

Quantification of a protein biomarker in human sputum using LC-MS: Issues, challenges and solutions

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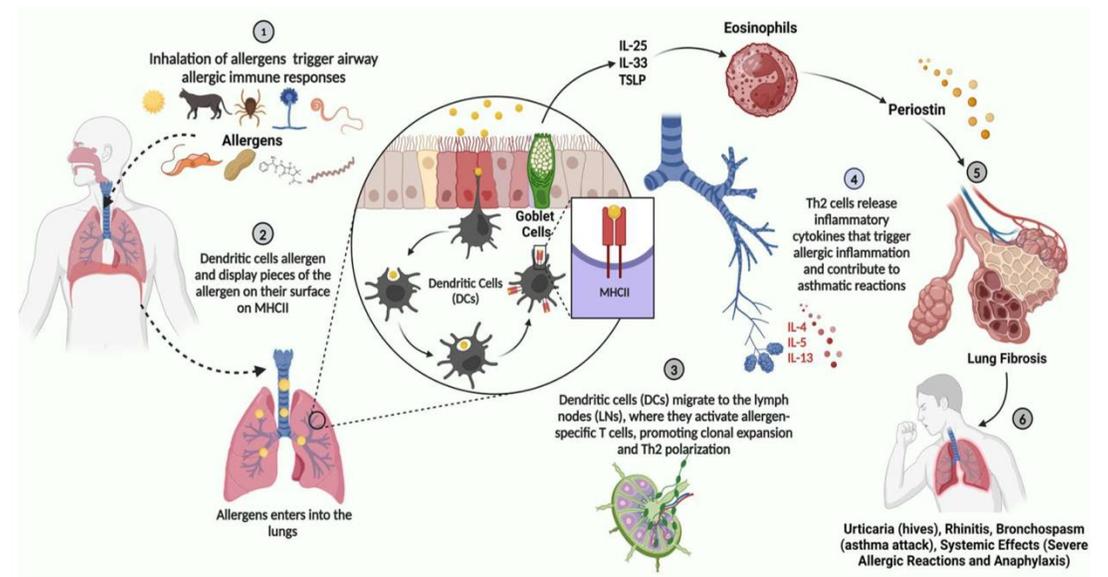
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Interleukins (ILs) as biomarkers in the clinic

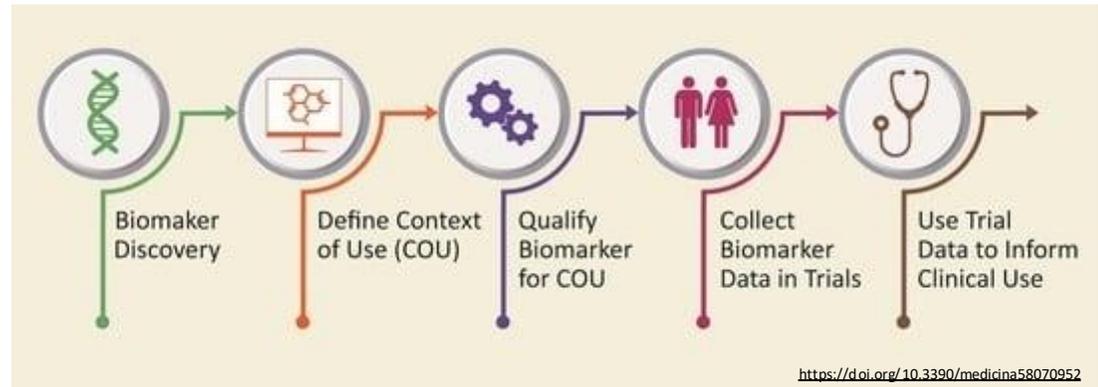
- ILs levels in sputum may reflect the inflammatory state of the airways
- ILs as pharmacodynamic and/or safety biomarkers
- Quantification of an Interleukin in human sputum

Schematic representation of the pathologic immune response in the airways



Exploratory biomarker assay for an Interleukin in human sputum

Context-of-Use

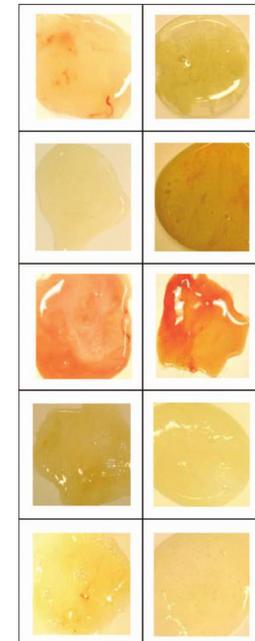


- Phase Ib study to gain proof of mechanism
- Induced sputum is sampled 4 times in each subject using a controlled protocol (2 pretreatment, and 2 post treatment)
- Interleukin biomarker measured to assess which biology is impacted pharmacodynamically from the treatment

