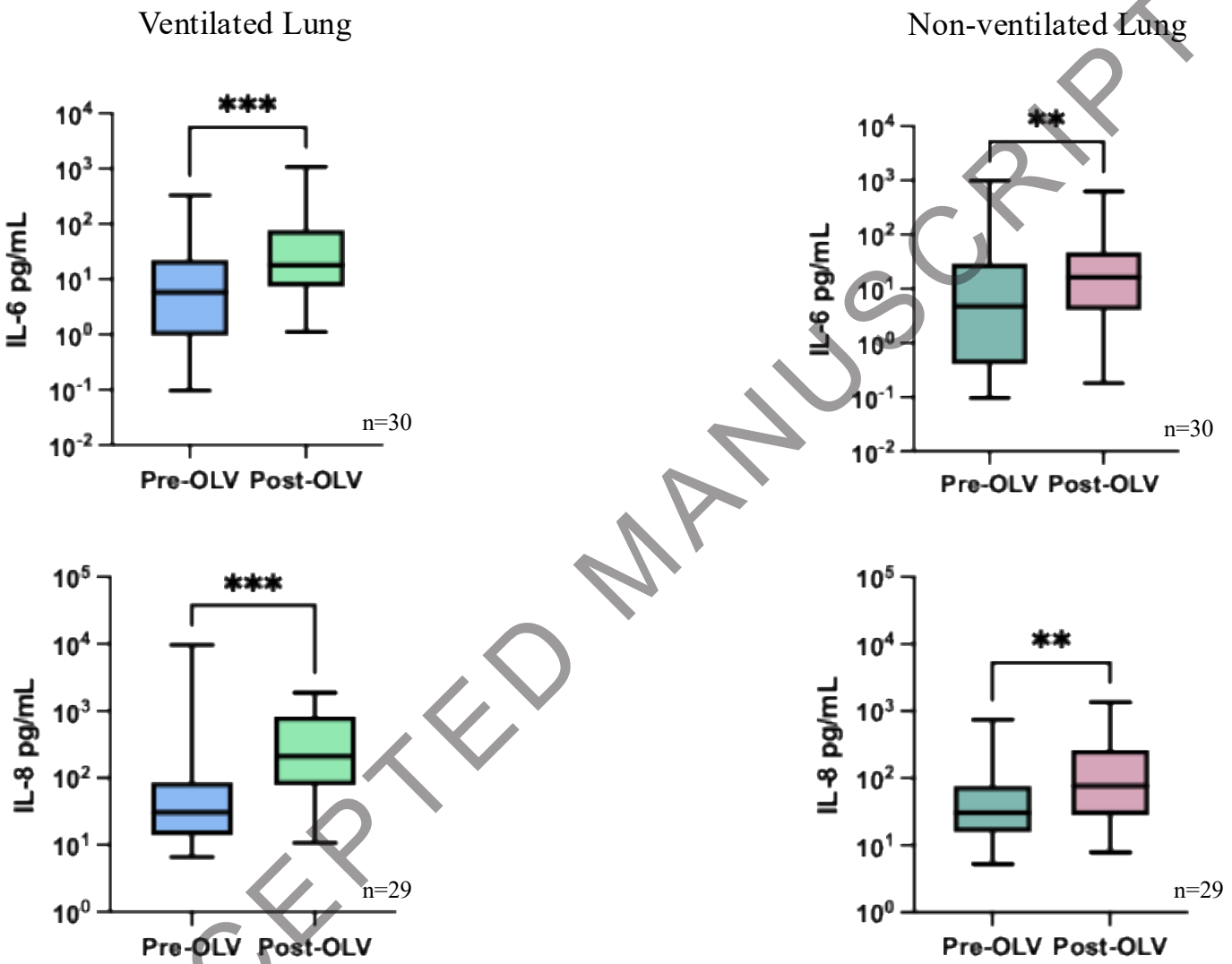


Fig 3. Soluble IL-6 and IL-8 before and after OLV.
Enzyme-linked immunosorbent assays were performed to quantify the proinflammatory cytokines interleukin (IL)-6 and IL-8 within cell-free bronchoalveolar lavage fluid of both lungs, pre- and post-one-lung ventilation (OLV). OLV led to an increase in IL-6 and IL-8 in both lungs, although the increase was greatest in the ventilated lung. Data were analysed by Wilcoxon matched-pairs signed rank test. ** p <0.01, *** p <0.001.

