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**Aspirin reduces lipopolysaccharide-induced pulmonary inflammation in human models of ARDS.**

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## **What is the key question?**

Experimental animal and human observational studies suggest aspirin may be beneficial in ARDS: however, whether aspirin can reduce inflammation and injury in the human alveolus has not been assessed prospectively.

## **What is the bottom line?**

In two human models of ARDS we show that aspirin reduces pulmonary neutrophil infiltration and alveolar inflammation and injury.

## **Why read on?**

Aspirin is effective in reducing neutrophil-mediated inflammation in the human alveolus: these findings support further clinical trial testing of aspirin to treat neutrophil-driven pulmonary diseases including ARDS.

Additional data and information are available in the online data supplement

## **ABSTRACT**

**Rationale:** Platelets play an active role in the pathogenesis of ARDS. Animal and observational studies have shown aspirin's anti-platelet and immunomodulatory effects may be beneficial in ARDS.

**Objective:** To test the hypothesis that aspirin reduces inflammation in clinically relevant human models of that recapitulate pathophysiological mechanisms implicated in the development of ARDS.

**Methods:** Healthy volunteers were randomized to receive placebo or aspirin 75mg or 1200mg (1:1:1) for seven days prior to lipopolysaccharide (LPS) inhalation, in a double-blind, placebo-controlled, allocation concealed study. Bronchoalveolar lavage (BAL) was performed 6 hours after inhaling 50 micrograms of LPS. The primary outcome measure was BAL IL-8. Secondary outcome measures included markers of alveolar inflammation (BAL neutrophils, cytokines, neutrophil proteases), alveolar epithelial cell injury, systemic inflammation (neutrophils and plasma CRP) and platelet activation (thromboxane B<sub>2</sub>, TXB<sub>2</sub>). Human lungs, perfused and ventilated *ex vivo* (EVLP), were randomized to placebo or 24mg aspirin, and injured with LPS. BAL was carried out 4 hours later. Inflammation was assessed by BAL differential cell counts and histological changes.

**Results:** In the healthy volunteer (n=33) model, data for the aspirin groups were

combined. Aspirin did not reduce BAL IL-8. However, aspirin reduced pulmonary neutrophilia and tissue damaging neutrophil proteases (MMP-8/-9), reduced BAL concentrations of TNF $\alpha$ , and reduced systemic and pulmonary TXB2. There was no difference between high and low dose aspirin. In the EVLP model aspirin reduced BAL neutrophilia, and alveolar injury as measured by histological damage.

**Conclusion:** These are the first prospective human data indicating that aspirin inhibits pulmonary neutrophilic inflammation, at both low and high doses. Further clinical studies are indicated to assess the role of aspirin in the prevention and treatment of ARDS.

## INTRODUCTION

The acute respiratory distress syndrome (ARDS) is characterized by an uncontrolled alveolar inflammatory response, the hallmarks of which are neutrophil infiltration, protein rich pulmonary oedema accumulation, hypoxia and the need for mechanical ventilation. Platelets may contribute to the pathophysiology of ARDS. Platelet activation, degranulation and aggregation within the lung can lead to further vasodilatation, with chemoattraction of neutrophils and monocytes, increasing coagulation and the deposition of a provisional matrix, and inducing fibroblast activation and proliferation, all features of ARDS [1,2].

Additionally the formation of neutrophil or monocyte platelet aggregates can drive inflammation: neutrophils seek out activated platelets within the circulation and interact with them via PSGL-1 – a glycoprotein ligand for P-selectin - to initiate inflammation[3]. This process is implicated directly in several *in vivo* models of acute lung injury, including acid-induced ALI and LPS induced injury [4,5].

Recently platelets have also been shown to play an important role in formation of neutrophil-extracellular traps (NETs) [6]. NETs formation allows activated neutrophils to release chromatin material interlaced with granular proteins and proteases, which traps pathogens but also damages endothelial cells and drives neutrophil recruitment to the lungs [7]. An increase in NETs has been detected in plasma of patients with transfusion related ARDS [7], and is also implicated in the pathophysiology of ARDS of other aetiologies [8,9].

Aspirin is widely used as a highly effective anti-platelet in the primary and secondary prevention of cerebrovascular, coronary artery and peripheral vascular disease. In

addition to its anti-platelet effects, it increases the production of resolvins and lipoxins, which may reduce inflammation and promote resolution [10]. Various experimental studies of ARDS have shown a beneficial effect of aspirin on reducing pulmonary inflammation [11]. Similarly, in observational studies, patients on aspirin therapy prior to hospitalization had a reduced incidence of ARDS [12,13] and organ dysfunction [14]]. In contrast the recent LIPS-A study, which prospectively treated patients at risk of developing ARDS with aspirin, showed no reduction in incidence of ARDS [15]. The study had limitations, with a lower incidence of ARDS than expected, and the population was much less ill, with a lower incidence of mechanical ventilation, acute kidney injury and mortality, than predicted. As such the study was underpowered for the primary and secondary endpoints. In addition over half the screened potentially eligible population was excluded due to pre-existing anti-platelet use or pre-existing bilateral infiltrates. The authors acknowledged that the study was underpowered and concluded the exclusion of large cohorts significantly limited the generalizability of the findings. In a single centre observational study we found use of aspirin (at anti-platelet doses) in patients with ARDS either prior to, or during their ICU stay was associated with a reduction in ICU mortality [16].

Most animal studies have used a higher dose of aspirin, while observational studies have reported a beneficial effect with 75 – 81mg of aspirin [12]. There have been no prospective experimental studies testing whether aspirin can directly affect inflammation within the human lung (LIPS-A addressed only markers of systemic inflammation [15]), or whether high or low doses are necessary to attenuate pulmonary inflammation in humans.

The objective of this study was to test if aspirin can reduce pulmonary inflammation in two human models of ARDS, and to identify if high dose aspirin was more effective than low dose in reducing pulmonary inflammation.

Inhalation of LPS has been previously shown to produce pulmonary inflammation, which is qualitatively representative of ARDS without any associated serious adverse outcomes [17], and has been used to test anti-inflammatory or pro-repair interventions to inform clinical trials for the prevention or treatment of ARDS [17]. The model of injuring isolated perfused and ventilated human lungs *ex vivo*, allows the testing of anti-inflammatory therapeutics in a human setting, with the advantages of being able to use a more injurious stimulus than in the healthy volunteer inhaled LPS model, and allowing histological examination [18 ,19, 20]. Instilling LPS in this *ex-vivo* lung perfusion (EVLP) model drives neutrophilic pulmonary inflammation, impaired fluid clearance and elevation in BAL concentrations of the pro-inflammatory cytokines that characterize ARDS [18].

In this study, we show that aspirin reduces neutrophilic pulmonary inflammation, and peripheral neutrophil activation as measured by NETs formation, and that low and high dose aspirin have similar effects within the pulmonary compartment.

## **METHODS**

### ***In vivo* model of pulmonary inflammation in healthy volunteers**

The study, ARENA (the effect of **A**spirin on **RE**ducing **iN**flammation in a human *in vivo* model of **A**cute lung injury), was registered at ClinicalTrials.gov (NCT01659307) and was approved by the local research ethics committee (ORECNI).



*Volunteers:* Thirty-five healthy non-smoking subjects, taking no regular medication, recruited by advertising, underwent screening assessment (history, physical examination, routine blood investigation, ECG, and spirometry).

Exclusion criteria were age <18 years, pregnancy, breast-feeding or female of childbearing potential not taking adequate contraception, participation in a clinical trial of an investigational medicinal product within 30 days, aspirin or non-steroidal anti-inflammatory use in the past 4 weeks, history of asthma, known aspirin or NSAID hypersensitivity, history of peptic ulcer disease, platelet count < 150 x 10<sup>6</sup>/ml, known aspirin resistance.

All subjects gave fully informed consent to partake in the study. The study was carried out in the Wellcome Trust-Wolfson Northern Ireland Clinical Research Facility (CRF) at Belfast City Hospital.

*Study design:* This was a randomized, double-blinded, placebo-controlled allocation concealed clinical study in which subjects were randomized to aspirin 1200 mg, 75 mg, or placebo (1:1:1). Randomization was undertaken by an independent clinical trials pharmacist, using the statistical software nQuery Advisor. Mixed block sizes (of 3, 6 and 9) and no stratification were used. Blinding was achieved by encapsulation with gelatin. The aspirin capsule contained the aspirin tablet with lactose powder. The placebo capsule contained lactose powder only. Both aspirin and placebo study drugs had an identical appearance. Subjects took the study medication for 7 days before inhalation of LPS. On day 7, 30 mins after the study medicine was taken under direct observation by the study team, subjects inhaled 50micrograms LPS (O26:B6 - Sigma) using an automated nebulizer (Spira dosimeter, Finland) as previously described [17].

Blood was collected immediately before the subject inhaled the LPS, and also at 6 hours and 24 hours after LPS inhalation. Bronchoalveolar lavage (BAL) was performed 6 hours after LPS inhalation, at which point we have previously identified a robust neutrophilic inflammatory response. Processing of BAL fluid (BALF) and blood was performed as described previously [17,21]. The study schedule is summarized in figure S1 in the supplementary appendix.

The primary outcome of the study was BAL IL-8 concentration. The sample size was calculated as 11 per group to detect a 30% change in BAL IL-8, with 80% power at 2-tailed significance of <0.05, with aspirin. This was based on a previous study where BAL IL-8 concentration after LPS inhalation by healthy volunteers was 389+/-94pg/ml [17], and evidence that aspirin reduced systemic IL-8 by up to 30% in a cohort of patients undergoing dialysis [22]. The main secondary efficacy outcomes were markers of alveolar inflammatory response (BAL neutrophil counts, BAL inflammatory cytokines), alveolar epithelial injury (albumin, protein permeability ratio, SP-D, and MMP-7) and plasma inflammatory response as assessed by CRP 24 hours after LPS inhalation.

### ***Ex vivo* lung perfusion (EVLP) model**

This model was adapted from the methods described by Lee *et al* [18]. Briefly, lungs obtained from IIAM (the International Institute for the Advancement of Medicine), with consent for use in research, were perfused, re-warmed with DMEM with L-glutamine and 5 % albumin (perfusate) and inflated with 10cm H<sub>2</sub>O continuous positive airway pressure. BAL was carried out at baseline once a temperature of 35°C was reached.

Whole blood (100ml) obtained from a healthy volunteer was incubated for 1hr with 24mg aspirin and added to the perfusate (giving a final concentration of aspirin of 21 micrograms/ml in the perfusate, which corresponds to peak plasma levels obtained after a 300mg dose[23]). Simultaneously LPS (6mg) was introduced into a pre-selected lobe. At four hours BAL was repeated. Experiments were carried out for 6 lungs in the placebo group and 5 lungs in the aspirin treated group.

The use of human lungs and blood was approved by Queen's University of Belfast School of Medicine, Dentistry and Biomedical Sciences Research Ethics Committee. The study schedule is shown in Supplementary figure 2. A detailed description of the EVLP model is included in the online supplement.

The investigator carrying out the laboratory analyses was kept blinded to the treatment assignment group of the normal volunteers or the *ex vivo* lungs throughout.

### **BAL cytokines and proteases**

These were measured using cytometric bead array or ELISA as previously described [17], and are detailed in the online supplement.

