Hypoxia increases the potential for neutrophil-mediated endothelial damage in COPD

Katharine M Lodge[1,2], Arlette Vassallo[1], Bin Liu[1], Merete Long[3], Zhen Tong[1], Paul R Newby[4], Danya Agha-Jaffar[2], Koralia Paschalaki[2], Clara Green[4], Kylie Belchamber[4], Victoria C Ridger[3], Robert A Stockley[5], Elizabeth Sapey[4], Charlotte Summers[1], Andrew S Cowburn[2], Edwin R Chilvers[1,2], Wei Li[1], Alison M Condliffe[3,1]

ONLINE DATA SUPPLEMENT

EXPANDED MATERIALS AND METHODS:

Materials

Enzchek® elastase activity kit was purchased from Life Technologies (Cat no. E12056). Resistin and NGAL ELISA kits were purchased from RayBiotech (Cat no. ELH-Resistin-1; ELH-Lipocalin2-1) for neutrophil supernatant assessment. Cyclophilin A ELISA kits were purchased from Elabscience (Cat no. E-EL-H6096) for neutrophil supernatant assessment and from Oxford Biosystems (Cat no. RD191329200R) for plasma assessment. NGAL ELISA kit was purchased from R&D Systems (Cat no. DLCN20) for plasma assessment. NE ELISA kit was purchased from Abcam (Cat no. ab119553) for plasma assessment. All other plasma proteins were measured by Meso Scale Discovery chemiluminescence immunoassay (VPLEX Angiogenesis panel 1, Cat no. K15190D-1; VPLEX Vascular injury panel 2, Cat no. K15198D-1; resistin, Cat no. K151FND-1; MPO, Cat no. K151EEC-1). Endothelial cell basal medium EBM-2 was purchased from Lonza and supplemented with Bullet kit (Lonza) to obtain endothelial growth medium EGM-2. Endothelial cell growth medium MV2 was purchased from PromoCell. FITC Annexin V apoptosis detection kit I was purchased from BD Biosciences (Cat no. 556547). RealTime-Glo™ Annexin V apoptosis assay was purchased from Promega (Cat no. JA1000). Human polyclonal anti-cyclophilin A and human polyclonal anti-annexin A1 were purchased from GeneTex (Cat no. GTX113520; GTX101071). Horse radish peroxidase-conjugated goat anti-rabbit IgG was purchased from Dako (Cat no. P0448). PAF was purchased from Tocris Bioscience (Cat no. 2940). Cytochalasin B and fMLP were purchased from Sigma-Aldrich (Cat no. C6762; F3506). PI3Kγ inhibitor (AS605240) was purchased from

Sigma-Aldrich (Cat no. A0233). PI3Kδ inhibitor (CAL-101) was purchased from ApexBio (Cat no. A3005).

COPD patient recruitment

Cohort 1:

Exacerbating COPD patients were recruited within 24h of presentation to Cambridge University Hospitals NHS Foundation Trust using electronic patient medical records (EPIC software 2014). Inclusion criteria were: age < 80 years, a physician diagnosis of COPD, a minimum of 10 pack-years smoking history, a current diagnosis of an exacerbation of COPD (infective or non-infective) by the admitting medical team, and a clear or unchanged chest radiograph. Exclusion criteria were: admission to hospital for other reasons apart from treatment of the COPD exacerbation, immunodeficiency or immunosuppressive medication (apart from acute oral corticosteroids for treatment of the exacerbation), long term oral corticosteroid use, active malignancy, anaemia at the time of blood sampling, current smoker > 5 cigarettes per day, or lack of capacity to consent. None of the patients studied required mechanical ventilation. Informed consent was provided for the collection of clinical data and samples. Peripheral venous blood was collected within 24h of hospital admission for neutrophil isolation and for plasma/serum preparation; blood was collected into sterile S-Monovette® tubes containing EDTA to obtain plasma or tubes containing polystyrene beads coated with silicate clotting activator to obtain serum. Age- (within 10 years) and sex-matched healthy volunteers were recruited for identical venous blood sampling. Healthy controls had to be free from respiratory symptoms, had no history of COPD, asthma, or any other chronic lung disease, had no active malignancy, were free of any acute or infective symptoms, and were not on any immunosuppressive medications.

