Neutral Sphingomyelinase-2, Acid Sphingomyelinase and Ceramide Levels in COPD patients compared to controls.

Simon Lea, Hannah Metcalfe, Jonathan Plumb, Christian Beerli, Chris Poll, Dave Singh & Katharine Abbott-Banner.

Supplementary materials

Methods

Alveolar macrophage culture

Briefly, areas of lung distant from the tumour were perfused with 0.1M NaCl. The resulting cell suspension was centrifuged (400g, for 10 minutes, at room temperature) and the cell pellet re-suspended in RPMI-1640. The cells were layered over a Ficoll-Paque (GE Healthcare, Buckinghamshire, UK) gradient. The mononuclear cells at the Ficoll interface were extracted, washed and re-suspended in complete media: RPMI-1640 (Sigma-Aldrich, Dorset, UK) containing 10% v/v foetal calf serum (FCS; Invitrogen, Paisely, UK); 2mM L-Glutamine (Invitrogen); 100U/ml penicillin and 100µg/ml streptomycin (Sigma-Aldrich). Cells were counted using trypan blue exclusion and seeded at $4x10^5$ per well of 24-well plate or $1x10^6$ per well of 6-well plate. Macrophages were allowed to adhere overnight (37^oC, 5% CO_2).

RT-PCR & QPCR

24-well plate cultures were washed 3x in PBS before being lysed in TRIzol (Invitrogen) and homogenized using a 21-gauge needle. Chloroform was added to the TRIzol samples (200 µl/ml) and samples centrifuged at 12,000 *g* for 15 min. The upper aqueous RNA phase was removed and purified using RNeasy Mini Kit (Qiagen, Crawley, UK) according to the manufacturers' instructions. RNA was quantified using an Eppendorf BioPhotomoeter V1.35 (Eppendorf, Cambridge, UK). cDNA was synthesised from 50 ng RNA using the Verso™ 2-step qRT PCR kit (Thermofisher, Surrey, UK). Thermal cycling was carried out on a Stratagene MX3005P (Agilent

Technologies, West Lothian, UK). 1 µl of cDNA was added to ABsolute Blue qPCR mix (Thermofisher) according to manufacturers' instructions in the presence of 6-carboxyfluorescein (FAM)-labelled primers for nSMase-2 (Human SMPD3 Catalogue no: Hs00920354_m1, Applied Biosystems), aSMase (Human SMPD1 Catalogue no: Hs01086851_m1, Applied Biosystems) or glyceraldehyde-3phosphate dehydrogenase (GAPDH) (Catalogue no: 4352934E, Applied Biosystems). Amplification conditions were 95˚C for 15min, then 40 cycles of 95˚C for 15 secand 60˚C for 1 min. Relative nSMase-2/aSMase expression was determined using the ΔΔCt method normalising to the endogenous control.

Immunohistochemistry

Tissue blocks were taken, as far distal to any tumour as possible, then formalin fixed and paraffin embedded, 4 µm sections were cut and lifted onto polysine coated glass slide (Surgipath, Peterborogh, UK). Optimal heat induced epitope retrieval (HIER) was carried out for nSMase 2 and aSMase by microwaving sections in citrate buffer pH6 (10mM Sodium Citrate) for 20 min at 800 W. Sections were incubated overnight at 4°C in either rabbit polyclonal anti-nSMase-2 antibody (ab85017, Abcam) which is a synthetic peptide conjugated to KLH derived from within residues 600 to the C-terminus of human nSMase-2 or mouse monoclonal anti-aSMase antibody (ab74281, Abcam) which is a synthetic peptide conjugated to KLH derived from within residues 1-100 of human acid SMase. nSMase-2 and aSMase primary antibodies were diluted 1/200 and 1/300 in 1.5% normal goat or normal horse serum (Vector Laboratories) respectively. Endogenous peroxidase was quenched by incubating sections in 3% H_2O_2 in methanol for 30mins at room temperature. nSMase-2 and aSMase were detected using biotinylated goat anti-rabbit immunoglobulin (Ig)G and biotinylated horse anti-mouse IgG secondary antibodies (Vector Labs) respectively in conjunction with an avidin-biotin peroxidase complex (Vector Labs) and diaminobenzidine (DAB) substrate (Vector Labs). Sections were counterstained with Meyer's haematoxylin (Sigma, Poole, UK). Omission of primary antibody from staining protocol and substitution of primary antibody with an isotype control antibody (Vector Labs) were used as negative controls.