### **BAL protein and permeability measurements**

Total protein was measured by Bradford assay. BAL albumin was measured by an immunoturbidimetric method by Randox Testing Services, UK. IgG was measured by ELISA (Immundiagnostik). Protein permeability ratio was expressed as ratio of BAL

IgG:total protein.

### **Neutrophil extracellular traps (NETs)**

To quantify NETs in human plasma, a capture ELISA based on NE associated with DNA was used [9]. A detailed description is provided in the online supplement.

### **Leukocyte platelet aggregates (LPAs)**

Neutrophil- and monocyte-platelet aggregates were measured in blood at baseline and 6 hours after LPS inhalation as previously described [5]. A detailed description is provided in the online supplement.

### **Phagocytosis assay**

Neutrophils isolated from peripheral blood were allowed to adhere to tissue culture plastic for 30 minutes prior to treatment with aspirin (0.1 or 0.5mM). Cells were then exposed to autologous serum-opsonized zymosan for 30 minutes, washed with PBS, air dried, fixed with methanol, and stained with Giemsa to determine the percentage of neutrophils containing two or more zymosan particles [24]. Further details are given in the online supplement.

### **Lung injury severity score**

Lung sections from the injured lobes in the EVLP model were examined histologically. Details of the scoring system [25] are given in the online supplement. The scorer was blinded to the group allocation.

## Statistical Analysis

In the healthy volunteer study, the *a priori* plan was to present and compare data from the combined aspirin-treated groups with placebo. A secondary analysis to determine if results in the high and low dose groups differed was carried out. There were no planned interim analyses. Aspirin and placebo groups were compared by Mann Whitney U test. Data for the EVLP study were analyzed using GraphPad Prism (Graph Pad Software; San Diego, CA). A p value of 0.05 or less was considered significant.

## RESULTS

35 healthy subjects were randomized; 2 withdrew before progressing to LPS inhalation and bronchoscopy (one decided not to proceed with the study, and one developed an upper respiratory tract infection, so LPS inhalation and bronchoscopy were considered inappropriate). Randomization continued until 33 volunteers completed the study protocol. There were 13 in the placebo cohort, and 10 in each aspirin cohort (n=20 aspirin in total), (CONSORT diagram figure 1). Baseline parameters including age, sex, BMI or lung volumes are shown in table 1.

**Table 1: Baseline characteristics of healthy volunteers inhaling LPS**

Characteristics	Placebo	Aspirin
Age	24 +/- 4	27 +/- 10
Sex (% female)	35%	53%
BMI	23 +/- 3	25 +/- 3
FEV <sub>1</sub> (litres)	3.8 +/- 1	4.1 +/- 1
FVC (litres)	4.4 +/- 1	4.9 +/- 1

Aspirin was well-tolerated with no increased incidence of adverse effects in the aspirin treated cohort. There were no significant changes in FEV<sub>1</sub>, FVC, oxygen saturation measured by pulse oximetry, or vital signs over the course of the study. There were no SAEs (serious adverse events) or SUSARs (suspected unexpected serious adverse reactions) to trigger an unscheduled data monitoring committee meeting. A detailed description of the monitoring and adverse events (supplementary table 1, ST1) experienced by the volunteers is included in the online supplement.

### **Aspirin reduces BAL neutrophilia but not BAL IL-8**

Aspirin pre-treatment did not significantly reduce the pre-specified primary outcome measure IL-8 (figure 2a). However, aspirin reduced neutrophil count in the BAL fluid of healthy volunteers who inhaled LPS (figure 2b). There was no significant change in BAL macrophage or lymphocyte counts (table 2), nor BAL neutrophil extracellular traps (NETs) – data not shown.

Table 2

<b>BAL</b>	<b>Placebo</b>	<b>Aspirin</b>	<b>Difference in median (95%CI)</b>	<b>p value</b>
Macrophages (x10 <sup>5</sup> /ml)	2.99 (2.30-4.24)	2.96 (1.44-3.71)	0.4 (-0.71, 1.49)	0.45
Lymphocytes (x10 <sup>5</sup> /ml)	0.40 (0.26-0.62)	0.35 (0.20-0.80)	0.05 (-0.18, 0.25)	0.65

### **Aspirin reduces neutrophil-derived enzyme secretion in the alveolar space.**

Having established that aspirin reduces BAL neutrophilia in the human lung in response to LPS, we investigated the effect on markers of neutrophil activity. The neutrophil-specific enzyme MMP-8 was significantly reduced in the aspirin treated group (figure 3a). MMP-9, also secreted by activated neutrophils (and other cells) was significantly reduced in the aspirin treated group (figure 3b). The major secreted inhibitors of these MMPs, TIMPs-1/-2, were unaffected by aspirin treatment (figure S3a and S3b). Both myeloperoxidase and neutrophil elastase showed a trend to reduction in response to aspirin (figure 3c and d) but this did not reach statistical significance.

### **Aspirin reduces BAL $TNF\alpha$ in healthy volunteers who have inhaled LPS.**

To investigate the effect of aspirin on the inflammatory milieu of the LPS-injured alveolus we measured key cytokines that drive injury in ARDS. Aspirin reduced BAL concentrations of the inflammatory cytokine  $TNF\alpha$  (table 3) There was a non-statistically significant trend towards reduction in all other measured pro-inflammatory cytokines particularly IL-6,  $p=0.07$  (table 3). The IL-1 $\beta$ :IL-1Ra ratio, a functional marker of unopposed IL-1 $\beta$  activity was reduced but again did not reach statistical significance (table 3). Aspirin had no effect on BAL CRP.

**Table 3 The effect of aspirin on BAL cytokines in healthy volunteers who inhaled LPS**

BAL analyte	Placebo	Aspirin	Difference in median (95% CI)	P value
TNF $\alpha$ (pg/ml)	106 (87-154)	78 (52-128)	33 (1.91, 63.74)	0.03
IL-1 $\beta$ (pg/ml)	43 (26-99)	37 (19-55)	13 (-6, 46)	0.17
IL-1 $\beta$ :IL-1Ra	0.02 (0.01-0.03)	0.01 (0.008-0.014)	0.01 (-0.001, 0.02)	0.07
IL-6 (pg/ml)	857 (606-1263)	648 (330-835)	258 (-40, 526)	0.07
IL-8 (pg/ml)	448 (321-657)	346 (275-508)	102 (-40, 253)	0.11
MCP-1 (pg/ml)	547 (412-1055)	502 (348-888)	83 (-168, 363)	0.45
CRP (pg/ml)	332 (93-1140)	308 (114-824)	9 (-351, 266)	0.99

### **Aspirin did not affect epithelial barrier function in response to LPS**

Aspirin had no effect on epithelial barrier function as measured by BAL total protein (not shown), albumin (figure 4a) or IgG:total protein ratio (figure 4b). Interestingly aspirin also had no effect on secretion of the type II epithelial specific marker SP-D (figure 4c), nor on MMP-7, an epithelial derived protease in the alveolus (figure 4d).

### **The effect of aspirin on systemic inflammatory response**

While there was a trend towards a reduction in peripheral neutrophil count, and in peripheral NET formation in the aspirin treated group 6 hours after LPS inhalation these



did not reach significance (figure 5a,  $p=0.068$  and figure 5b,  $p=0.086$ ). Aspirin did not reduce peripheral CRP (supplementary data figure S4), nor peripheral leucocyte platelet aggregate formation (supplementary figure S5). Importantly when human primary neutrophils were treated with aspirin *in vitro* aspirin did not impede neutrophil phagocytosis (supplementary data figure S6).

### **Aspirin reduces BAL and systemic thromboxane B2 levels.**

Consistent with its known effect of circulating platelets, volunteers in the aspirin treated groups had reduced plasma concentrations of thromboxane B2 (figure 6a). Interestingly aspirin treatment was also associated with a significant reduction in BAL thromboxane B2 concentrations also (figure 6b).

### **High versus low dose aspirin in the human healthy volunteer model.**

The *a priori* statistical analysis plan was to combine data from the aspirin treated cohorts and compare with placebo. A secondary analysis of high versus low dose aspirin showed no consistent difference between the two groups (table 4). For neutrophil derived enzymes, MPO was lower in the high dose aspirin group, while NE was lower in the 75mg aspirin group.

**Table 4: The effect of high vs low dose aspirin on BAL and plasma biomarkers**

	<b>Aspirin 75mg</b>	<b>Aspirin 1200mg</b>
<b>BAL</b>		
Neutrophils (x10 <sup>5</sup> /ml)	1.6 (1.3-2.8)	1.5 (1.2-1.7)
MMP-8 (ng/ml)	3.4 (2.3-4.8)	2.6 (1.9-4.0)
MMP-9 (ng/ml)	33.7 (27.1-50.1)	33.7 (16.1-44.4)
MPO (ng/ml)	38.6 (29.2-53.3)	25.2 (17.5-34.6)
NE (ng/ml)	10.6 (6.0-15.6)	21.1 (11.1-36.1)
TNF $\alpha$ (pg/ml)	77 (51-100)	90 (49-141)
IL-6 (pg/ml)	563 (368-880)	782 (284-901)
IL-8 (pg/ml)	308 (264-431)	397 (269-515)
MCP-1 (pg/ml)	497 (385-967)	502 (289-924)
IL-1 $\beta$ (pg/ml)	46 (30-66)	30 (16-38)
IL-1Ra (pg/ml)	3000 (2413-4182)	2266 (1700-3682)
TIMP-1 (pg/ml)	2155 (1431-3783)	2243 (1275-3789)
TIMP-2 (pg/ml)	566 (495-912)	574 (224-805)
SP-D (pg/ml)	27810 (23356-38705)	35242 (23800-41848)
CRP (pg/ml)	449 (97-943)	237 (116-867)
<b>PLASMA</b>		
CRP (mg/l)	40 (33-61)	32 (25-63)

Data are median (IQR)

## **Aspirin reduces BAL neutrophils and histological injury score in the *ex vivo* perfused and injured lung.**

Consistent with its effects in the healthy volunteer model of LPS inhalation aspirin reduced BAL neutrophilia in human lungs injured with LPS *ex vivo* (figure 7a), and reduced evidence of lung injury as assessed at histological examination (figure 7b and c). Aspirin did not improve alveolar fluid clearance, a marker of intact epithelial function, in the *ex vivo* injured lung (not shown).

## **DISCUSSION**

While prior pre-clinical and observational studies have indicated aspirin may have beneficial effects in ARDS these are the first prospective data indicating a beneficial effect on alveolar inflammation in human subjects, and the first data showing aspirin reduces histological alveolar injury in human lungs.

Aspirin did not significantly reduce the pre-specified primary outcome measure of BAL IL-8 concentration. In this study the standard deviation of BAL IL-8 in healthy donors after LPS inhalation was higher than we have found in a previous study and the study was therefore underpowered for this outcome measure. Interestingly while aspirin did not significantly reduce IL-8 it did reduce neutrophil infiltration to the alveolar space in both the healthy volunteer study and the EVLP model, suggesting other mediators of neutrophil chemoattraction were affected.