Demographic and clinical data were collected (Table E1). Due to the unpredictable recruitment of COPD patients, older age and short timeframe for venepuncture, it was not possible to obtain plasma or perform neutrophil isolation from matched healthy volunteers concurrently in parallel.

Cohort 2:

Non-exacerbating COPD patients were recruited under the remit of the ESADIL study, Imperial College London. Inclusion criteria were: age 21-85 years, a respiratory physician diagnosis of COPD, a minimum of 10 pack-years smoking history, post salbutamol FEV1/FVC<0.7, increase in FEV1<12% and <200 ml following β2-agonist inhalation, no history of allergic or other respiratory disease. For this study, “frequent exacerbators” were recruited, i.e. a history of >2 moderate exacerbations (requiring treatment with systemic corticosteroids or antibiotics) within the year prior to Covid-19 lockdown restrictions. Exclusion criteria were: pregnancy or breast-feeding, consideration that the volunteer was unfit for the study following medical review and physical examination by the responsible physician, or lack of capacity to consent. Informed consent was provided for the collection of clinical data and samples. Peripheral venous blood was collected for neutrophil isolation into Vacuette® tubes containing 3.2% sodium citrate. Demographic and clinical data were collected (Table E2)

Cohort 3:

Exacerbating patients were recruited within 24h of presentation to University Hospitals Birmingham NHS Trust (UHB). Inclusion criteria were: a respiratory physician diagnosed exacerbation (severe severity by definition as admitted), a clear or unchanged chest radiograph; with a prior secondary care confirmed diagnosis of

COPD based on clinical symptoms, relevant exposures (greater than 10 pack year history of cigarette smoking) and confirmatory spirometry while in the stable state (diagnosed and staged by GOLD criteria (Global Initiative for Chronic Obstructive Lung Disease 2019)). Quoted spirometry for patients was measured within one year of the exacerbation episode while in the stable state. Exclusion criteria were: admission to hospital for other reasons apart from treatment of the COPD exacerbation, immunodeficiency or immunosuppressive medication (apart from oral corticosteroids for treatment of the exacerbation), active malignancy, or lack of capacity to consent. Informed consent was provided for the collection of clinical data and samples. Peripheral venous blood was collected within 24h of hospital admission for plasma preparation using the BD Vacutainer system® (Becton, Dickinson and Company). Healthy controls were matched by age, sex and where possible smoking status and pack year history (they were all ex smokers or current smokers). They were recruited from the 1000 Elders cohort at Birmingham and members of staff from UHB and University of Birmingham. Healthy controls had to be free from respiratory symptoms, had no history of COPD, asthma, ILD or any other chronic lung disease, had no active malignancy, were free of any acute or infective symptoms, were not on immunosuppressive medications and had normal spirometry. Demographic and clinical data were collected (Table E2).

Isolation of murine neutrophils

Murine femoral bone marrow neutrophils were isolated by negative immunomagnetic selection using a MACS neutrophil isolation kit (Miltenyi Biotech) as per the manufacturers' instructions. Pelleted bone marrow cells were re-suspended in MACS buffer ($2.5*10^9$ cells/ml) mixed with neutrophil biotin-antibody cocktail (1 µl/$1*10^6$ cells). Washed cells were re-suspended in MACS buffer ($1.25*10^9$ cells/ml) mixed with anti-

biotin microbeads (2 µl per $1*10^6$ cells). Washed cells were subjected to magnetic separation using a QuadroMACS™ Separator and 3 ml LS columns, whereby the neutrophil-rich flow-through was collected.