Quantification of Antibody labelling

Digital micrographs were obtained using a Nikon Eclipse 80i microscope (Nikon UK Ltd, Surrey, UK) equipped with a QImaging digital camera (Media Cybernetics, Marlow UK) and ImagePro Plus 5.1 software (Media Cybernetics). Labelling in each patient was quantified using the manual tag tool in Image-Pro Plus software (MediaCybernetics) and reported as follows:

Small airways = number of intact airways with detectable immunoreactivity (%).

Subepithelium refers here to the area that extends from the distal edge of the basement membrane (up to 100μm) to the alveolar attachments

Quantification of individual cell counts, subepithelial area and length of alveolar wall was carried out using the measurement tools in the ImagePro Plus 5.1 software.

- The number of immunoreactive cells within a given subepithelial area were reported as cells/mm^2.
- The number of immunoreactive cells along a given length of alveolar wall were reported as cells/mm.

Alveolar macrophages = At least 200 alveolar macrophages, defined as mononuclear cells with well represented cytoplasm present in the alveolar spaces and not attached to the alveolar walls, were counted per section and reported as a percentage of alveolar macrophages labelled.

Preparation of samples for ceramide determination

6-well plate cultures were washed 3 x in PBS, detached in PBS by incubating on ice for 15min and gentle scraping with the fine end of a transfer pipette tip (Fisher). Detached cells were collected in 1.5ml protein lo bind tubes (VWR), centrifuged into a pellet (8000rpm, 10mins) and immediately stored at -80^oC prior to transport to

Novartis Pharma AG (Postfach, CH-4002 Basel, Switzerland) where ceramide species (C16, C18 and C20) were analysed by UHPLC-MS-MS.

Ceramide determination

For quantitative determination of C16-, C18- and C20-ceramides in cell samples, the methods described by [17-18] were modified and adapted accordingly.

Reference compounds were obtained from Avanti Polar Lipids (Alabaster, AL)

Calibration, quality control and recovery control samples of all analytes were prepared in 5% fat free BSA in PBS without cells. For sample preparation 100 µL aliquots of each calibration, quality control and recovery control sample was transferred to 2.0 mL Eppendorf LoBind tubes. To each cell pellet, 100 µL 5% fat free BSA in PBS and 10 ceramic balls (1.4 mm diameter) were added. Fifteen microliter C17-ceramide internal standard at a concentration of 666 ng/mL and 750 µL methanol/trichloromethane 2:1 v/v was added to each sample. After 20 min vortexing, samples were incubated for 15 h at 48 °C in a water bath. After cooling 75 µL of a 1 M potassium hydroxide solution in methanol was added, samples were mixed and incubated for 2 h at 37 °C. For extraction 100 µL acetic acid 10 % and 750 µL trichloromethan was added. After rotating for 15 min at room temperature and centrifugation for 3 min at 4°C and 600 x g, 1 mL of the organic layer was transferred to 1.5 mL Chromacol Gold HPLC vials and evaporated to dryness in a SpeedVac concentrator (app. 3 h at 43 °C).

The residues were dissolved in 100 µL 5 mM ammonium formiate with 0.2% formic acid in methanol. After sonication, votexing and centrifugation for 10 min each, the supernatant was transferred to new HPLC vials and stored at 15 °C in the autoinjector during analysis.