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A)

Postoperative Pulmonary Complication	
Yes	20 (48%)
No	22 (52%)
Nature of Complication	
Infection	16 (38%)
Respiratory failure	8 (19%)
Pneumothorax	1 (2%)
Pleural effusion	10 (24%)
Aspiration	0 (0%)
Extubation delayed / Required reintubation	7 (17%)
Required tracheostomy	2 (5%)
Acute respiratory distress syndrome	2 (5%)
Side of Complication	
All complications	20
Bilateral	8 (40%)
Ventilated lung	9 (45%)
Non Ventilated lung	3 (15%)
Change in BAL NEVs following OLV	
Patients without PPC (median NEV/µl (IQR))	211 (49 – 3775)
Patients with PPC (median NEV/µl (IQR))	2471 (154 – 37616)

B)

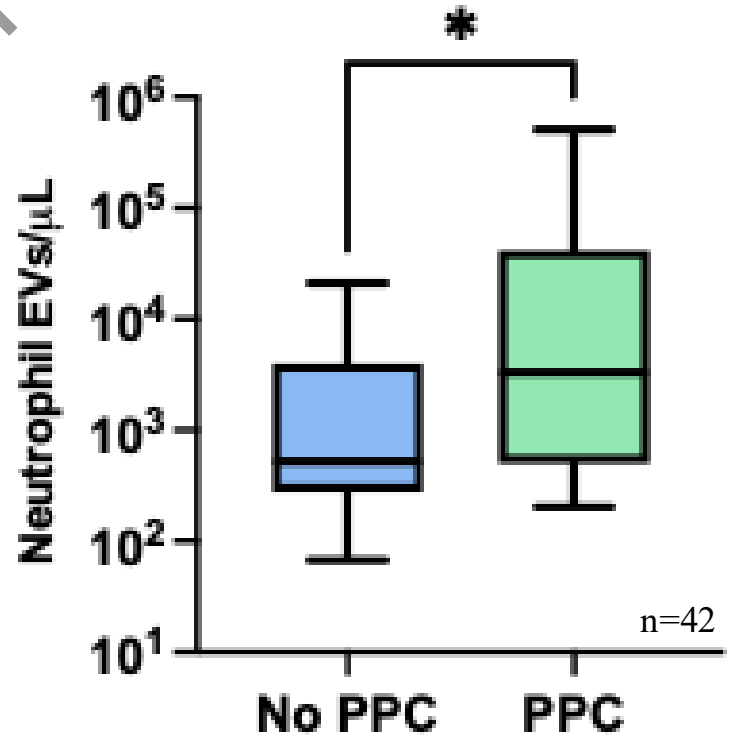


Fig 4. BAL NEVs as a biomarker for PPCs

A) Following esophagectomy, 48% of patients experienced a postoperative pulmonary complication (PPC), most commonly pulmonary infection. If a complication did occur, it was significantly more likely to affect the lung exposed to alveolar stretch during one-lung ventilation (OLV) (45% v 15%, p=0.038). Furthermore, in the ventilated lung following OLV, neutrophil-derived extracellular vesicles (NEV) increased significantly more in patients who experienced a PPC than those who did not (change in NEVs: PPC 2471 v no PPC 211 NEV/µl, p=0.044). B) Patients who sustained a PPC had significantly more bronchoalveolar lavage (BAL) NEVs within the ventilated lung after OLV compared to patients who didn't experience a PPC, which suggests that NEVs may be a clinically relevant biomarker for PPCs. Data were analysed by Chi-square test or Mann-Whitney test. * p <0.05.