Neutrophil shape change

Neutrophils were re-suspended in phosphate-buffered saline containing calcium and magnesium ($5*10^6$/ml) and treated with fMLP (100 nM, 30 min) or vehicle control. Shape change was analysed by flow cytometry. Fixed cells were analysed by flow cytometry (BD FACSCanto II), gating on single granulocytes, and measuring change in forward scatter (FlowJo v10).

Neutrophil supernatant NE and MPO activity

Supernatant NE activity was quantified by commercial Enzchek® elastase activity assay (Life Technologies), which measures the ability of NE to cleave non-fluorescent (quenched) BODIPY labelled-DQ-elastin substrate into fluorescent fragments, with fluorescence measured at 485/535 nm. Supernatant MPO activity was assessed by the H_2O_2-dependent oxidation of o-Dianisidine dihydrochloride as described (1).

Endothelial cell growth

Human pulmonary arterial endothelial cells (hPAECs, Lonza) were cultured in EGM-2 medium (Lonza) with 0.1% gentamicin/amphotericin B and 10% foetal calf serum (FCS). Human pulmonary microvascular endothelial cells (hPMECs, Promocell) were cultured in MV-2 medium (Promocell) with 5% FCS.

Endothelial Cell Survival

Supernatants from normoxic or hypoxic healthy donor neutrophils were incubated with confluent hPAECs in 12 well plates. After 6h, apoptosis of unfixed hPAECs was

assessed by flow cytometric measurement of FITC-Annexin V (AnV) and propidium iodide (PI) staining (BD LSRFortessa), with early apoptotic cells being AnV^+/PI^- and late apoptotic/necrotic cells annexin being V^+/PI^+.

Supernatants from normoxic or hypoxic healthy donor neutrophils were incubated with confluent hPMECs in 96 well plates. After 6h, apoptosis of unfixed hPMECs was measured by commercial RealTime-Glo^{TM} Annexin V apoptosis assay (Promega).

Supernatants from normoxic or hypoxic healthy donor neutrophils were incubated in the presence or absence of α1AT (46 µg/ml, 10 min) and then diluted 1:1 in serum-free EGM-2. Diluted supernatants were incubated with confluent hPAECs in 96 well plates. After 24h, fixed hPAECs were stained with rhodamine phalloidin and DAPI; cell detachment was assessed by immunofluorescence (Leica Sp5 confocal microscope) with quantification using ImageJ. After 48h, viability of unfixed hPAECs was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay: following incubation with MTT (500 µg/ml, 2 h), hPAECs were dissolved in 100 µl isopropanol, and absorbance at 550 nm was recorded.

Flow rig assessment of neutrophil-endothelial interaction

Endothelial–neutrophil interactions were assessed in a flow adhesion assay. Confluent hPMEC monolayers in microslides (m-Slide VI0.4, Ibidi) were treated with neutrophil supernatants containing corresponding healthy/COPD serum (2%) for 4h and then mounted onto the stage of a phase contrast microscope. The slides were connected to cell and wash reservoirs and to a withdrawal syringe pump by silicon tubes. hPMECs were washed (0.15% BSA in PBS+/+, 2 min) prior to perfusion with neutrophils ($1*10^6$ cells/ml, 4 min) at a wall shear stress of 0.1 Pa, then washed once more (0.15% BSA in PBS+/+, 2 min). Endothelial–neutrophil interactions were

captured 6 min after the initial cell bolus using time-lapse imaging. Quantification of the total number of rolling, adhered, and transmigrated neutrophils observed in the time-lapse captured images following cell injection was performed offline using ImagePro software. All flow-based adhesion assays were performed within a Perspex environmental chamber at 37˚C.