The analysis was performed on an Agilent 1290 Infinity UHPLC-System, directly coupled to the AP_ESI source of an Agilent 6490 triple quadrupole mass

spectrometer run in MRM. The separation of all 4 ceramides was performed on a Symmetry C18 2.1 x 50 mm column, filled with 100 Å 3.5 µm particles and held at 45 °C. A linear gradient applying different slopes from 5% to 97.5 % within 7.5 min and an increasing flow rate from 0.2 to 0.5 ml/min was applied. Buffer A was consisting of 95% methanol containing 0.2% formic acid and buffer B acetonitrile/2-propanol 8:2 containing 0.2% formic acid.

For all analytes the $[M\text{-}OH]^+$ precursor ion was selected and for quantification the EIC area of the product ion 264.1 m/z was used. Data processing was done with the MassHunter software applying an internal standard method. For all three ceramides a 2nd order fitted calibration curve from 1 to 5000 ng/ml with origin ignored and 1/x weighing was applied.

Table 2. Calibration and quality control parameters for the Ceramide determination

Ceramide	Recovery	LOQ	Precision	Accuracy	R^2
C16	92.0%	5 ng/ml	15.3% RSD	<8.2%	0.9999
C18	94.8%	1 ng/ml	9.6% RSD	<4.7%	0.9999
C20	98.1%	1 ng/ml	9.4% RSD	<5.8%	0.9998

Recovery = recovered ceramide amount from BSA matrix after sample preparation (n = 3 at c = 100 ng/mL)

LOQ = Limit of quantification (lowest measured calibration sample with accuracy <20%)

Precision = SD of n = 3 quality control samples at c = 100 ng/ml

Accuracy = maximal deviation of single calibration points from calibration curve

R^2 = regression coefficient of the calibration curve

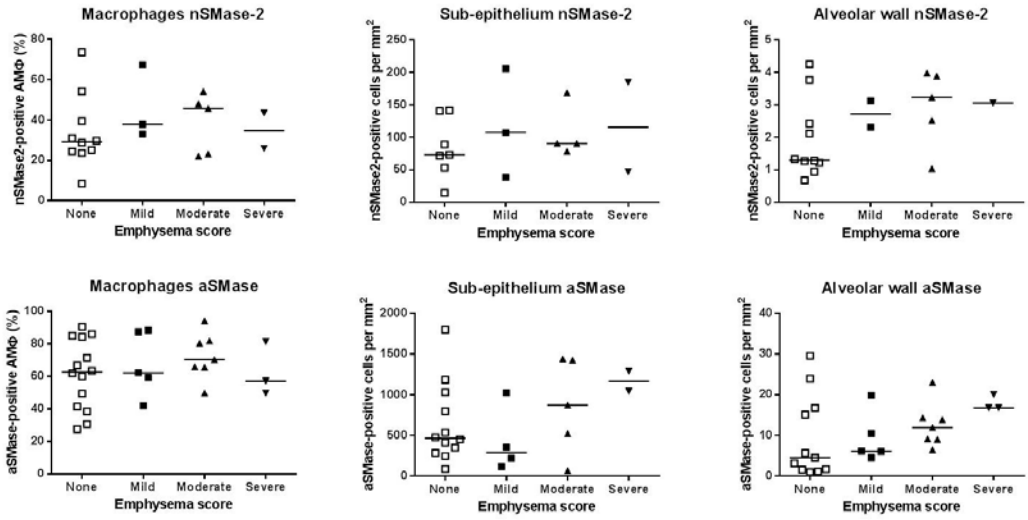

Figure S1. Quantification of aSMase and nSMase-2 protein within lung according to emphysema score.

IHC data were stratified according to an emphysema score generated by pathologists at UHSM on histological samples from a subset of patients. Associations were determined using Pearson's test.

