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Large-Scale Label-Free Quantitative Mapping of the Sputum Proteome

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Supporting Information

ABSTRACT: Analysis of induced sputum supernatant is a minimally invasive approach to study the epithelial lining fluid and, thereby, provide insight into normal lung biology and the pathobiology of lung diseases. We present here a novel proteomics approach to sputum analysis developed within the U-BIOPRED (unbiased biomarkers predictive of respiratory disease outcomes)

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international project. We present practical and analytical techniques to optimize the detection of robust biomarkers in proteomic studies. The normal sputum proteome was derived using data-independent HDMS^E applied to 40 healthy nonsmoking participants, which provides an essential baseline from which to compare modulation of protein expression in respiratory diseases. The "core" sputum proteome (proteins detected in ≥40% of participants) was composed of 284 proteins, and the extended proteome (proteins detected in ≥3 participants) contained 1666 proteins. Quality control procedures were developed to optimize the accuracy and consistency of measurement of sputum proteins and analyze the distribution of sputum proteins in the healthy population. The analysis showed that quantitation of proteins by HDMS^E is influenced by several factors, with some proteins being measured in all participants' samples and with low measurement variance between samples from the same patient. The measurement of some proteins is highly variable between repeat analyses, susceptible to sample processing effects, or difficult to accurately quantify by mass spectrometry. Other proteins show high interindividual variance. We also highlight that the sputum proteome of healthy individuals is related to sputum neutrophil levels, but not gender or allergic sensitization. We illustrate the importance of design and interpretation of disease biomarker studies considering such protein population and technical measurement variance.

KEYWORDS: *asthma, proteomics, biomarkers, U-BIOPRED, sputum, HDMS^E, unbiased, variance, allergic, neutrophil*

■ INTRODUCTION

Sputum induction is a widely applied method of sampling the epithelial lining fluid that lines the lower airways constituting the tracheobronchial tree. It enables assessment of both the cellular and extracellular environments in the lung[1−7] and is particularly useful in the study of inflammatory respiratory diseases, providing insight into the immune and structural cell populations and their secreted products. Initial studies of induced sputum focused on inflammatory cell counts and targeted quantification of soluble proteins by enzyme-linked immunosorbent assay (ELISA). Such analyses identified several induced sputum biomarkers as valuable in the description of inflammation in common chronic airway diseases including asthma and chronic obstructive pulmonary disease (COPD),[8,9] providing insight into determinants of disease severity[9] and relevant pathophysiological abnormalities, such as airway hyperresponsiveness[10] and changes in airway geometry.[11] Combined with studies of cell function such as chemotactic activity, quantification of cytokines and chemokines in the sputum fluid phase has provided a better understanding of the extent to which individual mediators contribute to inflammation, thereby providing initial stratification of respiratory disease.

Methods for global, unbiased analysis, that do not select a priori which analytes are measured, including transcriptomics, proteomics and lipidomics, appear useful for stratifying disease.[12−17] However, only a limited number of unbiased proteomic studies focusing on the lungs have been published to date. Ten years ago, we described the first sputum proteome, applying a shotgun method to an induced sputum sample from a female smoker with no detectable evidence of lung disease.[2] Since then, there have been a number of reports of this approach in COPD[18,19] including a study highlighting the utility of protein network analysis in sputum,[20] and a large study combining proteomic and transcriptomic analyses.[21] Likewise, limited studies of sputum have been performed to study asthma, and some have been relatively low throughput.[22,23] Apart from one study, by Titz et al.,[21] coverage of the sputum proteome remains low. Our previous study highlighted some overlap between sputum proteomes and proteomes of other sample types, namely bronchoalveolar lavage (BAL) and saliva. In recent years, attention has been drawn to the repeated failure of published biomarkers to translate to the clinic.[25−29] Such failure is often attributable to study design and validation, insufficient sample size and inappropriate experimental methodology. Problems with sample size are beginning to be addressed in proteomics,[30] particularly with the advent of data independent approaches such as MS^E,[31,32] which allow absolute comparison of samples without the inherent limitations associated with multiplexing, labeling or spectral counting-based quantitation,.[33−35] Despite the utility of MS^E for large clinical studies, there is very little information on the effect of measuring samples over extended periods and resulting data variability. The approach to quality control in the analysis of human BAL samples using repeated measurements and pooled samples published by the Moseley group at Duke University[36,37] is a standout example of the necessary approach required for clinical studies; however, sample sizes in these studies have been relatively small.

