Online Supplement

Low sputum MMP-9/TIMP ratio is associated with airway narrowing in smokers with asthma

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METHODS

Measurements

MMP-9 concentration and activity assays: Commercial assays were performed in accordance with the instructions provided. The activity assay was quantified by fluorescence resonance energy transfer (FRET) and was read kinetically over 4 hours as this showed better reproducibility than an end-point read-out. Samples were not chemically activated with amino-phenyl mercuric acetate to allow estimation of endogenous enzyme activity. Activity was calibrated to an activity standard and expressed as ng/ml.

Validation of MMP-9 assays: The commercial assays were supplied validated for plasma but not for sputum fluid therefore a comprehensive validation procedure for sputum was undertaken (by CG and JB). This involved the following stages:

Preparation of Validation Samples: Sputum supernatant was obtained from Analytical Biological Services Inc. (ABS), Wilmington DE 19801, USA). Two sets of validation samples corresponding to low and mid-range concentrations of MMP-9 in sputum were identified and frozen at -80°C. The high-range validation samples were from a single subject sputum spiked with exogenous human MMP-9 (kindly supplied by Jill Wright of Wyeth Discovery; Lot # 40011-70).

Determination of the Calibration Curve Range: The intra- and inter-assay imprecision of the read-back values for the seven calibrators were determined from five analytical runs. For each analytical run, seven calibrator concentrations were analysed in duplicate and a back-calculated concentration for each individual calibrator data point was obtained. The

absorbance data was logged before performing a four-parameter best-fit curve. The mean, SD, %CV, and %Bias of the back-calculated concentrations were calculated for each analytical run (to assess intra-assay imprecision) and for all analytical runs (to assess inter-assay imprecision).

Determination of Sample Dilution and the Reportable Range: To establish an optimum dilution factor for sputum samples and to assess dilution linearity, four samples from apparently healthy subjects and 2 endogenous and 2 spiked sputum supernatants were analysed using dilution ranges from 1:20 to 1:200 with calibrator diluent.

Determination of Intra- and Inter-Assay Imprecision: To determine the intra- and inter-assay imprecision of the method in sputum, the MMP-9 concentration was measured in three separate aliquots of each of the three validation samples (Low, Mid and High) in five independent analytical runs. For intra-assay precision, the %CV for each validation sample was determined in each analytical run. The inter-assay imprecision (%CV) was determined in five analytical runs. This generated fifteen (15) MMP-9 concentration values (3 aliquots in 5 runs) for intra-data and n=5 for inter-data. The overall imprecision was calculated using the thirty replicate values for each QC and the intra-assay imprecision was calculated based on six singleton replicates per run.

Determination of Analyte Stability: Sputum samples (ABS) were delivered frozen and on arrival defrosted, aliquoted and stored at -80°C. Therefore, samples had been through two freeze/thaw (F/T) cycles before stability assessment. For assessment of short-term stability, samples were defrosted and either; immediately refrozen; incubated at RT for 2, 6 or 24h and refrozen; or incubated at 4°C for 2, 6 or 24h and refrozen. All samples were refrozen for a minimum of 24h before assay. The MMP-9 concentration in samples which were immediately

refrozen (3F/T) yields the baseline MMP-9 concentration for this stability comparison. The stability of MMP-9 following 3 F/T, 4 F/T and 5 F/T (one, two and three additional F/T cycles respectively) was also determined in each matrix. A freeze thaw cycle was carried out by defrosting samples and leaving them at room temperature for a minimum of fifteen minutes. The concentration of MMP-9 in samples following two F/T cycles yields the baseline for comparison. The % change in the MMP-9 concentration for each sample was calculated by comparison to the measured baseline concentration.

Plate Effects: A single sample was prepared and run in all wells across the plate. A standard curve was run in parallel and used to back-calculate the values. Edge effects, gradients and %CV across the plate were calculated. *Accuracy/Recovery:* Two sputum samples were spiked with MMP-9 at four different concentrations (final theoretical spiking concentrations of 1600, 800, 400 and 200 ng/mL). The %Bias of spiked MMP-9 and recoveries were calculated.