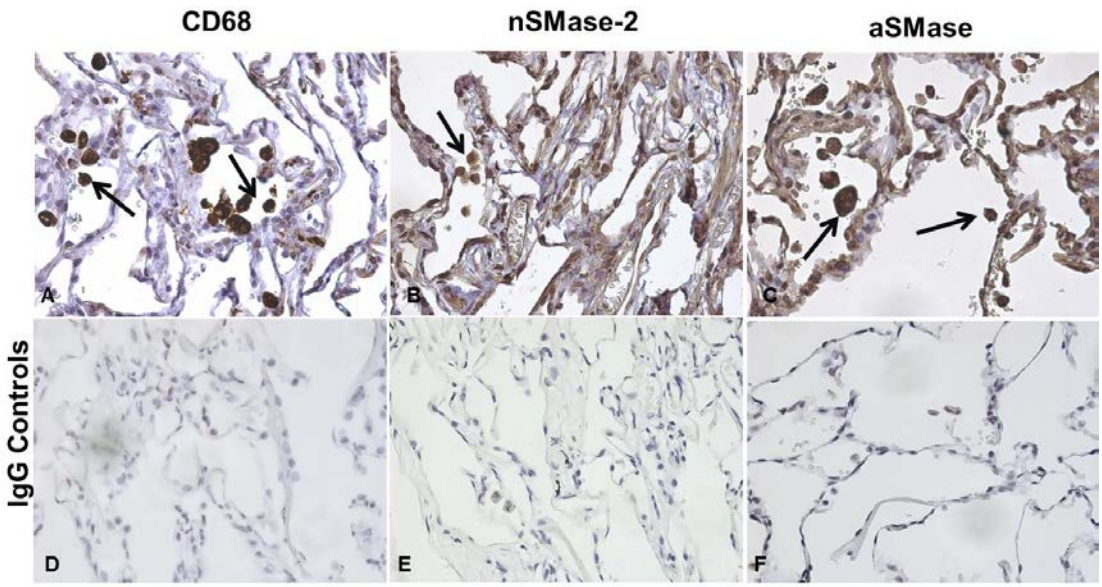

Figure S2. CD68, nSMase-2 and aSMase labelling in human alveolar macrophages. Representative images for CD68 (A), nSMase-2 (B) and aSMase (C) expression (Brown) within the peripheral lung tissue from COPD patients. Mononuclear cells with a clearly defined cytoplasm and situated within the alveolar space stained positive for CD68, nSMase-2 and aSMase in serial sections (black arrows) and were determined as alveolar macrophages. (D-F) Representative images of the negative controls. Magnification x200 (A-F).

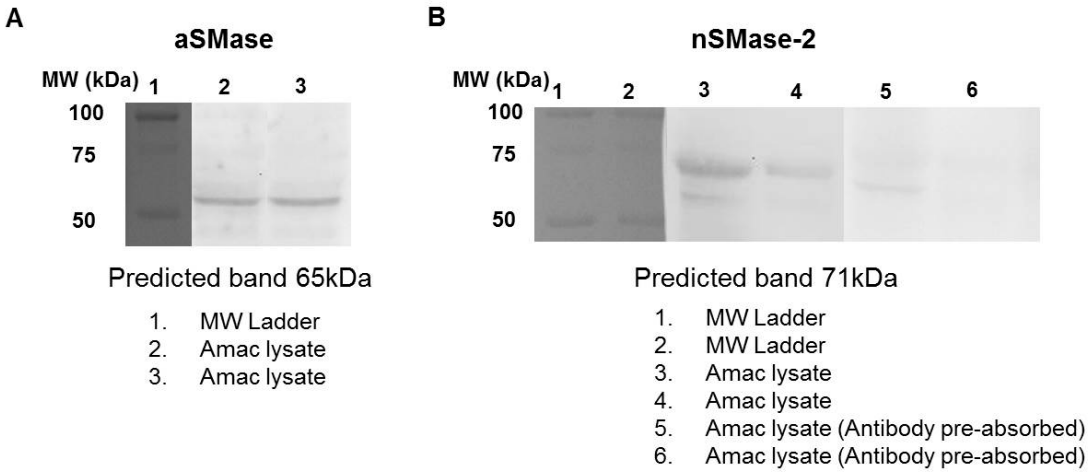

Figure S3. Determination specificity of aSMase and nSMase-2 antibodies.