Challenges for biomarker quantification in sputum

- **Heterogeneity:** Mucins, cellular and molecular components, microorganisms, collection method, patient's condition, saliva contamination
- **Proteolytic activity:** Proteases can degrade protein biomarkers
- **Solid matrix:** Pre-treatment prior to extraction and analysis
- **Data normalization:** Reliable normalizing factor for accurate concentrations
- **Standardized collection protocols:** Key for reproducible and comparable results

Human sputum

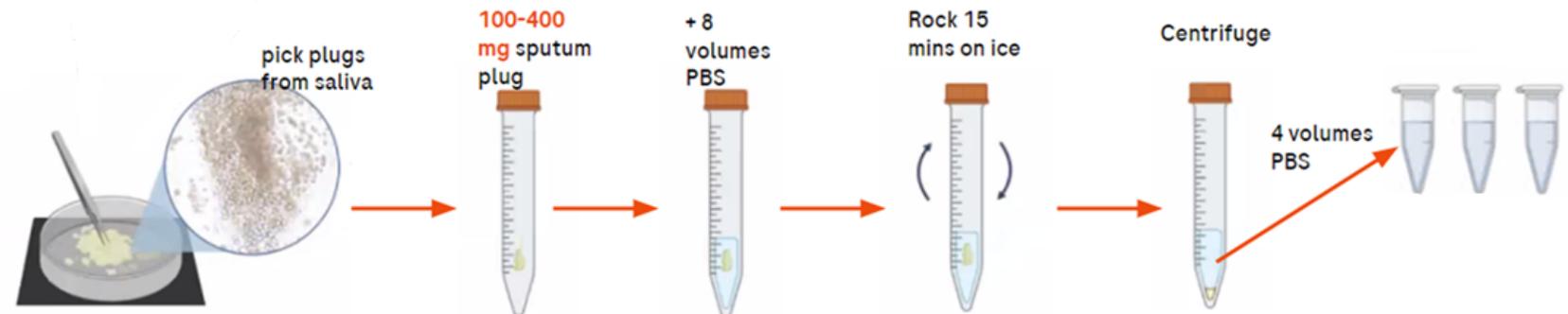


<https://doi.org/10.4187/respire.04547>

Sputum induction and processing protocol

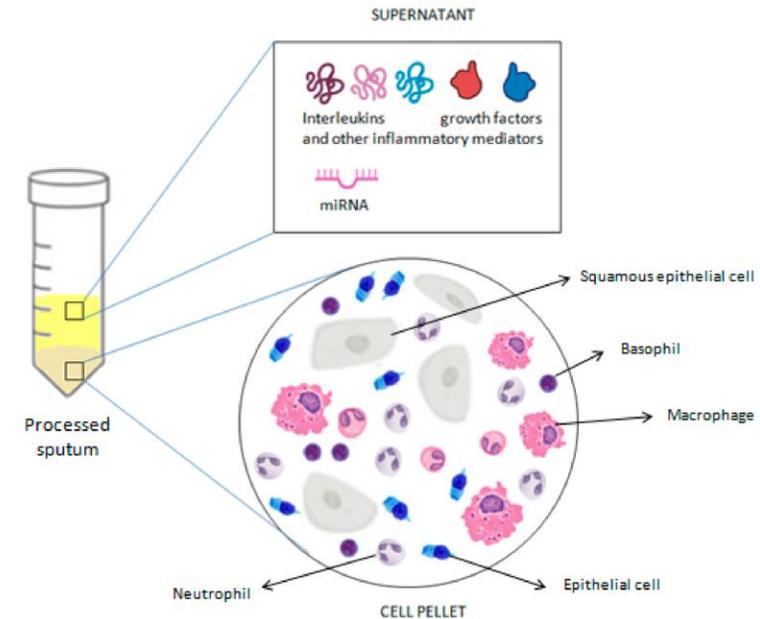
Comprehensive sputum collection and processing manual available for the clinical sites

- Sputum induced by inhalation of hypertonic saline (~20 min procedure)
- Selection of sputum plugs from saliva with inverted microscope
- Plugs reconstitution with 8 volumes of PBS, supernatant collection for protein biomarker analysis



Normalization strategies for protein biomarkers in sputum

- How to normalize protein biomarker concentrations in human sputum?
- Total protein, weight/volume or urea as common normalization factors
- Data interpretation may vary based on the adopted normalization strategy



Bioanalytical assay set-up for human sputum



- An LC-MS assay required due to specificity issues with the Ligand binding method
- LC-MS assays often tricky for Interleukins due to small size
- Assay based on fixed volume or fixed amount of total protein?
- Working with fixed volumes simplifies BA lab operations
- How to deal with different amounts of total proteins into each sample?