Measurement of tissue inhibitors of metalloproteinases (TIMP-1 and TIMP-2):

Serum TIMP1 and TIMP-2 were quantified simultaneously using the Fluorokine MAP Human TIMP Multiplex Kit (R&D Systems) according to the manufacturer's instructions. These kits were validated for serum and were designed for use with a Luminex® analyzer. Briefly, analyte-specific antibodies are pre-coated onto colour-coded microparticles. The microparticles, standards and samples are pipetted into wells and the immobilized antibodies bind the analytes of interest. After washing away any unbound substances, biotinylated antibodies specific to the analytes of interest are added to each well. The wells are washed to remove any unbound biotinylated antibody. Streptavidin-phycoerythrinconjugate (Streptavidin-PE) which binds the biotinylated detection antibodies, is then added to each well. A final wash removes unbound streptavidin-PE and the microparticles are resuspended

in buffer and read using the Luminex® analyzer. One laser is microparticle-specific and determines which analyte is being detected. The other laser determines the magnitude of the phycoerythrin-derived signal, which is in direct proportion to the amount of analyte bound.

Validation of TIMP-1 and TIMP-2 assays: The commercial assays were supplied validated for plasma but not for sputum fluid therefore a comprehensive validation procedure for sputum accuracy, spike recovery, freeze/thaw, cold-room and short-term room temperature stability, well effects, calibration curve range, assessment of inter- and intra-assay imprecision and sample dilution reportable range was undertaken (by CG and JB). This involved the following stages:

Calibration Curve Range and Limits of Quantitation

The intra- and inter-assay imprecisions of the read-back values for the seven calibrators for TIMP-1 and for TIMP-2 were determined from five (5) analytical runs. For each analytical run, seven (7) calibrator concentrations for each analyte were analysed in duplicate and a back-calculated concentration for each individual calibrator data point was obtained using a linear five parameter logistic best-fit curve. The mean, SD, %CV, and %Bias of the back-calculated concentrations were calculated for each analytical run (to assess intra-assay imprecisions) and for all analytical runs (to assess inter-assay imprecisions).

The LLQ is defined as the lowest calibrator concentration that can be measured with a %CV and %Bias $\leq$25%. The ULQ is the highest calibrator concentration that can be measured with a %CV and %Bias $\leq$25%.

The mean intra–assay imprecision (%CV) and accuracy (%Bias) values were estimated from five (5) analytical runs for each of the two sets of seven calibrators. The LLQ of the assays are

13.72 and 53.5 pg/mL for TIMP-1 and TIMP-2 respectively, at which concentrations the inter-assay %CV values were 1.0 and 3.6 and %Bias values were 0.5% and -0.9%, respectively. The ULQ of the assays are 10000 and 39000 pg/mL for TIMP-1 and TIMP-2, at which concentrations the %CV values were 19.2 and 9.4 and the %Bias values were greater than 25%.

Analytical Performance: Intra- and inter-assay imprecision: *To determine the intra- and inter-assay imprecision values of the method in sputum, TIMP-1 and TIMP-2 concentrations were each measured in two separate aliquots of each of the two (2) validation samples (Low and Mid range) in five (5) independent analytical runs. For intra-assay precision, the %CV for each validation sample was determined in each analytical run. The inter-assay imprecision (%CV) was determined in five (5) analytical runs. This generated ten (10) TIMP-1 and TIMP-2 concentration values (2 aliquots in 5 runs) for intra-data and n=5 for inter-data. For the highest calibrator for TIMP-2, data from four (4) analytical runs were obtained, giving eight (8) TIMP-2 concentration values (2 aliquots in 4 runs) for intra-data and n=4 for inter-data.*

The mean intra-assay imprecisions were assessed by running 2 characterisation samples (low and mid range samples) in duplicate. For TIMP-1, the mean %CV values were 2.6 % (mid) and 3.9 % (low) and ranged from 1.4% to 4.9% and 0.2 % to 9.4 % respectively. For TIMP-2, the mean %CV values were 4.4% (mid) and 5.5% (low), and ranged from 0.1 % to 7.5 % and 0.1 % to 11.8 % respectively. The inter-assay imprecision values for the low and mid-range samples were also calculated. For TIMP-1, the %CV values were 8.2 (mid) and 19.6 (low). For TIMP-2, the %CV values were 9.5 (mid) and 18.6 (low).