Western blot analysis of whole cell lysates from human alveolar macrophages to determine whether the antibodies used in IHC study detected proteins at their expected molecular weight. The bands detected for polyclonal antibody for nSMase-2 were diminished following peptide treatment.

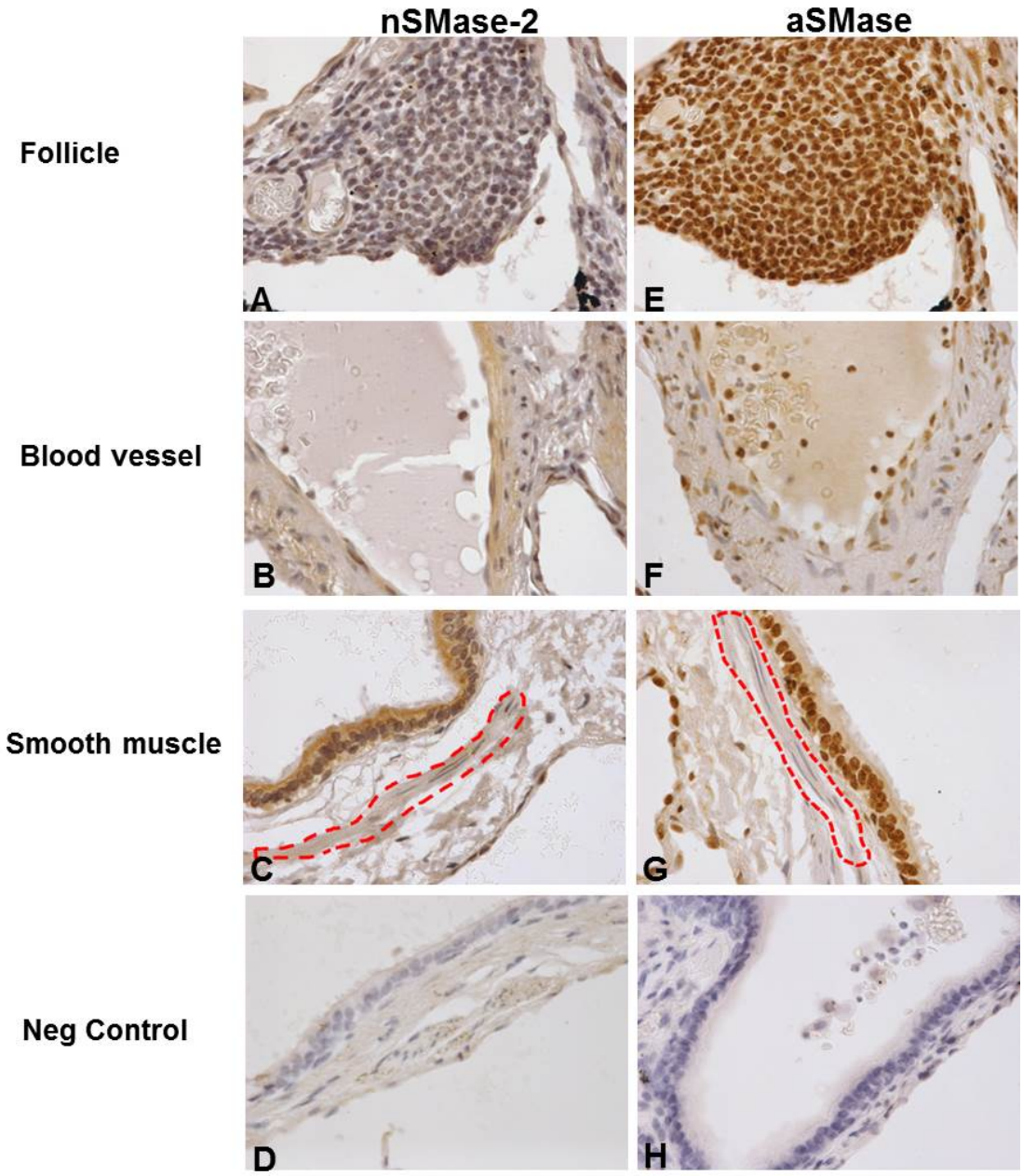

Figure S4. nSMase-2 and aSMase expression within human lung tissue

Representative images for (A-C) nSMase-2 and (E-G) aSMase within (A&E) inflammatory follicles, (B&F) blood vessels and (C&G) airway smooth muscle. Dotted line depicts the outline of the airway smooth muscle bundles. (D&H) Representative images of the negative controls. Magnification x600 (A-H)