In the current study, we have applied state of the art quantitative HDMS^E analysis to a large set of sputum samples to advance on the sputum proteome previously reported.[2] As part of the method evaluation, we explored the impact of granulocytic infiltration of the airways, participant gender and other common demographics on the sputum proteome. Given the high prevalence of allergic sensitization to common airborne allergens (e.g., house dust mite and pollens) in the general population, we also examined how atopy, defined by sensitization to at least one common aero-allergen, affects the sputum proteome. As a key component of the study, we assessed variability in proteomic measurements and considered the impact of such variability on biomarker discovery. Using repeated measurements, pooled samples, comparison between individuals and to serum samples from the same study participants, we assessed the likely source of variability in measurements on a protein by protein basis. We discuss the impact of variability on effective sample size and statistical power for comparative studies. Finally, we have performed an in-depth analysis of tissue and cellular origins of proteins from previous proteomic studies and defined the accessible functional proteomic space using functional enrichment analysis.

■ MATERIALS AND METHODS

Study Design and Participant Characteristics

The U-BIOPRED study was performed in 14 European clinical centers with extensive experience in sputum induction and processing. The clinical study has been described elsewhere[39] and the protocol was approved by all local Ethics Review Boards. Participants gave their written informed consent for extensive characterization using routine clinical protocols, including lung function tests, assessment of sensitization to common aero-allergens, and hematological and biochemistry blood tests (reported in detail in Shaw et al., 2015[38]). Samples were stored in a central biobank (CIGMR Biobank, University of Manchester)

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where they were blinded. Identity of the samples were unblinded only after all the mass spectrometric analyses and data preprocessing had been completed.

Forty healthy individuals (mean age 36.9 years, range 18−65, 70% male), provided sputum samples considered representative of the bronchial compartment, i.e., ≤40% contaminating squamous cells (Table 1). The frequency of atopy, demonstrated by positive skin or serum IgE specific for at least one common aero-allergen test, was 32.5%. The atopic and nonatopic participants did not differ in respect of sputum cell counts, including sputum eosinophils (<2% of total inflammatory cells in all participants), blood eosinophils, and lung function. As expected, total serum IgE concentrations were higher in atopic individuals ($p < 5 \times 10^{-5}$), but, surprisingly, serum LDH was also slightly higher in the atopic participants ($p = 0.01$) while alkaline phosphatase was lower ($p = 0.01$).

Table 1. Demographics and Sputum Cell Characteristics

N	40
Age (mean ± SEM)	35.4 ± 2.3
Weight (kg) (mean ± SEM)	85 ± 2.14
BMI (mean ± SEM)	25.62 ± 0.51
Gender (F/M)	29/11
Race (% white Caucasian)	90%
Smoking history	
Ex-smokers (count, % of total)	5 (12.5%)
Atopy positive (count, % of total)	13 (32.5%)
Positive IgE Assay (count, % of total)	7 (17.5%)
Positive Skin Prick Assay (count, % of total)	12 (30.0%)
FEV1/FVC predicted % (mean ± SEM)	83.58 ± 0.38
Sputum % Neutrophil (median, range)	37.95 (2.71−88.34)
Sputum % Eosinophil (median, range)	0.00 (0.00−2.57)
Sputum % Lymphocytes (median, range)	1.22 (0.00−7.76)
Sputum % Macrophages (median, range)	60.30 (7.11−96.10)
Sputum % Squamous epithelial (median, range)	14.70 (0.00−39.20)

Sputum Induction and Processing

Sputum induction with nebulized hypertonic saline (4.5% NaCl) and sample processing were performed in accordance with the recommendations of the European Respiratory Society Task Force on induced sputum methods.[39] Uniformity of methods was ensured by all study sites using standard operating procedures (SOP) and centralized training. For consistency required for comparison with proteomes in patients with disease (asthma or COPD), all participants were premedicated with the β2-agonist, salbutamol, given as standard, to prevent excessive bronchoconstriction in patients with airways disease.