Tandem Mass Tag-Mass Spectrometry (TMT-MS)

Neutrophil supernatants, generated from the normoxic/hypoxic (4h) activated (PAF, 1 μM, 5 min then fMLP, 100 nM, 10 min) neutrophils of five healthy donors, were concentrated using Vivaspin 2 MWCO 3000 columns (GE Healthcare). Sample preparation and TMT-MS were performed by the University of Cambridge Centre for Proteomics. An equal amount of protein per sample was labelled with a unique 10-plex isobaric tag (4 μl, 1 h, ThermoFisher Scientific). Samples underwent trypsin digestion (2.5 μg, overnight). Mixed unfractionated samples were subjected to liquid chromatography MS (LC-MS/MS) using a Dionex Ultimate 3000 RSLC nanoUPLC (ThermoFisher Scientific) system and a Lumos Orbitrap mass spectrometer (ThermoFisher Scientific). Raw data were searched against UniProt Human reference proteome (Proteome ID: UP000005640) using Mascot 2.6 (Matrix Science) and Proteome Discoverer™ version 2.1 (ThermoFisher Scientific). Protein identification allowed an MS tolerance of ± 20 parts per million and an MS/MS tolerance of ± 0.6 Da, along with permission of up to two missed tryptic cleavages. Fixed modifications were carbamidomethylation of cysteine and TMT 10-plex at lysine and N-termini; variable modifications were oxidized methionine, deamidated aspartic acid and asparagine, and TMT10-plex at threonine, serine and methionine. Quantification was achieved by calculating the sum of centroided reporter ions within a ± 2 millimass unit window around the expected *m*/*z* for each of the TMT reporter ions. For quantification,

the integration window tolerance was set to 20 parts per million. The confidence thresholds were set at a strict FDR of 0.01 and a relaxed FDR of 0.05. Finally, the following filters were applied during the Proteome Discoverer search: peptide score: 20, peptide rank: 1, peptide confidence: high.

Preparation of neutrophil-derived microvesicles (NMVs)

Neutrophils were re-suspended in normoxic or hypoxic IMDM ($1*10^7$/ml) and treated with fMLP (10 µM, 1 h). NMVs were generated by sequential high speed centrifugation. Following final centrifugation, NMV pellets and NMV-depleted supernatants were collected.

Quantification of NMVs

NMV pellets were re-suspended in 190 µl sterile filtered PBS with 10 µl counting beads (SPHERO™ Accucount blank particles 2.0-2.4µm, $5*10^5$/ml). NMV were quantified by flow cytometry: the NMV region of interest was standardised using fluorescent size calibration beads (Megamix, 0.5, 1 and 3 µm diameter), with 1,000 counting beads measured per sample. Plasma NMVs were stained with BV421-conjugated anti-CD66b antibody (1:10, 45 min) prior to washing with sterile-filtered PBS. The NMV pellet was re-suspended in PBS with counting beads ($3.3*10^5$/ml). Total MV and NMV ($CD66b^+$) numbers were quantified as above. For western blotting, NMV pellets or TCA-precipitated (10%) NMV-depleted supernatants were resuspended in lysis buffer, subjected to SDS-PAGE and probed for cyclophilin A (1:1000) and the microvesicle marker Annexin A1 (1:2000).

Quantification of neutrophil extracellular traps (NETs)

Neutrophils were re-suspended ($1*10^6$/ml) in normoxic or hypoxic IMDM with SYTOX™ green (5 µM) in 96 well plates and treated with PAF/fMLP or PMA (20 nM).

At baseline, cells in triplicate wells were permeabilised with 0.5% Triton-X. Fluorescence absorbance (indicating extracellular DNA) was measured at 485/535 nm.