Fixed trypsin to total protein ratio, or fixed trypsin amount?

- Variable total protein concentrations in sputum samples (0.059 – 2.94 mg/mL)
- Fixed trypsin to total protein ratio -> Different trypsin amounts into each sample?
- Fixed trypsin amount -> Different trypsin to total protein ratios across different samples?
- No fixed trypsin to total protein ratio valid for all samples

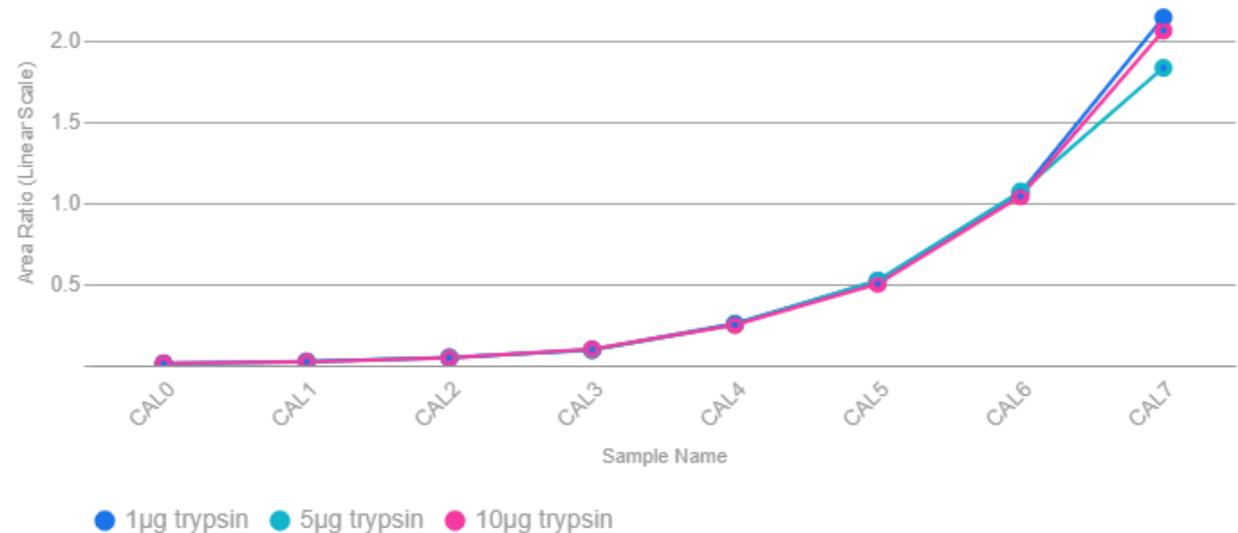
Fixed trypsin to total protein ratios across samples with different total protein amounts

Sample	Total protein (µg)	Enzyme / Total protein ratio	Trypsin amount (µg)	Interleukin concentration (ng/mL)	Accuracy
1	10.6	1:2	5	1.41	100 (Ref.)
		1:5	2	0.951	67.4
		1:10	1	0.254	18.0
		1:50	0.2	0.272	19.3
		1:100	0.1	0.0611	4.3
2	112	1:5	22.5	1.89	100 (Ref.)
		1:10	11.2	1.96	103.7
		1:20	5.1	1.78	94.2
		1:50	2.2	2.04	107.9
		1:100	1.1	1.22	64.6

May a fixed amount of trypsin be the way forward?