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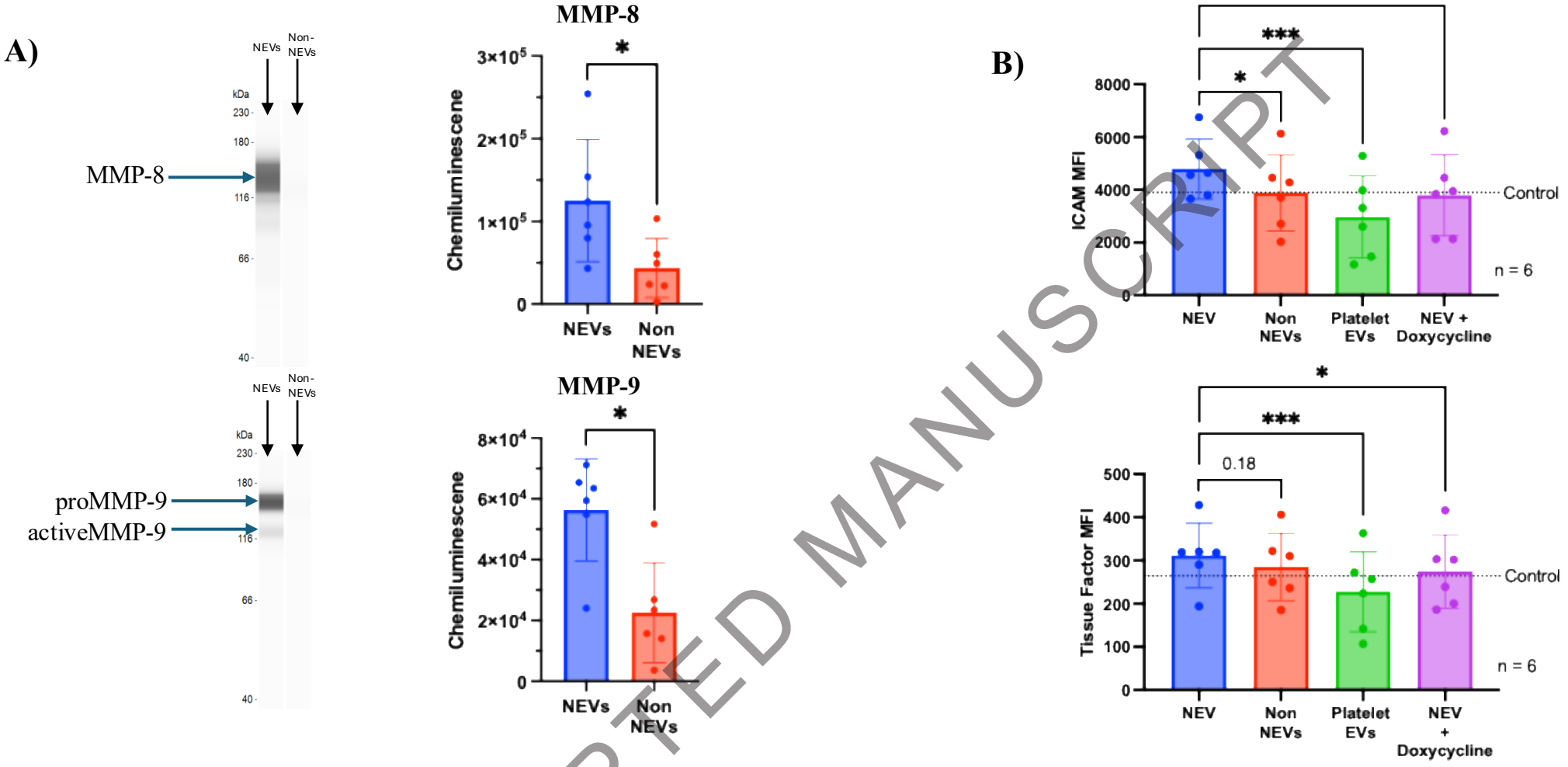


Fig 5. The bioactivity of MMP-8 and MMP-9 associated with stretch-induced BAL NEVs

A) Neutrophil extracellular vesicles (NEV) were isolated from the bronchoalveolar lavage fluid of the ventilated lung following one-lung ventilation. A standardised number of NEVs were lysed and probed for proinflammatory MMP-8 and MMP-9 using the Jess automated Western Blot system, which quantifies chemiluminescence allowing a relative comparison of protein abundance. The CD66b^{-ve} negative elute from NEV isolation i.e., non-neutrophil EVs, acted as controls. NEVs contained significantly more MMP-8 and MMP-9 than non-neutrophil EVs.