Sample Dilution and Reportable Range: *To show good dilution linearity and parallelism with the standard curve, we diluted one endogenous sample 1:10, 1:20, 1:40, 1:80 and 1:100 in Reagent Diluent. Results indicate good dilution linearity from 1:10 to 1:100 dilutions, with a 1:40 dilution appearing to be the best starting dilution for sputum samples. The reportable ranges for TIMP-1 and TIMP-2 are therefore 548.8 – 400 000 pg/mL and 2140 – 1 560 000 pg/mL respectively.*

Determination of Analyte Stability: *To assess the short-term stability of TIMP-1 and TIMP-2 in sputum, two sputum samples were incubated at 27°C for 2h and then assayed and compared to fresh sputum samples (basal). For TIMP-1, the percentage change from basal ranged from -9.9% to 11.8% and for TIMP-2, the percentage change from basal ranged from 5.8% to 7.2%. We conclude that TIMP-1 and TIMP-2 are relatively stable in sputum at RT for up to 2h. However, we recommend for standardisation of collection and handling protocols, that sputum be placed on ice immediately following collection and stored at -80 °C within two hours of processing*

Representative Range: To establish a representative range using this human TIMP multiplex assay, 37 sputum samples were measured. For TIMP-1, all 37 samples assayed gave values within the measurable range. The mean was 69053 pg/mL and ranged from 17927 – 183303. For TIMP-2, 35 of the 37 samples gave values within the measurable range. The mean was 12630 and ranged from 2421 – 64062 pg/mL.

Synopsis: This characterisation shows that the Fluorokine MAP Human TIMP Multiplex Kit (R&D Systems) assay is suitable for exploratory measurements of Human TIMP-1 and TIMP-2 in human sputum. The calibration curves showed good precision, from 13.72 pg/mL (LLQ) to

10 000 pg/mL (ULQ) for TIMP-1 and from 53.5 pg/mL (LLQ) to 39 000 pg/mL (ULQ) for TIMP-2. Sputum samples were used diluted 1:40 in RD to assay and experiments showed good dilution linearity around this dilution from 1:20 to 1:100. The intra-assay imprecision was estimated in a limited study and found to be satisfactory. TIMP-1 and TIMP-2 were found to be stable in the short-term in sputum.

RNA sample collection: Transcriptome analysis was performed on a selection of subjects, primarily healthy control subjects and asthma patients with severe disease; the numbers are indicated in Table E2. Airway cells were isolated from processed induced sputum [25] and RNA was isolated (AmbionmirVana RNA kit, Applied Biosystems, Warrington, UK) and stored at -80°C until use. Nasal respiratory epithelium samples were obtained using a plastic curette (Rhino-probe, Arlington Scientific, Inc.) to gently scrape the surface (3x3mm) of the mid-inferior portion of the inferior turbinate and the tip of the curette placed in RNA stabilising buffer (AmbionRNA*later,* Applied Biosystems, Warrington, UK).

RNA isolation: For sputum RNA, total RNA was isolated using the AmbionmirVana isolation kit according to the manufacturer's instructions, with inclusion of on-column DNAse treatment. For nasal curettage samples, total RNA was isolated using Qiazollysis buffer (Qiagen) according to the manufacturer's instructions, with the inclusion of on-column DNase-treatment and using a handheld micro-homogeniser. Typical yields from this technique were approximately 2ug total RNA/10^6 sputum cells and 4ug RNA per nasal curettage sample. Total RNA quality metrics (RIN) was assessed by Bioanalyser microfluidic chips (Agilent), and RNAs with RIN <6.4 discarded from further analysis. The median RIN for nasal epithelial cell RNA samples was 9.2 and for sputum cell RNA samples was 8.3.

RESULTS

Validation results for MMP-9 concentration

The Quantikine Human MMP-9 (total) ELISA Immunoassay (R&D Systems) was validated for quantifying MMP-9 in induced sputum fluid. The calibration curve showed good precision from 0.312 to 20 ng/mL, with an inter-assay imprecision of 0.0 to 2.7%CV. Samples were diluted 1:40 for assay therefore the reportable range of the assay for MMP-9 concentrations in human sputum samples is 12.5 to 800 ng/mL. The overall plate-effect CV was 3.3%. The mean intra- and inter-assay imprecision of the assay; determined using 3 validation samples were 3.9 to 7.2 % and 4.3 to 6.8 %CV, respectively, and the total imprecision (CV of 30 replicates) was 5.5 to 8.1%. The %Bias of MMP-9-spiked samples was ≤14% and recovery 96.4 to 113.6%. MMP-9 concentration in 1% DTT-treated sputum was stable at 4°C for 24 hours and at room temperature for 6h. MMP-9 was stable in sputum sample for up to 3 freeze-thaw cycles.