Table E1

Cohort 1	Healthy Donor N=14	COPD patient N=12
Age – mean year (SD)	66.07 (5.28)	70.74 (6.85)
Male sex – no. (%)	7 (50)	6 (50)
Smoking history – mean pack year (SD)	4.04 (11.33)	58.27 (32.95)
Current smoker – no. (%)	0 (0)	3 (25)
Current no. of cigarettes/day – mean (SD)	0 (0)	N=3 3 (2.51)
WCC – mean (SD) Neutrophil count – mean (SD) CRP – mean (SD)	Not tested	10.15 (4.46) 8.01 (4.29) 20.16 (22.0)
O_2 saturation on room air on admission – mean % (SD) O_2 saturation at time of venepuncture – mean % (SD)	Not recorded	86.3 (4.55) 91.75 (2.67)

Table E2

Cohort 2	COPD patient N=6
Age – mean year (SD)	75 (6.5)
Male sex – no. (%)	3 (50)
FEV1 % predicted (SD) (* spirometry within past year in the stable state)	53.17 (17.09)
FEV1/FVC* (SD)	49.61 (16.8)
Smoking history – mean pack year (SD)	45 (27.3)
Current smoker – no. (%)	1 (16.7)
Current no. of cigarettes/day	N = 1 10
O_2 saturation on room air at sampling – mean % (SD)	95.67 (2.42)
Timing of most recent exacerbation prior to sampling – mean months (SD)	3.4 (4.3)

Table E3

Cohort 3	Healthy Donor N=32	COPD patient N=32
Age – mean year (SD)	64.63 (8.80)	66.7 (7.03)
Male sex – no. (%)	20 (62.50)	21 (65.60)
FEV1 % predicted (SD) (* spirometry within one year of admission in the stable state)	109.99 (12.19)	70.80 (20.39)*
FEV1/FVC* (SD)	79.67 (5.37)	59.49 (12.64)*
Smoking history – mean pack year (SD)	12.13 (6.36)	28.74 (17.28)
Current smoker – no. (%)	10 (31.25)	13 (40.63)
Current no. of cigarettes/day – mean (SD)	N = 10 6.4 (1.96)	N = 13 11.61 (3.52)
O_2 saturation on room air on admission – mean % (SD)	95.53 (2.42)	89.23 (4.65)

Table E4

Cohort 1 comorbidities	Healthy Donor (total N=14) number of donors (%)	COPD patient (total (N=7) number of donors (%)
Type 2 diabetes	1 (7)	4 (57)
Hypertension	5 (36)	3 (43)
Ischaemic heart disease	1 (7)	2 (29)
Previous venous thromboembolism	0 (0)	2 (29)
Arrythmia	0 (0)	2 (29)
Obstructive sleep apnoea	0 (0)	2 (29)
Osteoporosis	0 (0)	2 (29)
Idiopathic angioedema	0 (0)	1 (14)
Obesity	0 (0)	1 (14)
Hypothyroidism	1 (7)	1 (14)
Alcohol excess	0 (0)	1 (14)
Chronic kidney disease	0 (0)	1 (14)
Motor neurone disease	0 (0)	1 (14)
Hypercholesterolaemia	4 (29)	0 (0)
Gastro-oesophageal reflux disease	3 (21)	0 (0)
Benign prostatic hyperplasia	2 (14)	0 (0)
Hyperthyroidism	1 (7)	0 (0)
Gout	1 (7)	0 (0)
Migraine	1 (7)	0 (0)

SUPPLEMENTARY FIGURE LEGENDS

Figure E1. Hypoxia increases elastase release from PAF-primed COPD neutrophils in a PI3Kγ-dependent manner.

Neutrophils from COPD patients were incubated under normoxia or hypoxia in the presence or absence of PI3Kγ-selective inhibitor (AS605240, 3 μM) or PI3Kδ-selective inhibitor (CAL-101, 100 nM) as indicated. After 4h, cells were treated with PAF (1 μM, 5 min) and fMLP (100 nM, 10 min) or vehicle control as indicated. Supernatant NE activity was measured and is expressed as fold change relative to hypoxic activated neutrophils (n=6). All samples were obtained from cohort 2. Results represent mean ± SEM, two way ANOVA. ** = $p<0.01$, *** = $p<0.001$