Aspirin reduced BAL concentrations of neutrophil specific protease MMP-8 as well as MMP-9. Both of these proteases are present in pre-formed states within neutrophils, in the secondary (MMP-8) and tertiary granules (MMP-9). While MMP-9 is also secreted

by other cells within the alveolus, including epithelial cells and macrophages [26], the neutrophil is recognized to be a major source of its production in ARDS [27]. The reduction in these proteases implies that in addition to reduced overall numbers of neutrophils in the alveolar space in the aspirin treated group, there are less degranulated neutrophils here also. A reduction in MMP-9 and MMP-8 may reduce tissue injury in the early stages of alveolar injury.

LPS inhalation drives activation of TLR-4 and down-stream activation of NF $\kappa$ B-dependent cytokine production. Aspirin significantly reduced secretion of the pro-inflammatory cytokine TNF $\alpha$ . Macrophage-derived TNF $\alpha$  has long been identified as a key factor driving activation of neutrophils in ARDS[28]. *In vivo* models show that selective inhibition of the pro-inflammatory signaling TNFR1 (p55) reduces neutrophil recruitment and injury in acid - and ventilator induced lung ARDS[29]. A trend to reduction in secretion of the other major pro-inflammatory cytokines implicated in ARDS (including IL-6 and IL1 $\beta$  net activity as assessed by IL-1 $\beta$ :IL-1Ra ratio) was seen but did not reach statistical significance. Aspirin in experimental studies has been shown to attenuate activation of NF $\kappa$ B by inhibiting I $\kappa$ B kinase [30], which may be a potential mechanism of reduction in the secretion of pro-inflammatory cytokines seen in this study.

Importantly, although aspirin had a clear effect on neutrophilic inflammation in this model it did not have any obvious effect on epithelial barrier function, as assessed by protein leak. Altered neutrophil infiltration to the alveolus in response to injury without changes in epithelial barrier function have been reported by other investigators [31,32] suggesting that the two processes, both pathophysiological hallmarks of ARDS, may be

differentially regulated. SP-D is reduced in the alveolar space in ARDS [33], and in our study aspirin had no effect on this type II epithelial cell marker implying aspirin does not exert its effect directly on this epithelial cell. In addition, the absence of an effect on MMP-7, the most abundant source of which in the lung is the epithelium [34], also suggests aspirin has little direct effect on the epithelium. Taken together the data suggest aspirin may have a significantly anti-inflammatory effect during the early injury phase of ARDS, without a direct effect on protein leak.

The *ex vivo* lung perfusion model has been used in previous studies as a human model of ARDS [18]. LPS administered intra-bronchially resulted in a significant increase in infiltration of neutrophils from the blood added to the perfusate into the alveoli. Neutrophil sequestration in the alveoli characterizes ARDS [35]. In addition to replicating the findings of reduced neutrophil recruitment to the alveolar space in the aspirin-treated group as seen in the healthy volunteer model, we were able to assess the effect of aspirin on whole tissue inflammation by histology. The scoring system used was a modification of that proposed by Matute-Bello *et al* [25]. Normal alveolar structure is characterized by thin alveolar walls with macrophages and neutrophils being rarely present, while in ARDS injured lungs show increased alveolar septal thickness, intra-alveolar haemorrhage, protein debris and neutrophilia. Pre-treatment with aspirin significantly reduced the severity of injury in this model, and likely reflects reduced neutrophil dependent injury. Similar to the human volunteer model aspirin had no effect on epithelial function as measured by AFC (alveolar fluid clearance).

Systemically a trend to reduction in neutrophil count occurred but did not reach statistical significance. Importantly aspirin did not impair neutrophil phagocytic uptake of

opsonized zymosan beads *in vitro*, implying aspirin may be used for anti-inflammatory effects while retaining the capacity to engulf pathogens. We recognize this assay did not measure aspirin's effect on phagocytosis *in vivo*, which may differ from its *in vitro* activity.

Recent studies have highlighted the importance of platelet-neutrophil interaction in neutrophil extracellular traps (NETs) formation. NETs have been shown to have bactericidal properties, which damage native cells[36] and have also been implicated in the pathology of ARDS. Platelet-dependent NETs formation drove pulmonary platelet sequestration, capillary permeability and pulmonary oedema in an *in vivo* model of transfusion related acute lung injury (TRALI) [7]. In our study median plasma NETs formation fell by approximately 30% in the aspirin-treated cohort, but did not reach statistical significance. It is possible that reduced plasma NETs may have contributed to the reduced neutrophil influx into the alveolar space.

Leukocyte-platelet aggregate (LPA) formation has been implicated in driving pulmonary and systemic inflammation [3], and although both monocyte- and neutrophil-platelet aggregates were demonstrable in this study, aspirin did not reduce their formation, unlike in the case of LPS challenge in mice [5]. It is possible that in LPS-stimulated inflammation in humans, similar to patients with atherosclerosis [37], aspirin does not reduce the platelet surface expression of p-selectin sufficiently to inhibit leukocyte-platelet aggregate formation.

Inhibition of COX-1 in platelets leads to reduced Thromboxane A secretion. Thromboxane A is rapidly metabolized to its more stable metabolite Thromboxane B2[38]. We demonstrated a reduction in thromboxane B2 in the plasma of patients

taking aspirin, confirming that the medication had been taken, and the cohort was not “aspirin-resistant” [39]. Reduction in systemic thromboxanes may be related to the trend to reduction in NETs formation in the aspirin-treated cohort, since NETs formation is thromboxane-dependent [7].

Interestingly we demonstrated reduced thromboxane B2 in the BAL of the aspirin treated volunteers, consistent with reduced platelet activation in the lung. Although thromboxanes can be produced by cells other than platelets [40], the quantities are much lower. The high concentrations measured in this study, given an approximate 100-fold dilutional effect of BAL, lead us to speculate that LPS inhalation may drive platelet-activation within the human alveolar compartment, as has been shown in the murine lung [5].

This is the first human study to compare two different doses of aspirin in the setting of pulmonary inflammation. The rationale for this was to assess whether low dose aspirin, (that primarily has an anti-platelet effect) or high dose, (that has a direct anti-inflammatory effect by reducing NF $\kappa$ B-dependent gene transcription in tissues) was effective in reducing pulmonary injury induced by LPS inhalation. There was no dose dependent effect within the aspirin sub-groups in the clinical study. The lower dose is sufficient to inhibit platelet activation and thromboxane release. Given that this was associated with reduced NETs and pulmonary neutrophilia we speculate that aspirin inhibits neutrophilic inflammation in a platelet-dependent manner potentially via reduced thromboxane-dependent NETs formation required for neutrophil migration to the lung. 1200mg aspirin was used as the higher dose and is associated with anti-inflammatory effects in humans, but was not additional in this model. Higher doses of up to 8g/day

have been used in rheumatic diseases for their anti-inflammatory effect, but are associated with increased gastro-intestinal side effects and salicylism. It is possible that a higher dose of aspirin in this model would have shown additional anti-inflammatory activity but we felt was unlikely to be clinically appropriate for critically ill patients. Given low dose aspirin is less likely cause adverse effects in the critically ill patient population, and shows efficacy in this model, future clinical trials testing aspirin in the prevention or treatment of ARDS should be designed using lower dose aspirin.

We acknowledge that our models have limitations. In the healthy volunteer model of LPS inhalation the nature of injury is mild and self-limiting compared with ARDS. BAL at a single time point does not capture the dynamic changes that take place in the inflamed alveoli in ARDS. The healthy participants lack co-morbidities and poly-pharmacy. This was a pre-treatment study, designed to test the proof-of-concept that aspirin could reduce pulmonary inflammation. Due to the short-term nature of the model, a post-injury treatment would not have allowed us to test the effect of aspirin, particularly low dose aspirin, as it takes more than 6 hours for 75mg aspirin to achieve full platelet inhibition, and further downstream effects. Pre-treatment is potentially relevant for those who can be identified as being at high risk of developing ARDS, and pre-existing aspirin therapy may have protective pulmonary effects for those undergoing surgery that pre-disposes to ARDS, for example coronary artery bypass grafting or abdominal aortic aneurysm repair. Our model does not address whether aspirin withdrawal in the peri-operative period increases the risk of pulmonary inflammation, and most studies looking at the effect of aspirin withdrawal peri-operatively have focused on cardiovascular and bleeding outcomes rather than pulmonary complications



[41, 42]. Although the EVLP model allows a greater induction of injury by using a higher LPS dose, it has a short life, preventing use of these lungs to study fibrotic or resolution phases of ARDS. The EVLP model lacks hepato-renal metabolism and a reticulo-endothelial system, which may modulate the drug and inflammatory response respectively. Additionally, the lungs have undergone a period of cold ischaemia, and had been rejected for transplantation so there is potential variability in the baseline degree of injury. We try to minimize this by using only lungs which have intact fluid clearance at baseline and no gross evidence of haemorrhage, contusion or consolidation. Furthermore, the underlying mechanisms leading to ICU admission and brain death, and the treatments the donor may have received in ICU may lead to altered response to either LPS or aspirin. However, again we aimed to minimize any confounding from this by randomization and blinding. In the EVLP model the blood volume is diluted (1/10), which has the potential to affect rheology, intercellular interaction, and cell recruitment to the lung. Unfortunately, it is not possible to obtain 1litre of of blood from a single donor for research purposes to avoid this. In both models, the injury LPS, is a bacterial antigen, rather than a live bacterium, and isolated TLR4 stimulation does not re-capitulate the myriad of stimuli that drive ARDS in patients.

Nevertheless both models have the advantage of being carried out in human tissues, proving prospectively that aspirin, at clinically relevant doses can attenuate neutrophilic human alveolar inflammation, and histological alveolar injury, but does not affect epithelial barrier function. These data support the further testing of aspirin in clinical trials as an anti-inflammatory strategy for treatment of neutrophilic driven pulmonary inflammatory diseases such as ARDS.

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## **Author contributions**

UH contributed to clinical study conduct, the *ex vivo* experiments, laboratory analyses, data analysis and manuscript preparation

AK contributed to laboratory analyses, data analysis and manuscript preparation.

MF contributed to *ex vivo* experiments, laboratory analyses, data analysis and manuscript preparation.

MS contributed to study design, obtaining funding, clinical study, data analysis and manuscript preparation.

AK contributed to laboratory analyses, data analysis and manuscript preparation.

CS contributed to laboratory analyses, data analysis and manuscript preparation.

EL contributed to laboratory analyses, data analysis and manuscript preparation.

ML contributed to laboratory analyses, data analysis and manuscript preparation.

RV contributed to study design, data analysis

J S contributed to laboratory analyses, data analysis and manuscript preparation.

AJ S contributed to laboratory analyses, data analysis and manuscript preparation.

JMcN contributed to study design, lab analysis, data analysis and manuscript preparation.

DFMcA contributed to study design, obtaining funding, clinical study, *ex vivo* experiments, data analysis and manuscript preparation.

CO contributed to study design, obtaining funding, clinical study, *ex vivo* experiments, lab analyses, data analysis and manuscript preparation.