Induced Mucoid portions of the induced sputum were selected with forceps to reduce salivary contamination, weighed and solubilized at room temperature with 6.8 mM dithioerythritol (DTE) in HEPES buffered saline, added at a 4:1 w/v ratio. The solution was filtered through a 100-μm filter, centrifuged at 400*g* to remove the cell pellet, further centrifuged at 12 000*g* to remove cell debris, both at 4 °C, and stored at −80 °C. The cell pellets were processed for quantification of alive or dead respiratory cells, squamous cells and differential inflammatory cell counts (by Diff-Quick rapid Romanowsky stain); eosinophil, neutrophil, macrophage/monocyte, lymphocyte and mast cell/basophil counts were reported as percentages of total inflammatory cells, while squamous cells were reported as a percentage of total cell counts.

Protein Isolation and Preparation for Analysis

Sputum samples were thawed to room temperature before taking 100-μL aliquots for extraction of lipids using a semiautomated Bligh−Dyer protocol (Bligh and Dyer, 1959) on a robotic liquid handling platform (Freedom EVO 100; TECAN, Männedorf, Switzerland). Briefly, each sample was made up to a volume of 800 μL with 0.9% saline solution before adding 2 mL of methanol (MeOH) and 1 mL of dichloromethane (DCM) and 10 μL of antioxidant (5 mg mL^{-1} butylated hydroxytoluene in MeOH). Samples were centrifuged at 1000*g* for 10 min at 10 °C to produce protein pellets which were snap-frozen in liquid nitrogen and stored at −80 °C.

In preparation for analysis, the frozen protein pellets were thawed to room temperature, dissolved in 150 μL of 50% trifluoroethanol, 50 mM ammonium bicarbonate and heated at 60 °C for 30 min. A pool for quality control was prepared with equal protein amounts from 40 different sputum samples (including healthy participants and participants with a diagnosis of asthma). Pool samples were processed and analyzed in parallel in batches containing 11 analytical samples and one pool. Dissolved protein pellets were reduced, alkylated and digested with trypsin. Peptide samples were filtered using a 10 KDa cutoff ultrafiltration device (Millipore) and the filtrate lyophilized in vacuo. Samples were dissolved in 3% acetonitrile (ACN), 0.1% trifluoroacetic acid (TFA) in preparation for reverse phase cleanup, performed according to the manufacturer's instructions using C18 spin tips (Protea Biosciences). Following elution, peptides were lyophilized and stored at −80 °C prior to analysis.

Serum Collection and Processing

Clotted venous blood samples were centrifuged at 1000*g* for 10 min, and collected supernatants stored at −80 °C. A pooled serum sample was created as for sputum. In order to increase the number of identifications, the 12 most abundant proteins were immunodepleted using disposable agarose columns (Pierce/Thermo-Fisher) and eluates reduced, alkylated, digested and lyophilized. Peptide extracts were then resuspended in 3% ACN, 0.1% TFA and desalted using 96 well RP solid phase extraction plates (3 M Empore). Eluates were transferred to separate microcentrifuge tubes, lyophilized and stored on ice until mass spectrometry.

Mass Spectrometry

Peptide extracts were resuspended in buffer A, (3% ACN, 0.1% Formic acid (v/v) and the concentration measured using a Direct Detect System (Millipore). An internal standard mixture of *E. coli* ClpB Hi3 standard (Waters), yeast enolase (ENO) and yeast alcohol dehydrogenase (ADH) was added to a final concentration in 20 μL of 250 ng/μL sputum peptide, 12.5 fmol/μL ClpB, 12.5 fmol/μL ENO, and 8.75 fmol/μL ADH (serum was 25% more concentrated).