Validation results for MMP-9 activity

The Fluorokine® E Human Active MMP-9 assay (R&D Systems) was validated for measurement of MMP-9 in induced sputum fluid. The calibration curve showed good precision from 0.25 to 16 ng/mL. Samples were diluted 1:100 for assay therefore the reportable range for MMP-9 activity is 25 to 1600 ng/mL. The mean intra- and inter-assay imprecision of the assay;

determined using 3 sputum validation samples, were 5.9 to 9.7 % and 4.7 to 10.9% CV respectively, and the total imprecision (CV of 30 replicates) was 9.5 to 10.9%. The % Bias of MMP-9-spiked samples was ≤11% and recovery 101.3 to 110.4%. MMP-9 activity in 3 out of 4 sputum samples treated with 1% DTT was stable at room temperature for up to 6h, and the 4^{th} sample increased by 72% at 6 hours. It is possible that DTT used to disperse the sputum plug during sample processing is responsible for this potential disaggregation effect on the enzyme and observed increase in enzyme activity. However, because typical DTT treatment for sputum mucus plug processing was only for 15 minutes and because 3 out of 4 samples were minimally affected therefore the effects were mitigated by performing assays using freshly thawed and diluted samples.

Table E1: Expression levels of mRNA for *MMP-9*, *TIMP1* and *TIMP2* in nasal respiratory epithelium and sputum leucocytes in patients with sthma and healthy controls.

	Severe asthma			Healthy control			
	Never smoker	Smoker	p-value[1]	Never smoker	Smoker	p-value[2]	p-value[3]
Epithelial cell RNA Number	14	17		17	14		
MMP-9	0.24 (0.14, 0.63)	0.05 (0.02, 0.15)	**p=0.001**	0.26 (0.13, 0.55)	0.05 (0.04, 0.10)	**p<0.001**	**p=0.952**
TIMP1	5.74 (3.66, 7.00)	4.78 (4.07, 5.22)	p=0.372	4.18 (3.58, 5.05)	4.42 (3.69, 5.32)	p=0.766	p=0.592
TIMP2	2.65 (2.39, 3.72)	2.70 (2.53, 3.44)	p=0.952	2.61 (2.11, 3.25)	2.86 (2.34, 3.26)	p=0.796	p=0.984
MMP9/TIMP1	0.05 (0.04, 0.09)	0.01 (0.00,0.03)	**p<0.001**	0.06 (0.03, 0.10)	0.01 (0.01, 0.03)	**p<0.001**	p=0.921
MMP9/TIMP2	0.10 (0.05, 0.14)	0.02 (0.01,0.06)	**p<0.001**	0.10 (0.06, 0.16)	0.02 (0.01, 0.03)	**p<0.001**	p=0.984
Sputum cell RNA Number	15	15		15	13		
MMP-9	2.43 (0.69, 2.73)	3.81 (2.26, 7.34)	**p=0.005**	1.65 (1.02, 3.44)	4.11 (1.62, 6.20)	**p=0.048**	p=0.490
TIMP1	15.9 (10.4, 18.1)	21.4 (19.5, 28.5)	**p<001**	15.6 (12.2, 18.4)	22.5 (14.2, 26.7)	**p=0.030**	p=0.381
TIMP2	13.7 (12.1, 16.2)	19.6 (15.9, 21.1)	p=0.056	17.9 (16.8, 20.4)	19.0 (16.8, 21.5)	p=0.712	p=1.00
MMP9/TIMP1	0.13 (0.06, 0.18)	0.19 (0.09,0.27)	**p<0.001**	0.13 (0.07, 0.19)	0.18 (0.10, 0.27)	p=0.231	p=0.927
MMP9/TIMP2	0.13 (0.05, 0.18)	0.27 (0.12,0.38)	**p=0.046**	0.10 (0.06, 0.19)	0.21 (0.08, 0.36)	p=0.072	p=0.549

Median (IQR); Expression levels are relative fluorescent units after global normalisation, divided by 1000.Boldface p values indicate $p < 0.05$.
[1] Comparison of smokers with severe asthma versus never smokers with severe asthma; [2] Comparison of healthy smokers versus healthy never smokers; [3] Comparison of smokers with severe asthma versus healthy smokers
Abbreviations: MMP, matrix metalloproteinase; TIMP, tissue inhibitors of metalloproteinases