B) To determine the bioactivity of NEV-associated MMP-8 and MMP-9, an in vitro alveolar model was developed by coculturing primary human alveolar epithelial cells (HAEC) and monocyte-derived macrophages (2:1). Isolated NEVs were incubated with the coculture for 4 hours, following which HAEC activation was measured by quantifying HAEC adhesion molecules, intercellular adhesion molecule-1 (ICAM-1) and tissue factor (TF) by flow cytometry. To assess NEV bioactivity specifically, non-NEVs (CD66b^{-ve}) from the same patient were also assessed, alongside isolated platelet EVs and cells incubated with media alone (control). In an additional well, the coculture was preincubated with doxycycline, a pan-MMP inhibitor, before NEV stimulation to determine the specific bioactivity of NEV-associated MMPs. NEVs caused significant ICAM-1 and TF upregulation. This bioactivity was ablated by MMP inhibition, implicating MMPs in inducing inflammation. Data were analysed by Wilcoxon matched-pairs signed rank test or ANOVA with Dunnett correction for multiple comparisons. * p <0.05, *** p <0.001. n=6.

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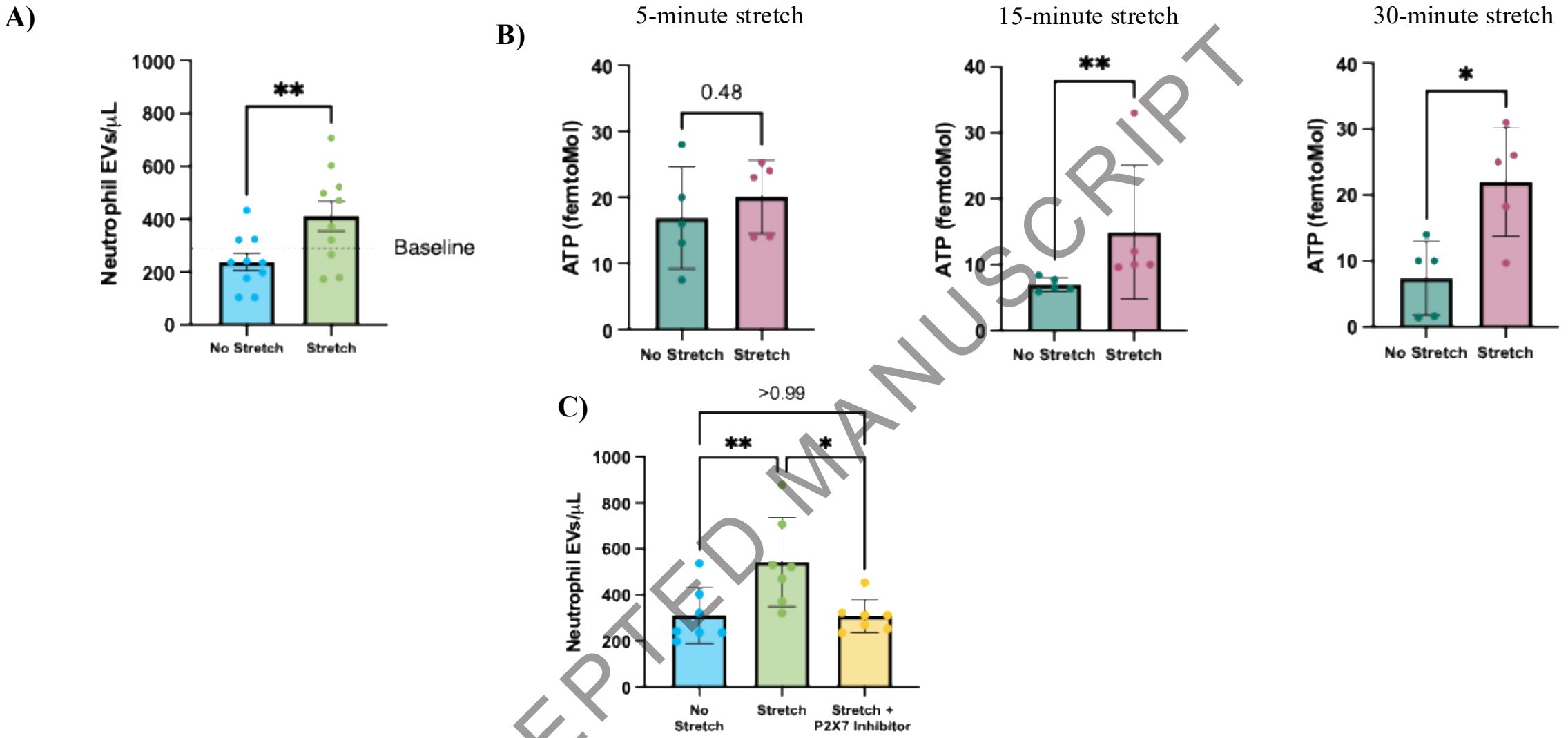