Calibrators digested with increasing trypsin amounts

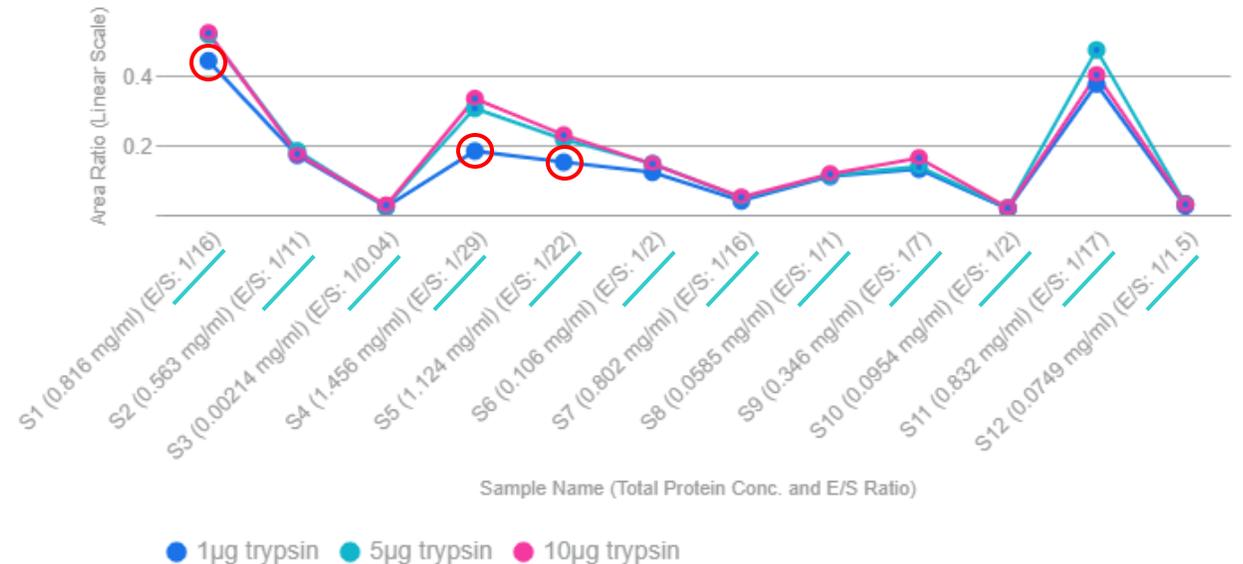
- Calibrators in surrogate matrix: 0.1% human plasma in PBS (total protein content ~0.07 mg/mL)
- No difference in IL concentrations with the different trypsin amounts tested



May a fixed amount of trypsin be the way forward?

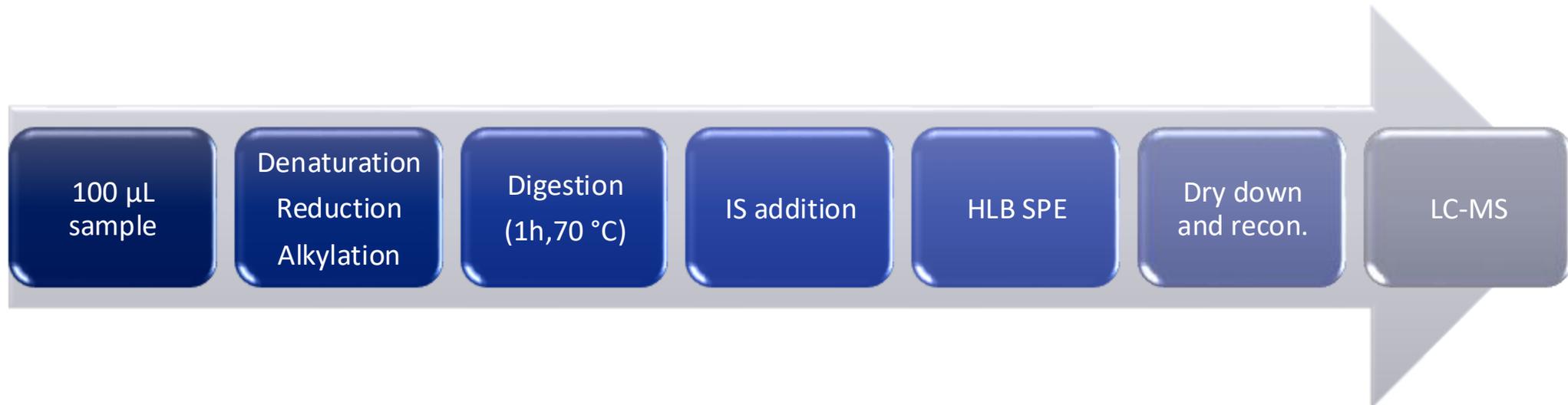
Sputum samples digested with increasing trypsin amounts

- Human sputum samples with different total protein concentrations
- Higher trypsin amounts lead to higher IL concentrations in samples with higher total protein content
- 5 μg trypsin seems to be sufficient for the tested samples



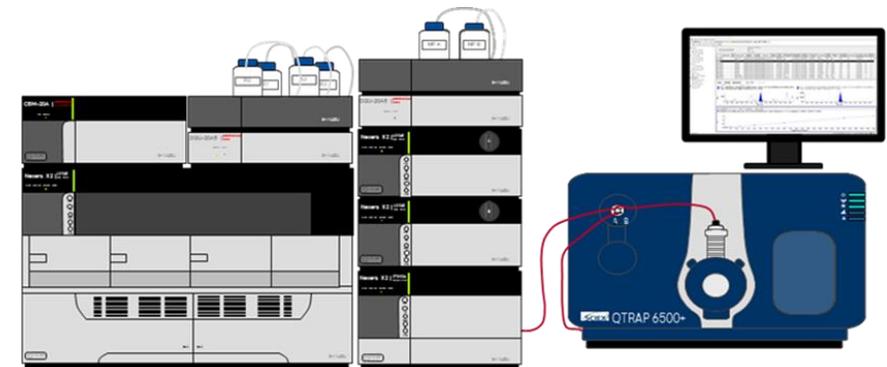
Bioanalytical assay format

- Concentration range: 125 – 25000 pg/mL, 100 μ L assay volume, 5 μ g trypsin
- Surrogate matrix for human sputum: 0.1% human plasma in PBS
- Assay type: Bottom-up approach (one day assay)