Figure E2: Supernatants from hypoxic neutrophils cause increased human pulmonary microvascular endothelial cell (hPMEC) apoptosis

Neutrophils from healthy donors were incubated under normoxia or hypoxia for 4h, then treated with PAF (1 μM, 5 min) and fMLP (100 nM, 10 min) or vehicle control. Supernatants from normoxic *vs* hypoxic, PAF/fMLP *vs* vehicle control-treated neutrophils were incubated with confluent hPMEC for 6h. Apoptosis was measured using RealTime-Glo™ Annexin V assay (n=11). Results represent mean ± SEM, two way ANOVA. ** = $p<0.01$, **** = $p<0.0001$

Figure E3: Hypoxia does not alter NMV release or cyclophilin A content

Neutrophils ($1*10^7$/ml) from healthy donors were incubated under normoxia or hypoxia for 3h, then treated with fMLP (10 μM, 1h). Cell supernatants were sequentially

centrifuged to pellet NMVs. A: NMVs were re-suspended in sterile PBS with AccuCount blank particles (50 beads/µl) and analysed by flow cytometry (n=7). B: Plasma NMVs from healthy controls or exacerbating COPD patients (cohort 1) were stained with BV421-conjugated anti-CD66b and analysed by flow cytometry, with NMVs ($CD66b^+$) expressed as % of total MVs (n=6). C&D: NMV lysates or NMV-depleted TCA-precipitated supernatants from the same neutrophils were subjected to SDS-PAGE and probed for annexin A1 and cyclophilin A by western blotting. Protein content was quantified by Image J analysis of band densitometry. Representative image (C) from n=4 experiments (D). Results represent mean ± SEM, unpaired t test.

Figure E4: Hypoxia does not enhance NET generation

Neutrophils from healthy donors were incubated under normoxia or hypoxia. A: Cells were treated at baseline with PAF (1 µM), or vehicle control (n=5) B: Cells were treated at baseline with PMA (20 nM) or vehicle control or, following 4h incubation, with PAF (1 µM) then fMLP (100 nM, 10 min) (n=3). NET production was quantified by fluorescence absorbance with Sytox Green (5 µM). NETosis is expressed as total extracellular DNA or as % of total DNA from triton-X (0.5%) lysed neutrophils. Results represent mean ± SEM, two way ANOVA. *** = $p<0.001$

Figure E5: Plasma from COPD patients has unchanged content of angiogenesis biomarkers

Plasma from healthy donors or exacerbating COPD patients was assessed for content of serum amyloid (SAA; A), C-reactive protein (CRP; B), angiopoietin 2 (C), vascular endothelial growth factor-A (VEGF-A; D), VEGF-C (E), VEGF-D (F), Tie2 (G), placental growth factor (PIGF; H), VEGF receptor-1 (I), fibroblast growth factor (FGF; J) by multiplex chemiluminescence immunoassay (n=36 healthy, n=36 COPD; 4

samples from cohort 1 and 32 samples from cohort 2). Results represent mean ± SEM, Mann-Whitney test. * = $p<0.05$, ** = $p<0.01$, **** = $p<0.0001$

SUPPLEMENTARY TABLE LEGENDS

Table E1. Cohort 1 Clinical and Demographic data. Data were collected from the Cambridge University Hospitals NHS Foundation Trust electronic patient medical record system (Epic software 2014) at the time of recruitment.

Table E2. Cohort 3 Clinical and Demographic data. Data were collected from the Imperial College London NIHR Biomedical Research Unit study records at the time of recruitment.

Table E3. Cohort 2 Clinical and Demographic data. Data were collected from University Hospitals Birmingham NHS Trust patient medical records at the time of recruitment.

Table E4. Cohort 1 Comorbidity data. Data were collected from the Cambridge University Hospitals NHS Foundation Trust electronic patient medical record system (Epic software 2014) at the time of recruitment. Data are presented for all donors who underwent neutrophil isolation.