## Figure legends:

**Figure 1.** CONSORT diagram of healthy volunteer trial.

**Figure 2.** Aspirin does not significantly reduce BAL IL-8 in the healthy volunteer model of LPS induced lung injury, but does reduce neutrophil recruitment to the alveolar space.. Healthy human subjects were randomized to placebo (n=13) or aspirin (n=20) for 7 days prior to LPS inhalation (50mcg) and underwent bronchoalveolar lavage 6 hours later. (a) IL-8 was measured in BAL fluid, but was not significantly reduced,  $p=0.11$  (b) Neutrophils in BAL fluid were counted on cytospin, and were reduced by over a third in the aspirin treated group (estimated difference in median= $0.8 \times 10^5$ /ml, 95% CIs 0.06, 2.1; \* $p=0.03$  for aspirin versus placebo).

**Figure 3.** Aspirin reduces neutrophil proteases in the BAL fluid of healthy volunteers who have inhaled LPS. BAL as in figure 2(a) was analyzed for neutrophil granular enzymes. (a) Aspirin significantly reduced BAL MMP-8 [difference in median 2.2ng/ml, 95% CIs 0.15, 4.45; \* $p=0.04$  for aspirin (n=20) vs placebo (n=13)]. (b) Aspirin significantly reduced BAL MMP-9, [difference in medians 14.2ng/ml, 95% CIs 1.0, 29.8; \* $p=0.04$  for aspirin (n=20) vs placebo (n=13)]. (c) Aspirin did not significantly reduce MPO, (n=20) vs control (n=13), [difference in median 12.4ng/ml, 95% CIs -6.6, 52.6;  $p=0.19$ ]. (d) Aspirin (n=20) did not statistically significantly reduce BAL neutrophil elastase compared with placebo (n=13), [median difference 4.5ng/ml, 95% CIs -3.0, 26.4;  $p=0.30$ ].

**Figure 4.** Aspirin does not reduce alveolar leak nor markers of epithelial injury/activation in BAL in healthy volunteers who have inhaled LPS. BAL as in figure 2 was analysed for (a) albumin as a marker of alveolar barrier integrity and (b) IgG:total protein ratio. Neither was affected by aspirin pre-treatment. For albumin difference in medians was 9.7mg/l, 95% CIs -23.5, 41.2. For IgG:total protein difference in medians was 0.02, 95% CIs -0.01,0.04. (c) Aspirin did not affect BAL SP-D, a marker of type II alveolar epithelial cell activation difference in medians 8.4ng/ml, 95%CIs -0.5-19.5. (d) Aspirin did not reduce BAL MMP-7, and epithelial cell derived protease in the alveolar space; difference in medians 0.2ng/ml, 95% CIs -0.7,1.0). n=13 for placebo, n=20 for aspirin for all measures.

**Figure 5.** The effect of aspirin on peripheral neutrophil count. (a) Peripheral neutrophil count in healthy volunteers 6 hours after LPs inhalation is not significantly reduced by aspirin, difference in medians  $1.47 \times 10^6$ /ml, 95% CIs -0.1,3.4,  $p=0.07$ . (b) Neutrophil extracellular trap (NET) formation in blood 6 hours after LPS inhalation was not statistically significantly reduced in the aspirin treated cohort (n=20) versus placebo (n=13), difference in medians 0.9, 95% CIs -0.1,2,1;  $p=0.09$ . Data are expressed as multiples of mean OD values obtained for NETs in normal blood.

**Figure 6.** The effect of aspirin on BAL and plasma thromboxane B2 (TXB2) after LPS inhalation (a) Plasma thromboxane B2 was reduced by aspirin treatment (n=12 placebo, n=20 aspirin).Difference in medians 3077pg/ml, 95% CIs 755,7850;  $*p=0.003$ . (b) BAL

thromboxane B2 was significantly reduced in the aspirin- treated cohort (n=20) compared with placebo (n=13). NB One outlier from the placebo group is not shown within the range of the graph (value>7000pg/ml), but is included in statistical analysis presented. Difference in medians 41pg/ml, 95% CIs 14.7, 64.3; \*p=0.003. [BAL TXB2 is also significantly reduced in response to aspirin with this outlier excluded.]

**Figure 7.** Aspirin significantly reduces neutrophilic inflammation in the human EVLP model. Human lungs were ventilated and perfused *ex vivo*. Blood was pre-incubated with aspirin (n=5) or placebo (n=6) for 1 hour before adding to the lung perfusate and simultaneously injuring the lung with LPS (6mg). Bronchoalveolar lavage was carried out 4 hours after injury.(a) Aspirin significantly reduces BAL neutrophilia. Neutrophils in BAL fluid were counted in cytopins. \*p=0.03 for aspirin (n=5) vs placebo (n=6), MWU test.(b) Ventilated perfused isolated human lungs that were injured *ex vivo* with LPS had reduced injury as measured by lung injury score when treated with aspirin (n=5) vs placebo (n=6) . y axis, AU – “arbitrary units”, \*p=0.05 MWU test (c) Illustrative histological sections of lung are shown (i), (iii) are placebo-treated lungs at low and high power respectively; (ii), (iv) are aspirin-treated lungs at low and high power. Scale bar represents 100 micrometres. Alveolar septal wall thickening, neutrophil recruitment and proteinaceous debris staining are less marked in the aspirin than the placebo treated group. Quantification of lung injury score was carried out in a blinded manner to counter any potential bias in image selection.

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**Aspirin reduces lipopolysaccharide-induced pulmonary inflammation in human models of ARDS.**

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## **Additional details of the ARENA study**

(*in vivo* model of pulmonary inflammation in healthy volunteers)

### **Bronchoscopy procedure**

Bronchoscopy was carried out using standard guidelines with a standardized method of administration of midazolam and fentanyl in all volunteers (47) . Topical anaesthesia was achieved with the application of 10% lignocaine spray to the back of the throat and further lignocaine 1 – 2 % was given via the bronchoscope as a “spray as you go technique” to the vocal cords, trachea, main and sub segmental bronchi. Oxygen saturation of > 95% were maintained by oxygen supplementation during the procedure. 180ml of normal saline were instilled into the right middle lobe in aliquots of 60 ml and aspirated with gentle suction. The BAL was immediately stored on ice for transfer to the laboratory for further processing.

After the bronchoscopy the volunteer’s vital signs were monitored every fifteen mins for a period of 1hr and allowed to go home if they met the departmental guidelines:

Assessment of clinical status by the nurses including level of alertness was satisfactory.

There was no evidence of any unexpected effects.

A friend or relative was required to collect them, help them home, and stay with them for 24 hr.

Participants were given a 24 hr contact number and encouraged to contact the research team in the event of any problems or concerns or the appearance of any new symptoms.

Participants received instructions that they are not to drive, operate any machinery, sign legal documents or drink alcohol.

Participants were informed that there is a possibility that they may develop fever, flu like symptoms, mild sore throat, hoarseness of voice, chest discomfort or nausea and vomiting over the ensuing 24 hr and they may cough up a small amount of blood. They were asked to contact the research team if any of these symptoms appear.

The volunteers were followed up 24hr following LPS inhalation, for repeat blood sampling and spirometry. Volunteers were also contacted 7 days after LPS inhalation to enquire of any issues arising from the study.

### **Adverse events**

All adverse events, as defined in the EU Clinical Trials Directive 2001/20 (i.e. “any untoward medical occurrence in a participant to whom a medicinal product has been administered including occurrences which are not necessarily caused by or related to that product) were documented. There was no significant difference in the vital signs, forced expiratory volume in one second (FEV<sub>1</sub>) or forced vital capacity (FVC) following LPS inhalation during the course of the study. The mild adverse events experienced by the healthy volunteers are outlined in table S1 and were followed up until they had

resolved. One subject had a transient reduction in measured Hb from 13.0 to 12.3g/dl after taking study medication; there were no symptoms of blood loss. On repeat sampling within 7 days Hb had returned to baseline measurement.

### **Rationale for choice of dose of aspirin**

75mg of aspirin is the standard dose of aspirin used as an anti-platelet agent in the UK, and therefore used in this study.

Anti-inflammatory activity has been shown with doses of >1000mg/day. Aspirin is commercially available in 300mg tablets and therefore a dose of 4x300mg (1200mg) was used as a dose that had known anti-inflammatory activity, without any potential to induce salicylism in the healthy volunteer cohort.

### **BAL fluid processing**

BAL fluid aliquots were mixed and 20 $\mu$ l extracted. Cell count was carried out using an automated cell counter, Countess (Invitrogen; Life technologies, Paisley, UK). 2 cell counts within 10% of each other were obtained, and a mean total cell count was taken. Cell-free supernatant was obtained by centrifugation of BAL at 500g for 5 mins, and the supernatant aliquoted and frozen at -80°C for further analyses.

Cytospins were prepared and stained with Speedy-Diff (Clin-Tech Ltd, UK). A single cytologist independent of the research team performed the differential cell count to ensure maintenance of blinding. Cytospins for differential white cell count were prepared and stained with Speedy-Diff (Clin-Tech Ltd, UK) as previously described.

### **BAL analytes**

BAL total protein was measured by Bradford assay. Myeloperoxidase (MPO), IL-1Ra, TIMP-1, TIMP-2 and C-reactive protein were measured by ELISA (R&D Systems; Abingdon, UK) according to the manufacturer's instructions. Cytokines (IL-1 $\beta$ , TNF- $\alpha$ , IL-6, IL-8, IL-10 and MCP-1) and Matrix Metalloproteinases (MMP-1,2,3,7,8,9) were measured using a cytometric bead array (R&D Systems; Abingdon, UK) according to the manufacturer's instructions.

### **Measurement of NETs**

Neutrophil elastase-deoxyribonucleic acid (NE-DNA) enzyme-linked immunosorbent assay (ELISA): To quantify neutrophil extra-cellular traps (NETs) in human plasma, a capture ELISA was developed based on NE associated with DNA. For the capture antibody (ab), 0.8  $\mu$ g/ml anti-NE mAb (Sc-9521 Santa Cruz) was coated onto 96-well plates (dilution 1:250 in 50  $\mu$ l) overnight at 4°C. After washing 3 times (300  $\mu$ l each), 50  $\mu$ l of samples was added to the wells and incubated for 2 hours. After 3 washes, a peroxidase-labeled anti-DNA mAb (Cell Death ELISAPLUS, Roche; dilution 1:100) was added for 2 hours incubation. After 3 washes (300  $\mu$ l each), 100  $\mu$ l peroxidase substrate (ABTS) was added. Absorbance was measured after 1hour incubation at room temperature in the dark. Data were expressed as the ratio of OD for measured NETS in the sample to OD for measured NETS in normal plasma.

### **Leukocyte-platelet aggregate measurements**

Blood was collected (without a tourniquet) into sodium citrate tubes (BD) and transported to the laboratory at room temperature within 30 min. Blood was mixed with

Tyrode's buffer (Sigma) plus platelet inhibitors (apyrase, PGI<sub>2</sub> and heparin) and labelled antibody and then incubated for 15mins in the dark, before addition of phosflow lyse/fix buffer (BD Biosciences), centrifugation and re-suspension of the pellet in Tyrode's buffer. Flow cytometry was performed using a FACS Canto II instrument (BD) with FACS Diva software 6.1.3. Forward and side scatter measurements were made with gain settings in linear mode, distinguishing monocyte and neutrophil populations. A minimum of 5000 monocytes and neutrophils were acquired for each determination. A three-color analysis (PE for CD14, FITC for CD15 and APC for CD41 and Isotype control) was used for simultaneous detection of leucocyte-platelet aggregates. CD14+ (monocytes) and CD15+ (neutrophils) were further gated into a CD41-positive platelet-bound populations, CD41-negative gate was set based on the APC-conjugated Isotype control. Events staining positively for both platelet and monocyte antigens (CD41+CD14+) or platelet and neutrophil antigens (CD41+CD15+) were considered to represent blood leucocyte-platelet aggregates. Data were analysed using FlowJo 7.6.5 software, by a blinded operator.