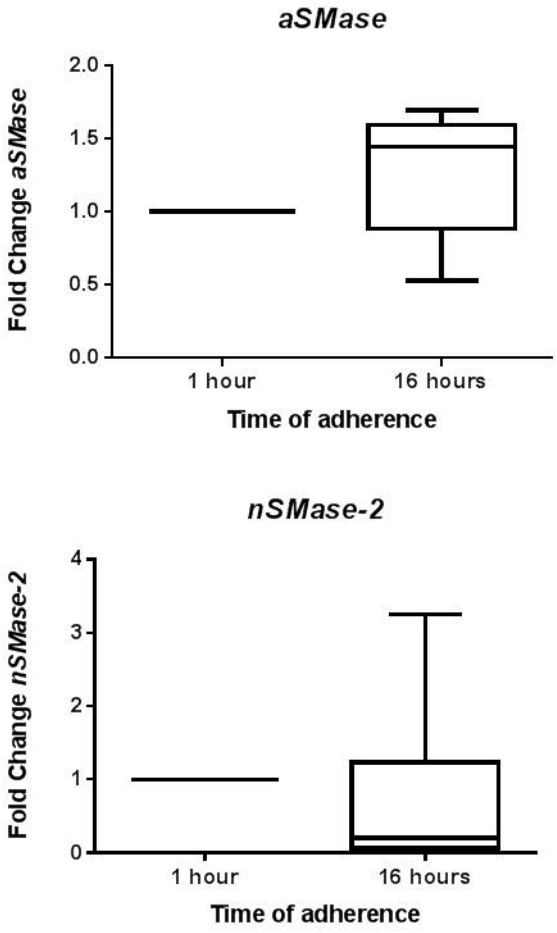

Figure S5. Qunatification of *aSMase* and *nSMase-2* mRNA within alveolar macrophages.

RT-PCR analysis of mRNA extracted from alveolar macrophages (n=6) isolated following either 1h or 16h adherence.

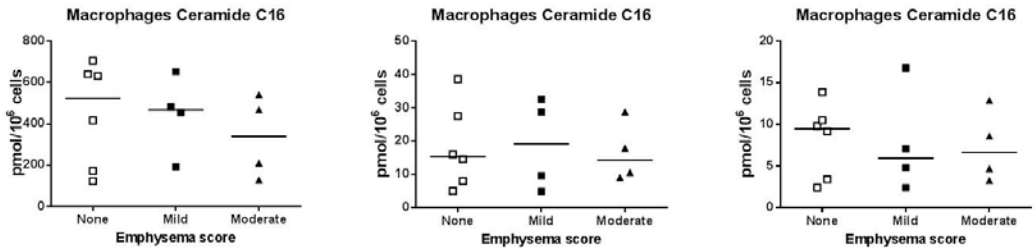

Figure S6. Quantification of ceramide isoforms according to emphysema score. RT-PCR data were stratified according to an emphysema score generated by pathologists at UHSM on histological samples from a subset of patients (n=14)