Fig 6. EV production and ATP release following cell stretch of an in vitro alveolar model

A) To establish mechanisms of stretch-induced NEV biogenesis, primary human alveolar epithelial cells (HAEC) were cultured with neutrophils isolated from the blood of healthy volunteers. This coculture was exposed to high cyclical stretch (21%) for 30 minutes. Neutrophil and epithelial cell extracellular vesicles (EV) were quantified using flow cytometry. Cell stretch induced the production of neutrophil-derived EVs (NEV). B) Next, the cyclical stretch of an HAEC monoculture led to a rapid release of adenosine triphosphate (ATP), a damage-associated molecular pattern known to stimulate the release of leukocyte EVs. C) Finally, to test the functional importance of ATP in the production of NEVs, separate HAEC-neutrophil cocultures were exposed to cyclical stretch, with or without prior incubation with KN-62, a P2X7 inhibitor. The inhibition of ATP acting at P2X7 receptors led to the ablation of the stretch-induced NEV production after 30 minutes, highlighting the crucial role of ATP in NEV biogenesis. Data were analysed by Wilcoxon matched-pairs signed rank test, t-test or non-parametric ANOVA (Friedman) with Dunn's correction for multiple comparisons. * p <0.05, ** p <0.01. n=5-10.

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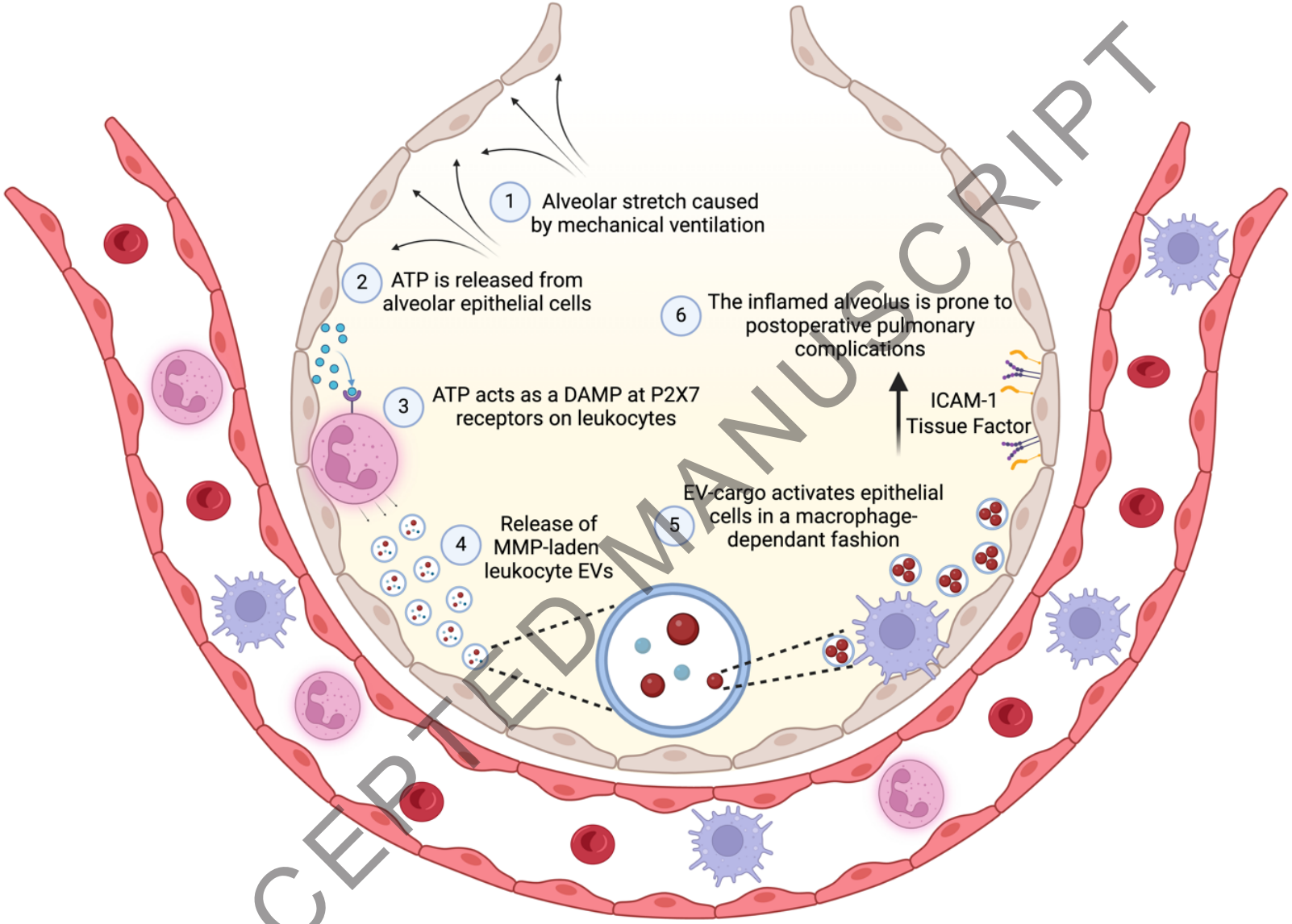