3 samples were excluded from the placebo and 4 samples were excluded from the aspirin group prior to unblinding and data analysis, due to omission of fluorochrome-labelled antibodies during sample processing and lack of distinct cell population on gating.

### **Ex vivo lung perfusion model**

Human lungs were supplied by International Institute for the Advancement of Medicine (IIAM) originating in the United States of America and transported via commercial flights. Lungs rejected for transplant were retrieved from brain dead donors. These were flushed with University of Wisconsin (UW) solution at the site of retrieval and were transported on ice. The methodology for flushing of the UW solution was consistent in the lungs used in this study.

The exclusion criteria for these lungs were as follows: donor age <14 years; warm ischaemic time >1hour; cold ischaemic time >48 hours; serology positive for HIV, HCV, HBV; aspiration pneumonia, emphysema, methicillin resistant *Staphylococcus aureus* in sputum, pulmonary artery cuff < 1cm; pneumothorax; infiltrates on chest radiograph or computerized tomography scan.

These lungs were inspected for any contusion, laceration, palpable masses or injury to the pulmonary artery cuff. A cannula was placed in the pulmonary artery and secured in place by a purse string suture. This was then connected to the efferent tube of the roller pump. A pulmonary artery catheter inserted through the side of the cannula was used to measure the pulmonary artery pressures. Similarly, an endotracheal tube was placed in the main bronchus and secured using a purse string suture, which was then connected to the ventilator circuit. The lung was suspended in an acrylic container from a weight transducer (SS12LA variable force transducer Harvard apparatus, UK). The acrylic container was placed in a water bath to rewarm the perfusate over the period of 1 hour. Both the pulmonary artery pressure and lung weight data were acquired using Biopac



MP100WSW digital data acquisition system (Harvard apparatus, UK). A temperature probe was placed in the pulmonary vein to monitor perfusate temperature. "Perfusate" consisting of 1 L of Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen, UK) without Phenol red and supplemented with L-glutamine (Invitrogen, UK) and 5% bovine albumin (Sigma, UK), was placed in the base of the reservoir. Initial perfusion was begun with 0.05 L/min and gradually increased to 0.4 L/min maintaining a pulmonary artery pressure of 10 - 15 mmHg. Once a temperature of 30°C was reached, the lungs were gently inflated with an ambu bag. Continuous positive airway pressure of 10cm H<sub>2</sub>O was applied with 95%O<sub>2</sub>/5% CO<sub>2</sub> using a ventilator (Dräger). Once a temperature of 36°C was reached a catheter (PE 240-Harvard apparatus) was inserted into the sub-segment lobe via the endotracheal tube and gently advance until resistance was encountered. 125 ml of warmed normal saline with 5% albumin was instilled, and baseline samples were taken at 5 and 35 mins and placed on ice for for analysis. Protein concentration in BAL fluid was determined by Bradford assay. Baseline alveolar fluid clearance (AFC), a measure of intact epithelial function, is calculated according to the formula.  $AFC (\%/hr) = 2[1 - (C_i/C_f) \times 100]$ , where C<sub>i</sub> = protein concentration at time=0, and C<sub>f</sub>=protein concentration after 1/2 hour. Lungs which do not have intact AFC (defined as >10%/hr ) are not used in the LPS injury model, as this is taken to represent baseline alveolar injury.

The catheter was then re-sited in a different lobe, 6 mg of LPS from *Escherichia coli* O111:B4 (Sigma, UK) with 10 ml of normal saline were injected via the catheter to induce injury. 100 ml of blood from healthy volunteers were incubated with either aspirin-24 mg (intravenous formulation - Bayer) or saline (Baxter) for a period of 1 hr

and then introduced into the perfusate. The addition of the study drug was carried out in a blinded fashion. LPS injury in the EVLP model produces robust repeatable injury at 4 hours. We and others have previously shown that we can maintain intact alveolar epithelial clearance in the EVLP model for 4 hours in the absence of injury. Beyond this the lung becomes increasingly oedematous even in the absence of injury. At the end of 4 hr, BAL was carried out using 125ml normal saline as above. 2 cm<sup>3</sup> tissue sections were taken and placed in 10% formalin before embedding in paraffin blocks. These were subsequently sliced and stained with Eosin and Haematoxylin for assessment of histological injury.

### **Lung injury severity score**

Histological injury during the acute phase of ARDS is characterized by the presence of alveolar septal oedema, intra-alveolar haemorrhage, intra-alveolar proteinaceous debris and alveolar neutrophilia. The first three parameters were assessed in haematoxylin and eosin (H and E) stained sections by light microscopy (Leica microsystems, Germany) at a total magnification of 880x. 5 separate images of adjacent fields of view were acquired. Alveolar septal oedema was quantified by measuring the thickness of the alveolar septa using Image-J software from each of the 5 acquired images. The alveolar septum with the maximum thickness was selected from each image. Alveolar septa adjacent to blood vessel or airway were excluded due to thickening resulting from collagen deposition, which occurs, in normal circumstances. Each image was compared to the average thickness of the alveolar septa obtained from a control lobe (0.52 from n

= 3) and scores were assigned according to the increase in thickness based on the table below. Proteinaceous debris was scored as per supplementary table 2 (ST2).

The presence of intra-alveolar haemorrhage was assigned a score of 0, 1, 2 or 3 based on a semi quantitative assessment of none, mild, moderate or severe. BAL neutrophil counts were taken as representation of the neutrophil in the alveolar space. The cumulative score from these 4 parameters was used to compare the severity of alveolar injury between the two groups. The scorer was blinded to the group allocation. This scoring scheme was modified from the scoring system described by Matute-Bello *et al*

### **Isolation of peripheral blood neutrophils**

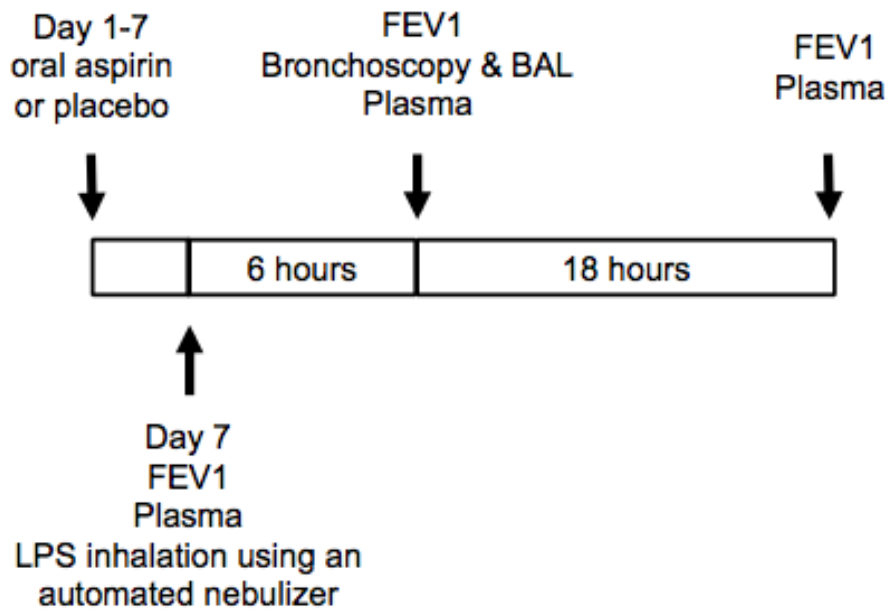
Neutrophils were isolated from whole blood of healthy volunteers by dextran sedimentation and fractionation through isotonic discontinuous Percoll gradients as previously described. Neutrophils were only if purity was >95% (assessed by morphological analysis). Ethical approval was granted by the County Durham and Tees Valley Research Ethics Committee (12/NE/0121).

### **Phagocytosis assay**

Freshly isolated neutrophils were allowed to adhere to tissue culture plastic in Iscove's modified Dulbecco's medium (IMDM) containing 1% autologous serum for 30 minutes at 37°C in 5% CO<sub>2</sub> prior to treatment with Salbutamol (10µM), Acetylsalicylic acid (0.1 or 0.5mM) or a combination for 30 minutes at 37°C in 5% CO<sub>2</sub>. Cells were then exposed to

autologous serum-opsonized zymosan for 30 minutes, washed with PBS, air dried, fixed with methanol, and stained with Giemsa. The percentage of neutrophils containing two or more zymosan particles was quantified using a Nikon TMS phase contrast inverted microscope with bright field. Duplicate counts were performed on four randomly selected fields with a minimum of 100 cells per field

**Figure S1.** Study schedule for the healthy volunteer model (ARENA)



**Figure S2.** Study schedule for the EVLP model

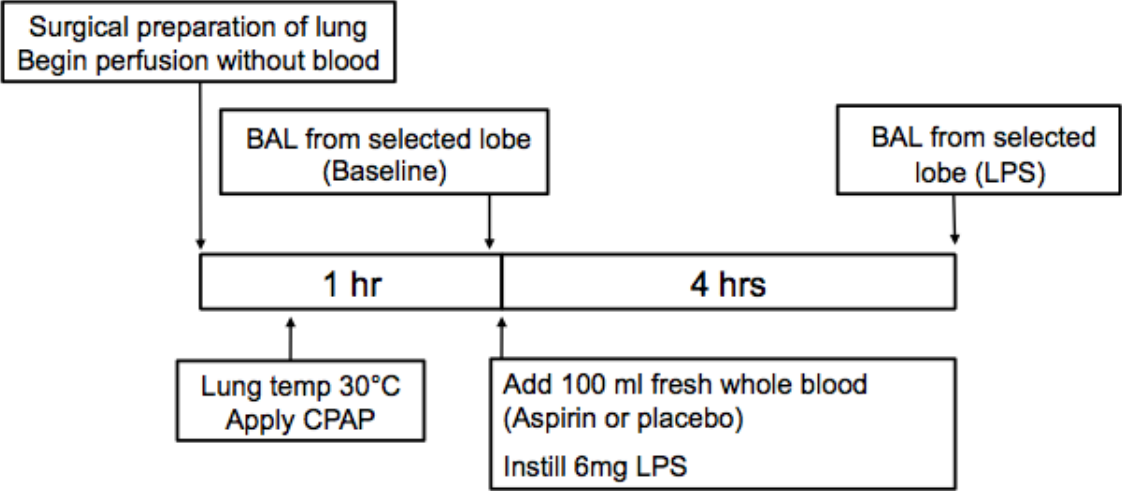
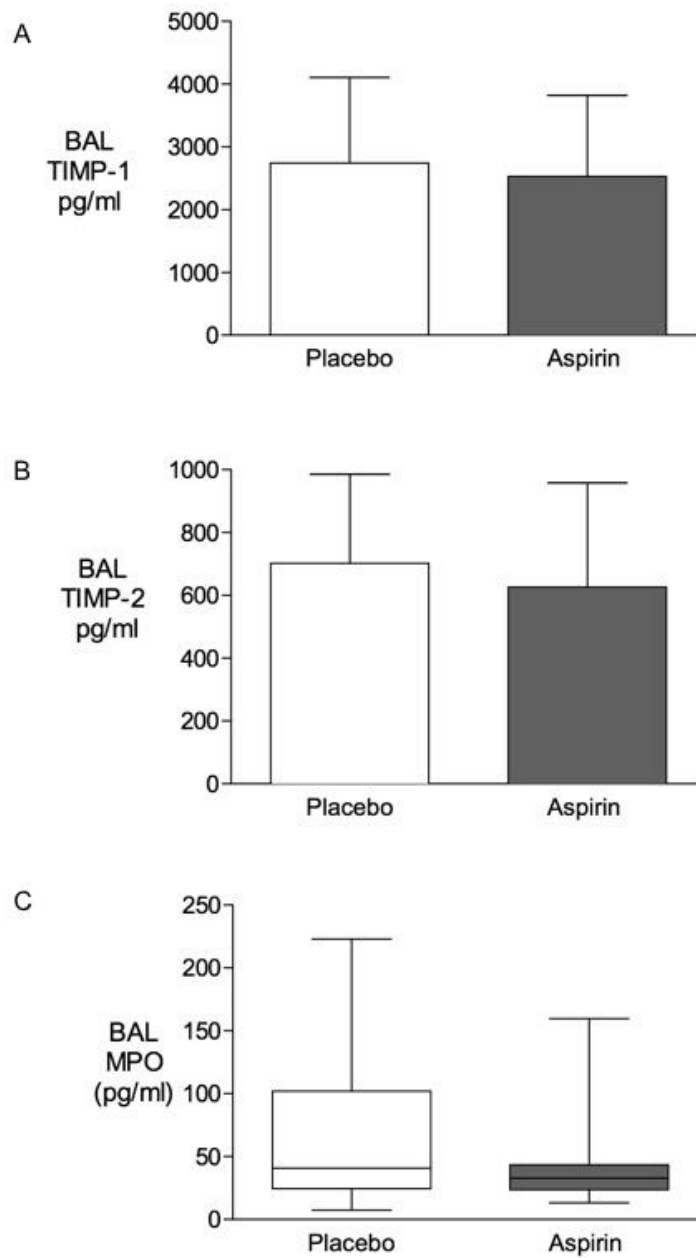