LC-MS/MS method

- UHPLC conditions: Column C18+, 2.1 x 150mm; Flow rate 0.5 mL/min; Run time 17 min
- Mobile phases: 0.01% Acetic acid in water (A); 0.01% Acetic acid in ACN (B)
- MS conditions: SCIEX 6500+ QTRAP in positive ion mode



Liquid chromatography mass spectrometry (LC-MS)

Parallelism experiments in human sputum

Key requirement for any biomarker methods to be considered quantitative

- Two parallelism methods used

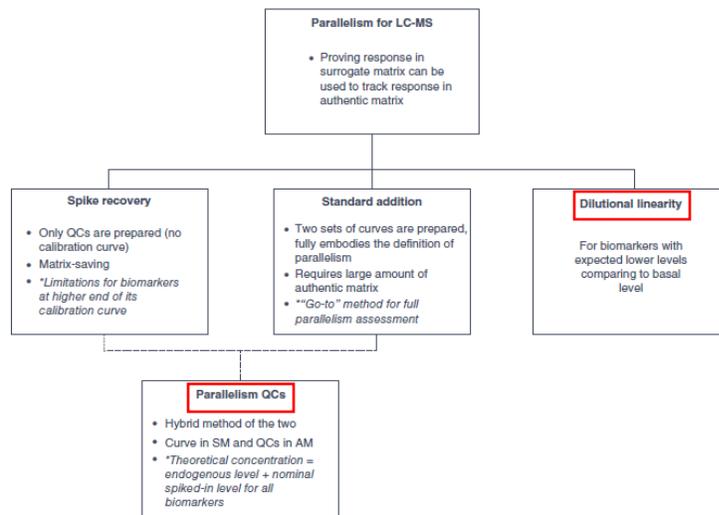
- Sputum samples with different total protein concentrations **selected**

Research Article

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Bioanalysis

Comparison of three parallelism assessment methods of biomarker quantification by LC-MS/MS: a case study of the bioanalysis of creatinine in human urine samples



Flowchart for parallelism assessment using LC-MS

Sample Name	Total Protein (mg/mL)
Sputum 1	0.000214 (BLQ)
Sputum 2	0.059
Sputum 3	0.075
Sputum 4	0.10
Sputum 5	0.11
Sputum 6	0.35
Sputum 7	0.54
Sputum 8	0.56
Sputum 9	0.74
Sputum 10	0.76
Sputum 11	0.80
Sputum 12	0.82
Sputum 13	0.83
Sputum 14	0.95
Sputum 15	0.96
Sputum 16	1.01
Sputum 17	1.12
Sputum 18	1.19
Sputum 19	1.46
Sputum 20	2.94

Parallelism by dilution linearity

Sputum samples with high Interleukin endogenous concentrations diluted with surrogate matrix

- Parallelism by dilution linearity successful for samples with different total protein concentrations

Sample Name	Total protein (mg/mL)	Enzyme / Total protein ratio	Dilution Factor	Measured IL Conc. (ng/mL)	Back calculated IL Conc. (ng/mL)	Accuracy (%)
Sputum 12	0.82	1:16	1	5.16	5.16	Ref.
			2	2.54	5.08	98
			5	1.06	5.31	103
			10	0.531	5.31	103
Sputum 13	0.83	1:16	1	4.22	4.22	Ref.
			2	2.17	4.34	103
			5	0.966	4.83	115
			10	0.490	4.90	116
Sputum 17	1.12	1:22	1	2.26	2.26	Ref.
			2	1.10	2.20	97
			5	0.458	2.29	101
			10	0.262	2.62	116
Sputum 10	0.76 (Lowest)	1:16	1	2.72	2.72	Ref.
			10	0.233	2.33	85.8
Sputum 14	0.95	1:19	1	7.36	7.36	Ref.
			20	0.364	7.28	98.9
Sputum 20	2.94 (Highest)	1:59	1	12.1	12.1	Ref.
			20	0.655	13.1	108

Parallelism by spiking (Parallelism QC)

Sputum samples with low Interleukin endogenous concentrations spiked-up with analyte