Fig 7. Proposed pathway detailing how mechanical ventilation-induced NEVs render patients prone to PPCs

Following alveolar stretch induced by mechanical ventilation, adenosine triphosphate (ATP) is released from alveolar epithelial cells, specifically. ATP interacts with resident and recruited leukocytes such as neutrophils to instigate the production of extracellular vesicles (EV) which are laden with proinflammatory cargo e.g., matrix metalloproteinase (MMP)-8 and MMP-9. Cargo associated with EVs can then induce alveolar inflammation, rendering patients prone to postoperative pulmonary complications. DAMP: damage-associated molecular pattern, ICAM-1: intercellular adhesion molecule-1.

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Ventilatory parameter or patient observation	One-lung ventilation (median, (IQR))	Two-lung ventilation (median, (IQR))	p-value
Tidal volume (ml) (per lung)	351 (314 – 371)	239 (226 – 268)	-
Tidal volume per predicted body weight (ml/kg PBW) (per lung)	5.1 (4.7 – 5.6)	3.7 (3.3 – 3.9)	-
Mechanical Power $_{Surrogate}$ (J/min) (per lung)	8.3 (7.3 – 11.0)	5.5 (4.6 – 6.6)	-
Positive End Expiratory Pressure (cmH_20)	5.9 (3.9 – 7.0)	6.0 (5.0 – 7.9)	**0.006**
Peak Pressure (cmH_20)	25.0 (20.0 – 26.0)	19.0 (18.0 – 20.0)	**<0.001**
End-tidal carbon dioxide (kPa)	5.5 (4.9 – 6.2)	4.9 (4.6 – 5.3)	**<0.001**
Respiratory rate (per minute)	18 (16 – 20)	16 (15 – 16)	**<0.001**
Heart rate (beats per minute)	77 (70 – 94)	74 (65 – 80)	**<0.001**
Systolic blood pressure (mmHg)	106 (99 – 112)	108 (102 – 108)	0.321
Diastolic blood pressure (mmHg)	60 (56 – 65)	60 (55 – 63)	0.307
Mean arterial pressure (mmHg)	76 (74 – 80)	77 (73 – 80)	0.748
Fraction of inspired oxygen (%)	77 (66 – 92)	42 (35 – 48)	**<0.001**
Peripheral oxygen saturations (%)	95 (92 – 97)	100 (99 – 100)	**<0.001**

Table 1. Mechanical ventilation and patient observation data of patients undergoing esophagectomy during the period of OLV and TLV
During the perioperative period, mechanical ventilator and patient observation data were recorded minutely by the computer software Cerner Anaesthesia. Data was subsequently extracted, separated into the periods of one- and two-lung ventilation, and then averaged. Please note that tidal volume and surrogate mechanical power values are provided 'per lung' because throughout one-lung ventilation, the entirety of the ventilator's energy is delivered to an individual lung instead of being distributed across both lungs. Therefore, the lung ventilated during one-lung ventilation receives 'proportionally' greater tidal volume and mechanical power than during two-lung ventilation. Data were analysed by Wilcoxon matched-pairs signed rank test. n=40. IQR = interquartile range, ml = milliliter, kg = kilogram, PBW = predicted body weight, J = joule, min = minute, cmH_20 = centimetre of water, kPa = kilopascals, mmHg = millimetre of mercury.

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