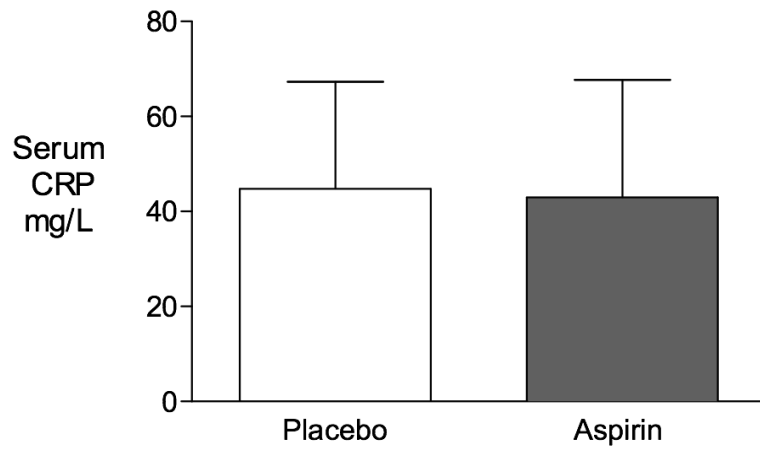
Figure S3. The effect of aspirin on BAL Tissue Inhibitors of Metalloproteinases, TIMP-1 and TIMP-2. Aspirin had no effect on (a) TIMP-1 and (b) TIMP-2.

**Figure S3.** The effect of aspirin on Tissue Inhibitors of Metalloproteinases and Myeloperoxidase in the ARENA study. Aspirin did not reduce secretion of (A) TIMP-1,  $p=0.6$  or (B) TIMP-2,  $p=0.5$  (B) after LPS inhalation. (C) Data for MPO showing extreme outlier in the aspirin cohort included: data are median, IQR and min to maximum,  $p=0.19$ .



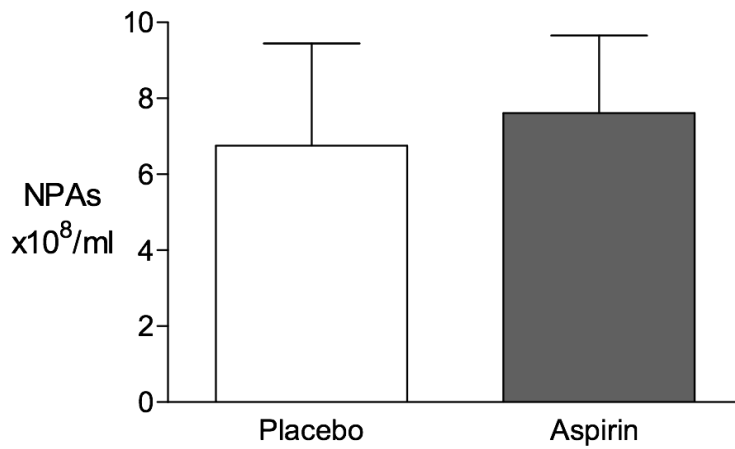


**Figure S4.** The effect of aspirin on systemic CRP in the ARENA study. Aspirin does not reduce serum CRP 6 hours after LPS inhalation. Difference in median 2mg/l, 95% CIs -12,17.

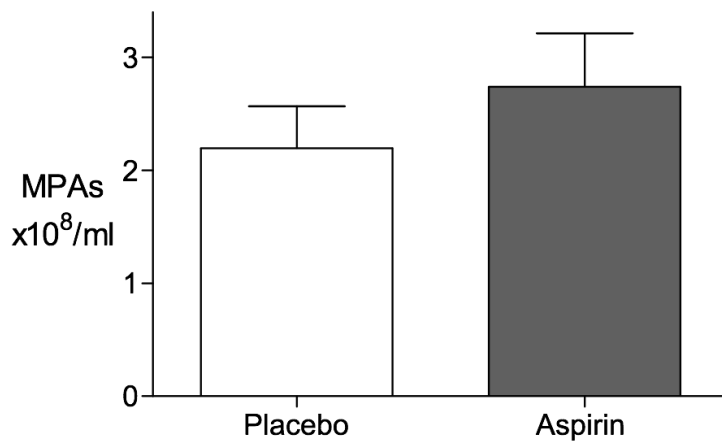


**Figure S5.** The effect of aspirin on leukocyte-platelet aggregate formation in the ARENA study. Aspirin did not affect the number of leukocyte platelet aggregates in peripheral blood 6 hours after LPS inhalation, as measured by (A) neutrophil platelet aggregates (NPAs), and (B) monocyte platelet aggregates (MPAs). n=10 for placebo, n=16 for aspirin.

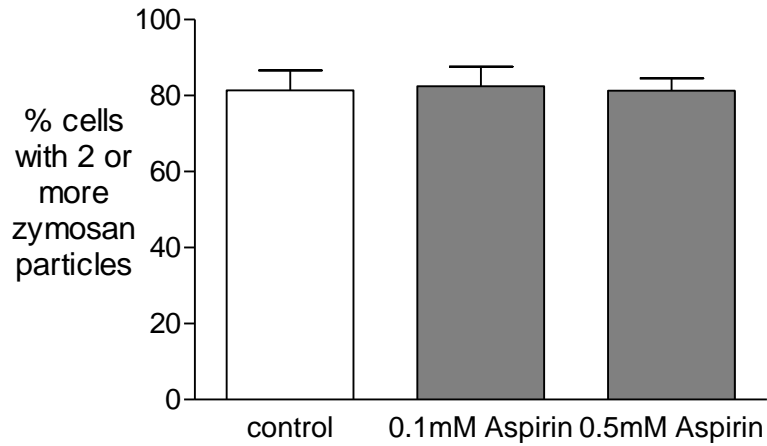
(A)



(B)



**Figure S6.** The effect of aspirin on neutrophil phagocytosis. Neutrophils were pre-incubated with aspirin 0.1 or 0.5mM for 30 minutes, before addition of serum-opsonized zymosan. Results are expressed as % of neutrophils with  $\geq 2$  ingested zymosan particles. Aspirin had no effect on uptake of zymosan particles by neutrophils. Values are mean+SD, n=6.



	Placebo (n=13)	Aspirin (n=20)	p value
FEV <sub>1</sub> decr >10%	3	1	ns
Nausea/vomiting	2	3	ns
Throat discomfort	0	2	ns
Chest discomfort	1	3	ns
Fever	1	3	ns
Development of URTI	1	0	ns
Transient reduction in measured Hb (<1g/dl)	0	1	ns

Supplementary table 1 (ST1) Adverse events in the ARENA study. Data were compared using Fisher's exact test. ("FEV<sub>1</sub> decr >10%" relates to a greater than 10% reduction in forced expiratory volume in 1 second post bronchoscopy compared with baseline).

Parameter	Score per field			
	0	1	2	3
Proteinaceous debris	None	1	> 1	-
Alveolar septal thickening	< 2x	2x – 4x	4x – 8x	>8x
Intra-alveolar haemorrhage	None	Mild	Moderate	Severe
Neutrophils in alveolar space (BAL counts; cells/ml)	< 1 x 10 <sup>6</sup>	1 - 10 x 10 <sup>6</sup>	10 - 20 x 10 <sup>6</sup>	> 20 x 10 <sup>6</sup>