- Parallelism by spiking successful for samples with different total protein concentrations

Sample Name	Total protein (mg/mL)	Enzyme / Total protein ratio	Spiking Level	Theoretical IL Conc. (ng/mL)	Measured IL Conc. (ng/mL)	Accuracy (%)
Sputum 2	0.059 (Lowest)	1:1	Endogenous	N/AP	0.924	N/AP
			QC Low/Mid	3.32	3.19	96.0
			QC High	18.4	18.5	100
Sputum 4	0.100	1:2	Endogenous	N/AP	0.177	N/AP
			QC Low/Mid	2.72	2.40	88.4
			QC High	18.1	24.0	133
Sputum 3	0.075	1:1.5	Endogenous	N/AP	0.296	N/AP
			QC Low/Mid	1.52	1.44	92.0
			QC High	18.2	16.5	90.5
Sputum 10	0.76	1:15	Endogenous	N/AP	2.72	N/AP
			QC High	18.3	16.4	89.7
Sputum 14	0.95	1:19	Endogenous	N/AP	7.36	N/AP
			QC High	18.8	19.9	106
Sputum 20	2.94 (Highest)	1:59	Endogenous	N/AP	12.1	N/AP
			QC High	18.8	16.1	85.5
Sputum 1 (5 µg trypsin)	0.000214 (BLQ)	>>1:1	Endogenous	N/AP	0.160	N/AP
			QC Low/Mid	2.71	2.01	74.2
			QC High	18.1	14.4	79.8
Sputum 1 (10 µg trypsin)	0.000214 (BLQ)	>>1:1	Endogenous	N/AP	0.168	N/AP
			QC Low/Mid	2.71	1.94	71.7
			QC High	18.1	14.5	80.2

Parallelism experiments in human sputum: Outcome

- Assay quantitative within the selected bioanalytical range for the Interleukin (125 – 25000 pg/mL), in sputum samples with a defined total protein concentration range (0.059-2.94 mg/mL)
- The high absolute trypsin amount (5 μ g, E/S ratio between 1:60 and 1:1) ensures consistent digestion efficiency, overcoming the challenge of different total protein amounts
- Clinical study samples with higher total protein to be diluted prior to analysis
- Method currently in use at Roche to support a drug development program targeting the airways
- Best normalization strategy to be defined upon revision of first generated data in placebo vs treated patients (ongoing)

Fit-for-purpose validation

Validation parameters tested

- **Linearity**
- **Sensitivity**
- **Carry-over**
- **Precision & Accuracy**
- **Selectivity**
- **Stability**
- **Matrix Effect**

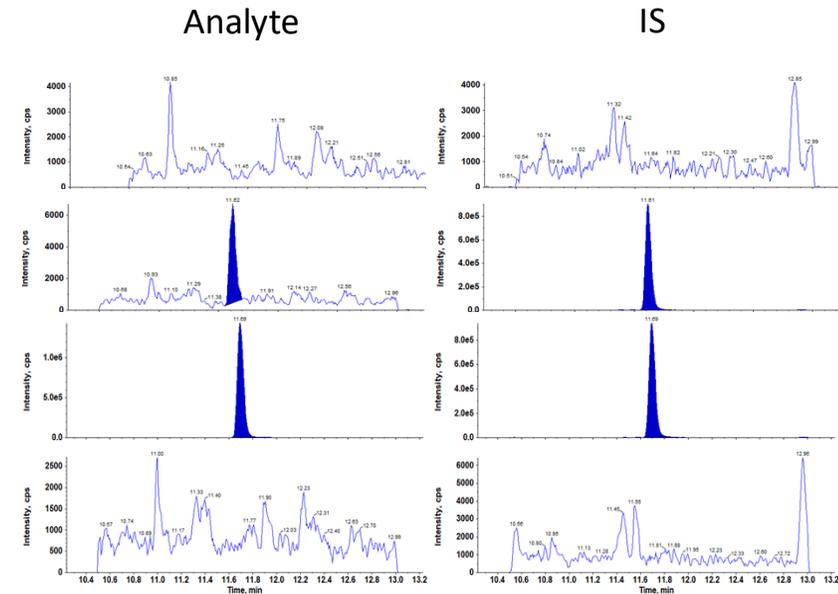
Chromatograms in surrogate matrix

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LLOQ

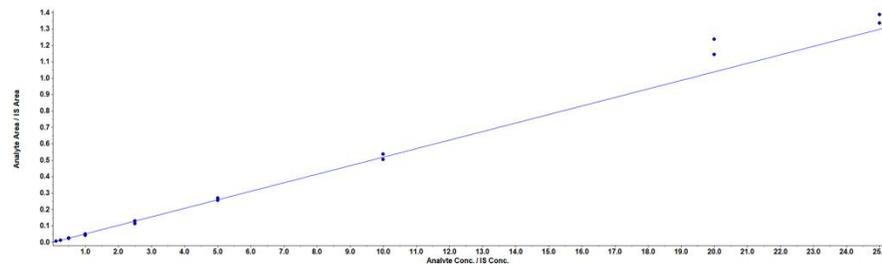
ULOQ

Carry-over blank



Calibration curve

■ "Linear" Regression ("1 / (x * x)" weighting): $y = 0.052x + -0.000671$ ($r = 0.9943$)