Supplementary table 2 (ST2) Lung injury scoring system

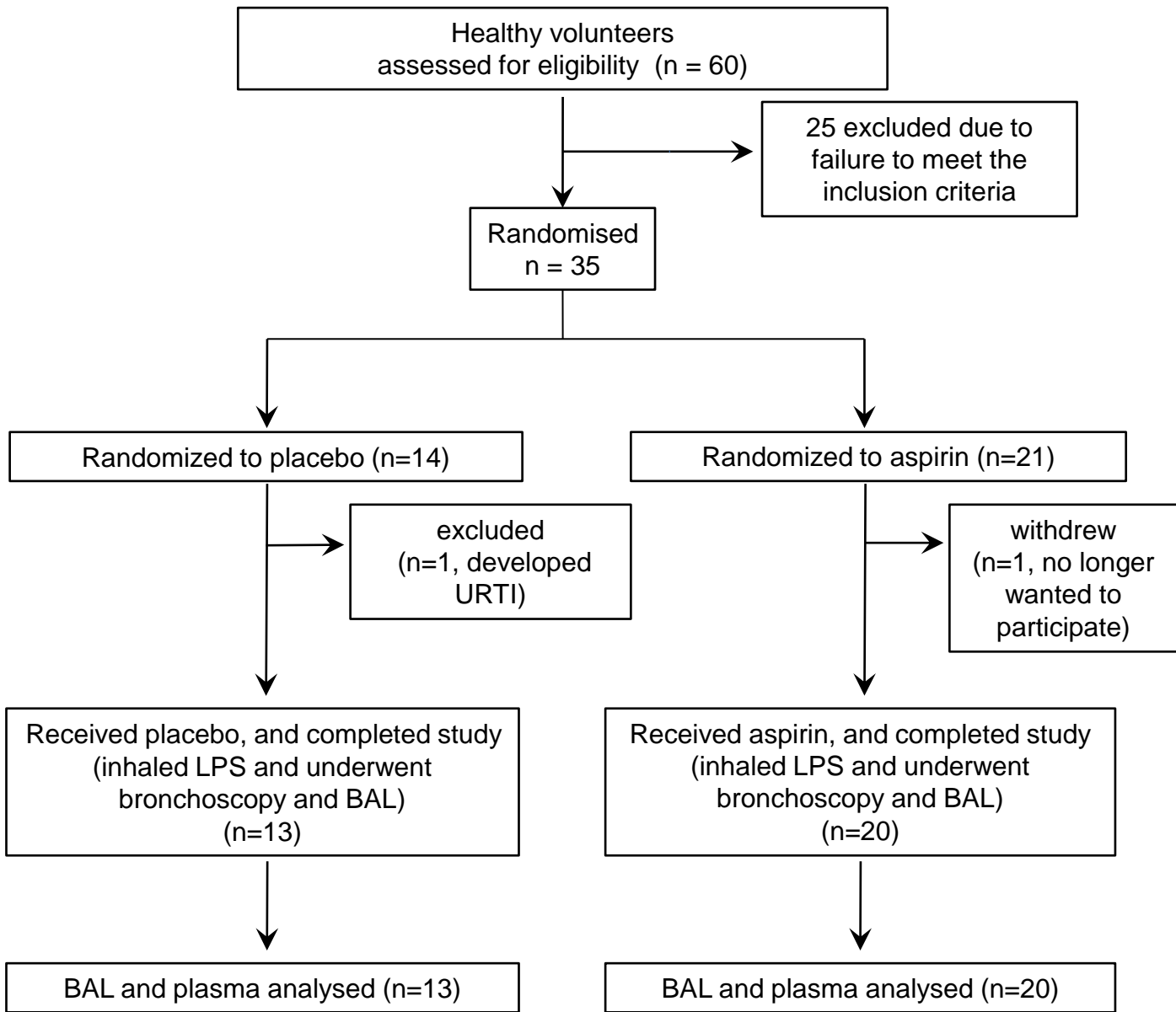
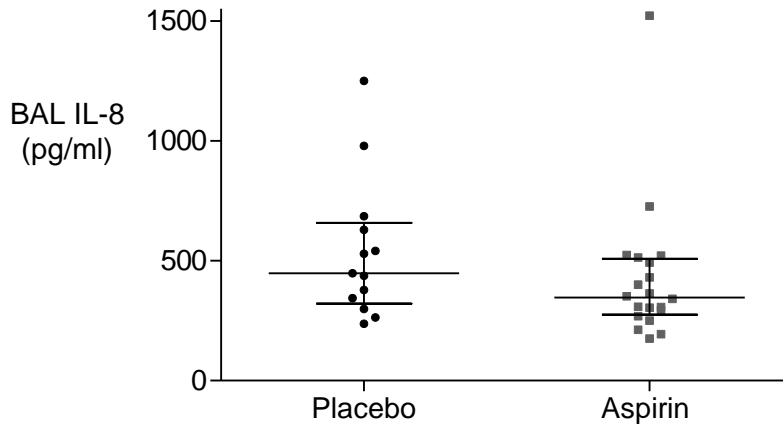


Figure 1

A



B

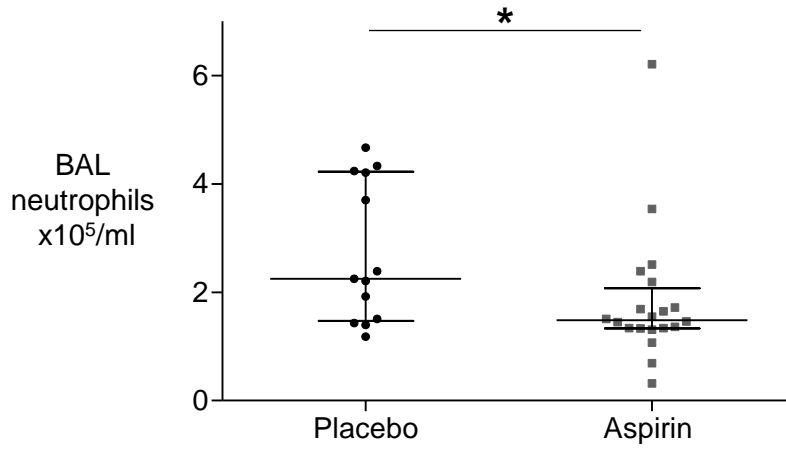


Figure 2

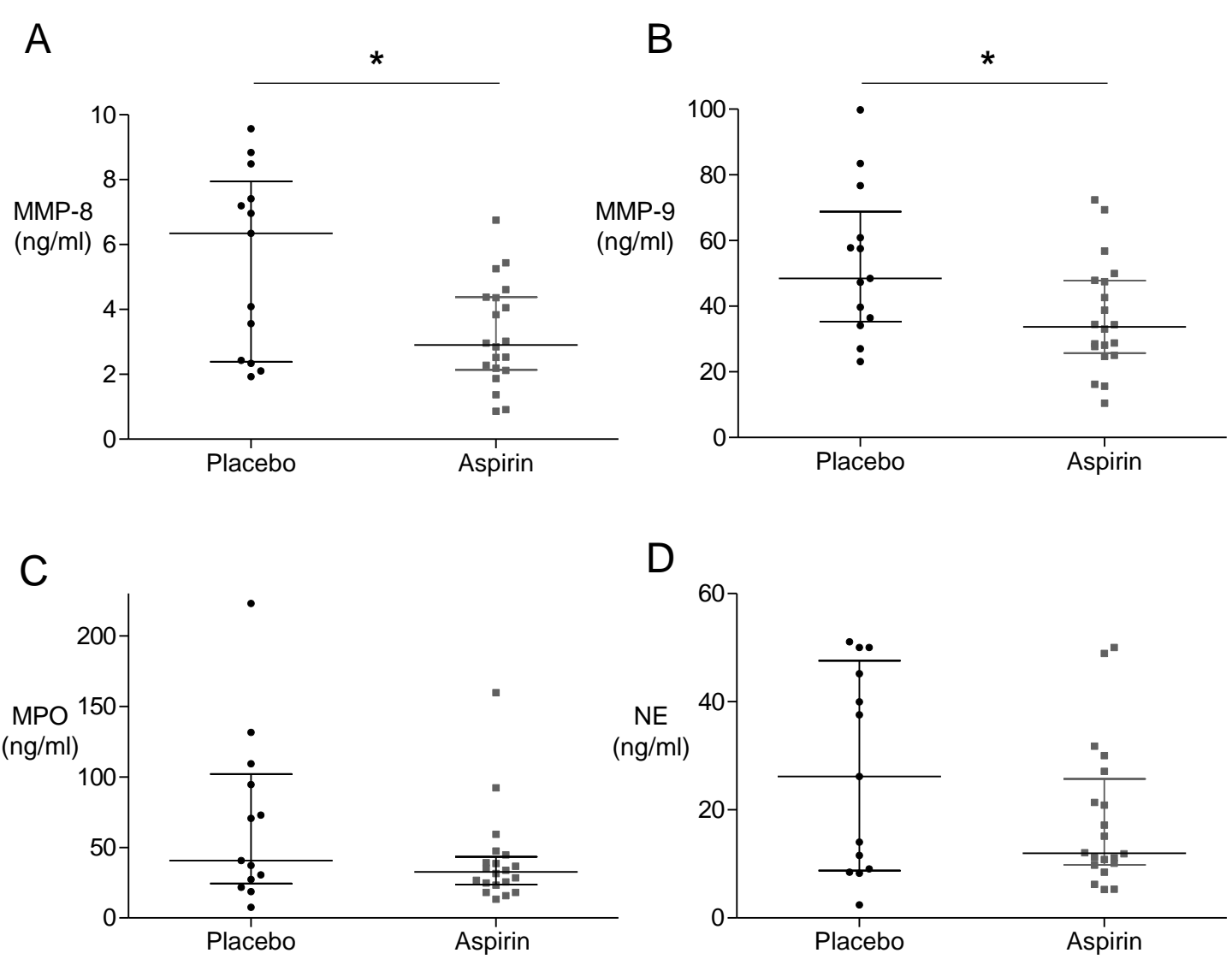


Figure 3



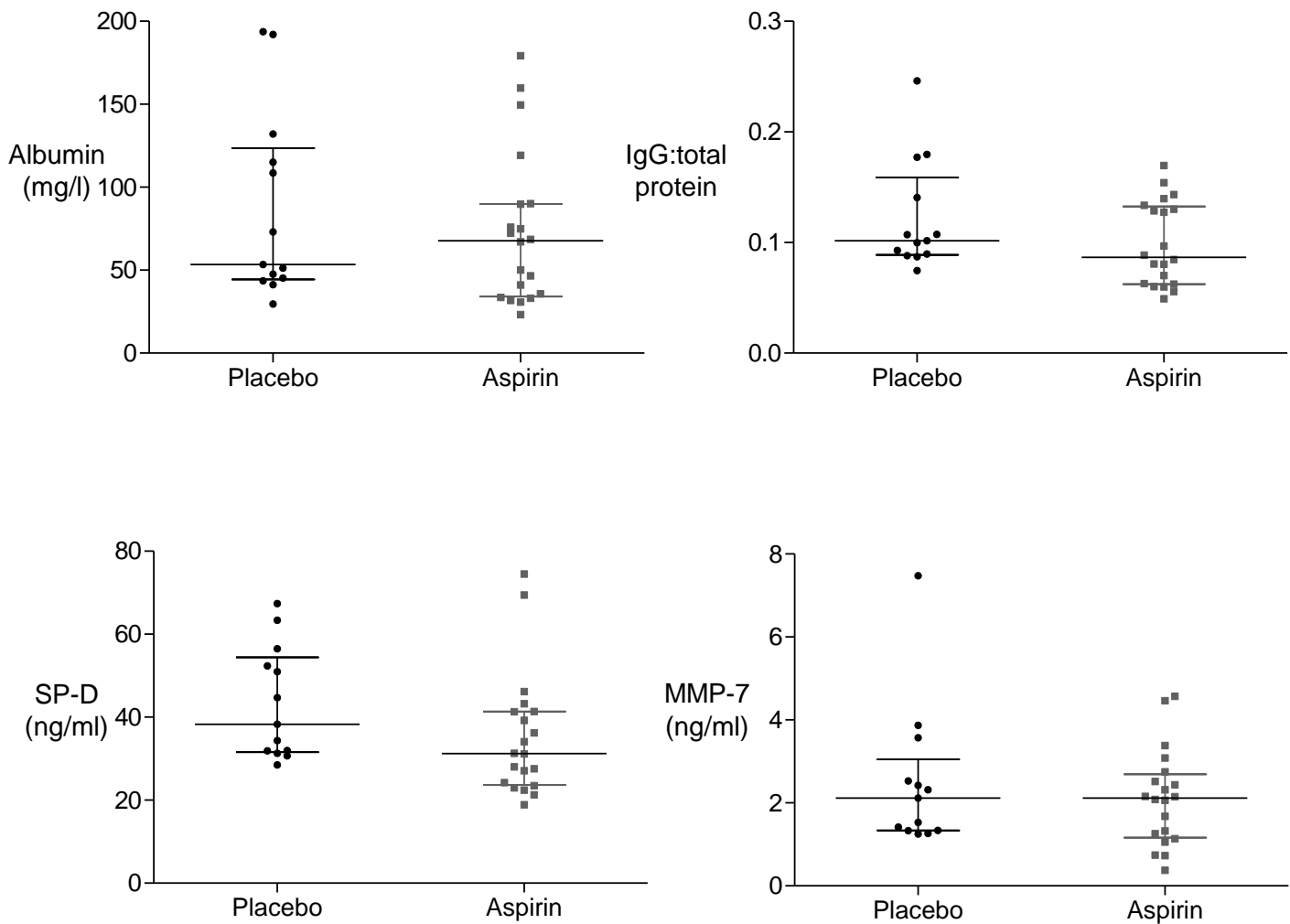
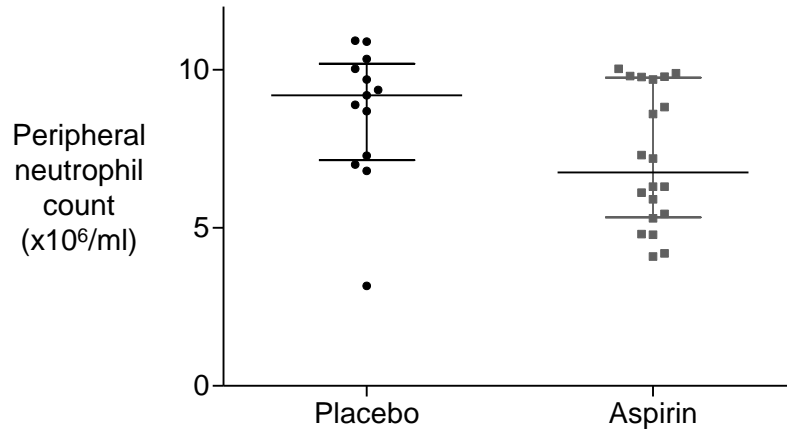


Figure 4

A



B

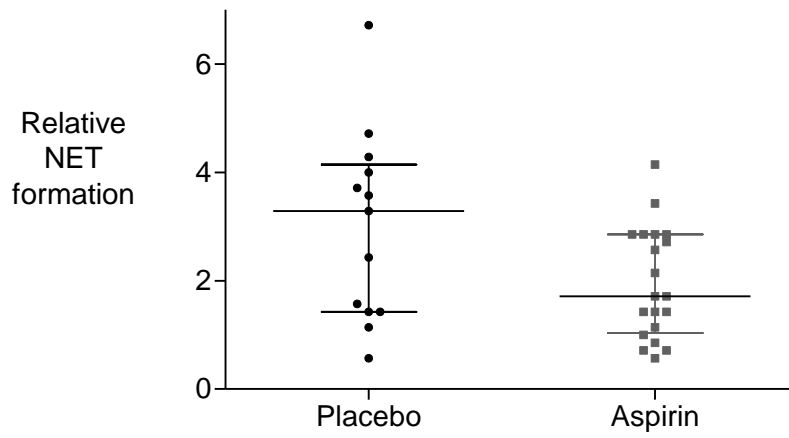
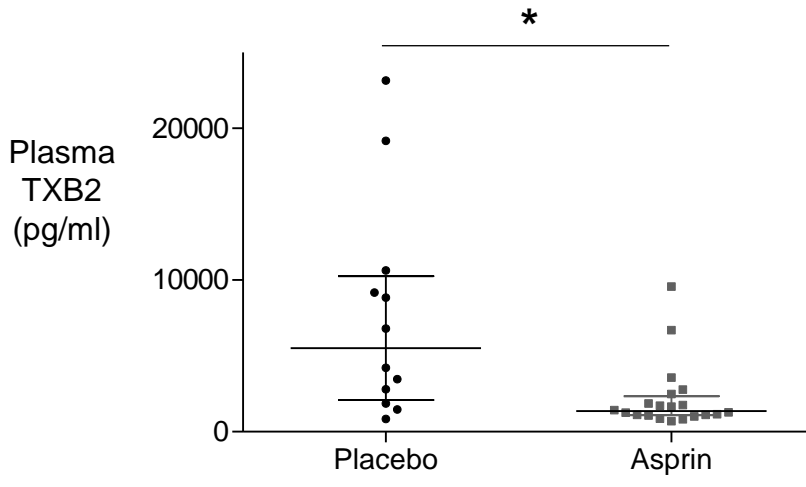


Figure 5

A



B

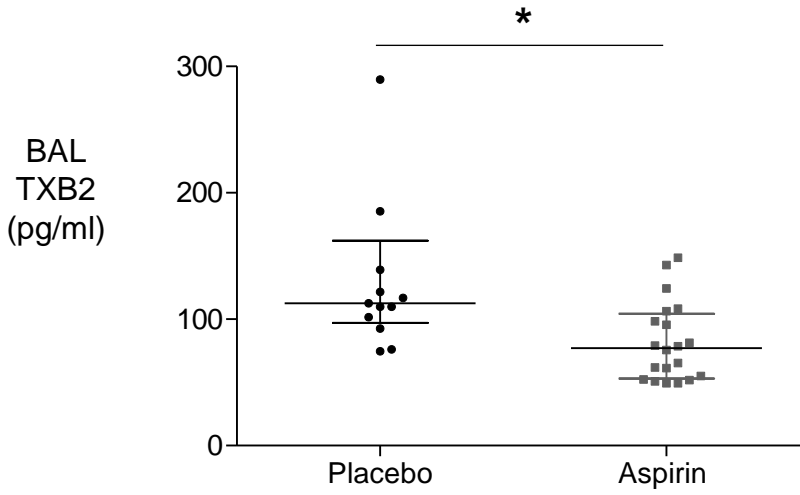


Figure 6

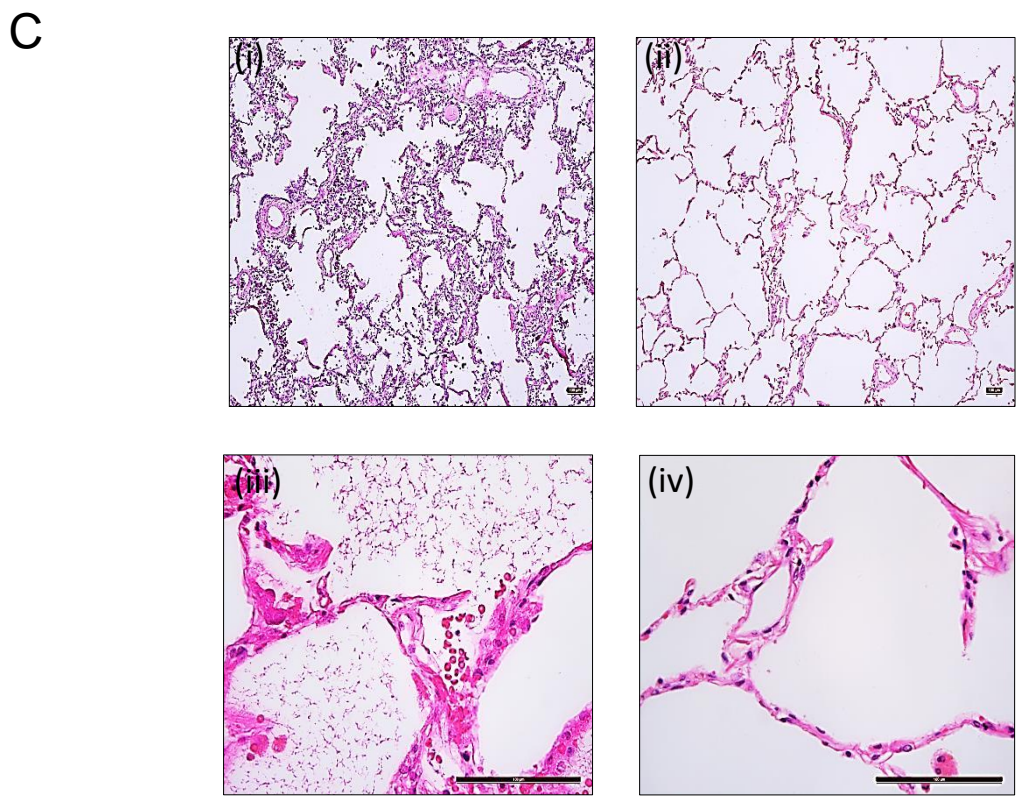
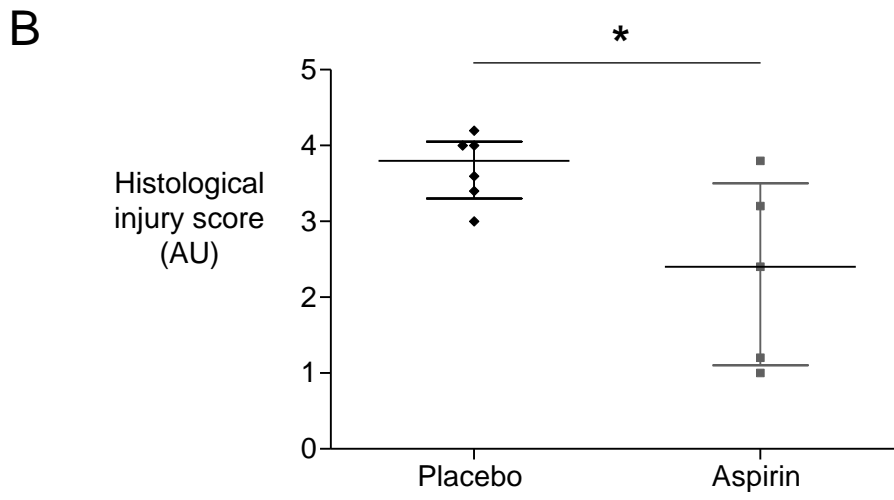
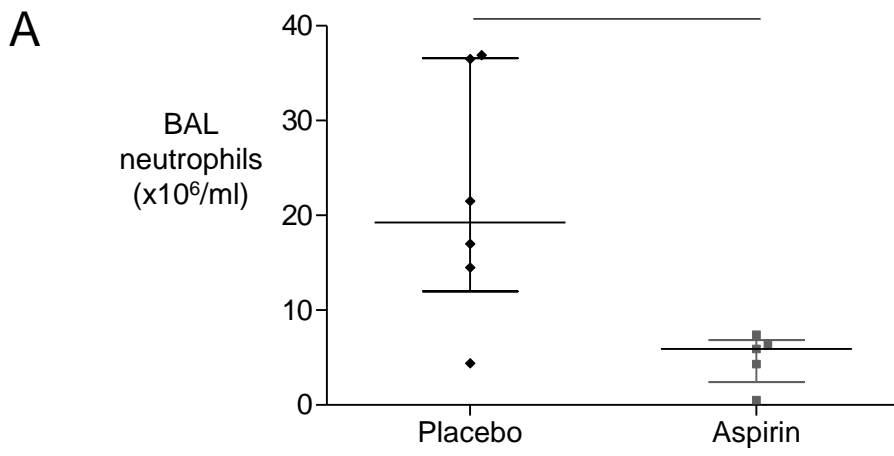


Figure 7