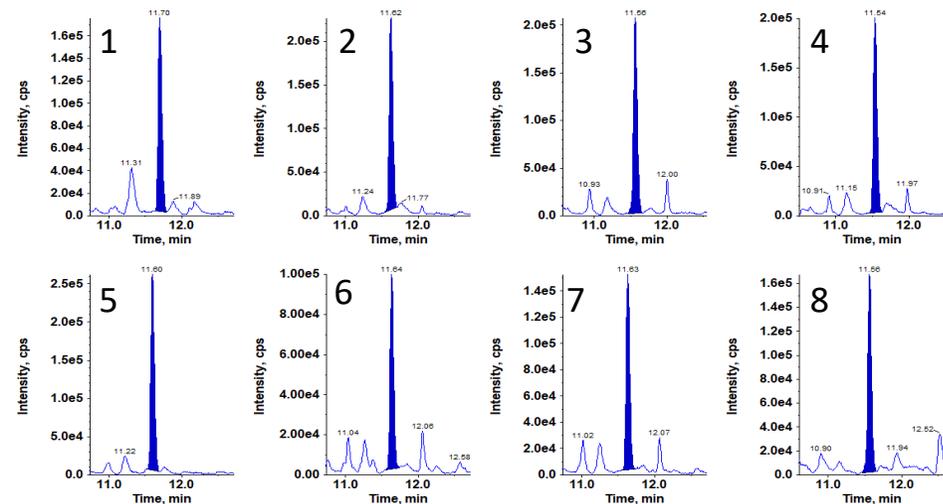


Fit-for-purpose validation

Endogenous level determination, Selectivity

- Sputum endogenous Interleukin levels

Sample	IL Conc. in sputum supernatant (ng/mL)	IL Conc. in sputum (ng/g)
1	6.23	56.1
2	7.36	66.2
3	5.87	52.8
4	6.02	54.2
5	9.25	83.3
6	2.72	24.5
7	4.98	44.8
8	12.1	109



- Selectivity

Sample	Area Ratio Quantifier / Qualifier MS Transition	Selectivity (%)
1	1.50	106
2	1.38	97.6
3	1.43	101
4	1.48	105
5	1.46	103
6	1.46	103
7	1.40	99.0
8	1.50	106
CAL 1	1.52	N/AP
CAL 2	1.45	
CAL 3	1.43	
CAL 4	1.36	
CAL 5	1.41	
CAL 6	1.40	
CAL 7	1.44	
CAL 8	1.43	
CAL 9	1.41	
Average	1.41	

Fit-for-purpose validation

Precision and Accuracy, Stability

P&A

- Surrogate matrix
- Human sputum

Sample Name	Analyte Conc. (ng/mL)	Values	Calculated Conc. (ng/mL)	CV (%)	Accuracy (%)
QC LLOQ SM	0.125	6 of 6	0.116	10.8	92.6
LQC SM	0.375	6 of 6	0.327	3.01	87.1
MQC SM	7.50	6 of 6	6.96	17.4	92.8
HQC SM	18.75	6 of 6	19.5	4.18	106
LQC sputum (20x diluted with SM)	0.365	6 of 6	0.366	4.46	100
MQC sputum (endogenous)	7.30	6 of 6	7.81	3.59	107
HQC sputum (spiked-up)	18.5	6 of 6	20.1	2.39	109

Bench-Top Stability
6h @ RT

- Surrogate matrix
- Human sputum

Sample Name	Analyte Conc. (ng/mL)	Values	Calculated Conc. (ng/mL)	CV (%)	Accuracy (%)
BTLow SM	0.375	3 of 3	0.306	10.8	81.5
BTHigh SM	18.75	3 of 3	19.2	3.01	104
BTLow sputum (20x diluted with SM)	0.365	3 of 3	0.354	4.46	97.0
BTMid sputum (endogenous)	7.30	3 of 3	7.48	3.59	103
BTHigh sputum (spiked-up)	18.5	3 of 3	19.8	2.39	107

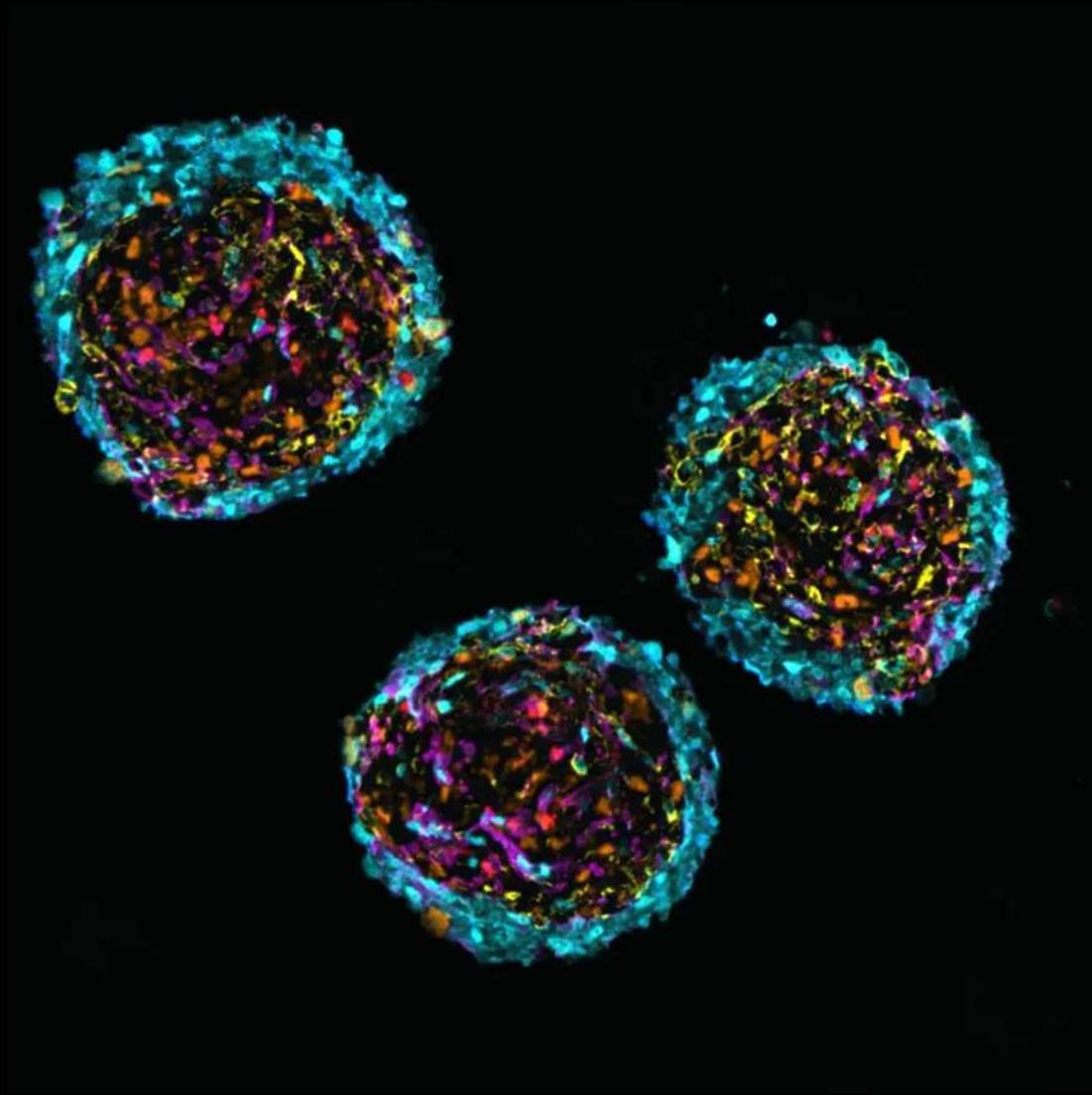
Summary and conclusions

- Quantification of protein biomarkers in human sputum poses unique challenges (matrix heterogeneity)
- A highly standardized sample collection and processing protocols is key for accurate and reproducible results
- If sensitivity allows, LC-MS is a valuable alternative to LBA as it may overcome assay specificity issues
- Setting-up a quantitative assay based on fixed volume/weight is a valuable and practical strategy
- High trypsin amount may overcome the challenge of different total protein amounts in individual samples
- Quantitative readout of the assay to be demonstrated by bracketing parallelism experiments within the selected bioanalytical range and total protein concentration range in the sputum samples
- Normalization strategy may influence data outcome and it requires understanding of biology and disease mechanism

Thank you!

Brian Dan
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Luca Ferrari
Richard Welford
Ronja Peter

The Project Team



Doing now what patients need next