Samples were analyzed in duplicate, sequentially (not spread across batches), via HDMS^E on a Waters Synapt G2S high definition mass spectrometer coupled to a nanoAcquity UPLC system. 4 μL of peptide extract was injected onto a C18 BEH trapping column (Waters) and washed with buffer A for 5 min at 5 μL/min. Peptides were separated using a 25 cm T3 HSS C18 analytical column (Waters) with a linear gradient of 3−50% ACN + 0.1% formic acid over 50 min at a flow rate of 0.3 μL/min. Eluted samples were sprayed directly into the mass spectrometer operating in MS^E mode. Data were acquired from 50 to 2000 m/z with the quadrupole in RF mode using alternate low and elevated collision energy (CE) scans, resolution of 35 000. Low CE was 5 V and elevated CE ramp from 15 to 40 V. Ion mobility separation was implemented prior to fragmentation using a wave velocity of 650 m/s and wave height of 40 V. The lock mass Glu-fibrinopeptide, $(M + 2H)^{+2}$, $m/z = 785.8426$) was infused at a

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concentration of 100 fmol/μL at a flow rate of 250 nL/min and acquired every 60 s.

Database Searching and Curation

Raw data were processed using a custom package (Regression tester) based upon executable files from ProteinLynx Global Server 3.0 (Waters). The optimal setting for peak detection across the data set was determined using Threshold inspector (Waters) and these thresholds were applied: low energy = 100 counts; high energy = 30 (for serum this was set to 25) and a total energy count threshold of 750. Database searches were performed using regression tester and searched against the Uniprot human reference database (20/11/2014; 20 229 entries) with added sequence information for internal standards. A maximum of two missed cleavages was allowed for tryptic digestion and the variable modification was set to contain oxidation of methionine and carboxyamidomethylation of cysteine. Precursor and product ion mass tolerances were calculated automatically during data processing and the false discovery rate (FDR) was set at 4%. We report only proteins identified in at least two patient samples, which results in a FDR below 1%.[40] Only proteins identified in each technical replicate of at least two patient samples were considered; thus, the false positive rate is minimized, since chemical noise is random in nature and does not replicate across injections. Quantity was estimated in absolute amounts using the Top 3 method.[32,41] The ion accounting output files[42] were compiled and summary information generated from search log files using custom Python scripts. Information contained in ion accounting files were collated into a single .csv document using a custom Python script.

Data Filtering and Normalization

Protein identifications collated from the ion accounting files were further quality filtered by allowing only identifications with the following criteria: identification in at least two separate samples (not including replicate injections), a process that required at least three high quality unmodified peptides using the Top 3 method, and 2 peptides with at least 4 fragment ions for each protein. All other protein identities were removed. Proteins were first ranked according to coverage across the samples, and then each protein entry was ranked according to the order in which they were run. QC information was added for each sample (batch information, protein concentration, ion counts). First, differences in run-to-run intensity (loading) were adjusted by normalizing each run to the sum of top 3 intensities of the proteins up to the point where the sample set reached 10% missing data (we refer to this as "top-90 normalization"). ComBat was used to adjust for batch to batch variation.[43]

Inforsense software (ID Business Solutions, Guildford, UK) was applied to generated heat maps for the top 150 proteins using both "top 3 peptide intensity sum" (a proxy for concentration) and peptide concentrations (expressed in fmol) on column calculated from internal standards. Sample-wise correlation plots were created using Inferno RDN (http://omics.pnl.gov/software/infernordn).[44] Heat maps and correlation plots were inspected for poor samples or injections; those with very low or no ID's and/or poor correlation were removed from the data set.

Samples were analyzed in duplicate and the average intensity values used for analysis. For the purpose of quality control, several analyses were performed. Replicate injections were inspected for consistency in quantitation. To achieve this, an average of the two injections "top 3 peptide intensity sum" was used and a distance matrix calculated by taking the Euclidian distance between the two injections as a function of the average of the injections. These values were visualized in a heat map, enabling rapid inspection of duplicates with high variance, which likely indicated a technical issue between injections (e.g., sprayer dropout, or failure to inject the correct volume). Data were corrected by applying the following universal rule (Rule 1) for samples with >2-fold between-injection difference in average intensity of all proteins: "report injection one intensity values for all proteins, unless a specific protein was only quantified in injection two, then include this value for increasing coverage". Injection 1 was selected for consistency as it is not possible to distinguish which run more accurately represents the true abundance.

While the above method was useful in identifying whole samples with poor repeatability between injections, there were cases where the concentrations of individual proteins were highly variable. To assess these cases, a log was created using a custom script, which highlighted those proteins where the ratio between injections was >1.5. Proteins with high frequency of poor measurement stability across all samples were processed according to Rule 2: "if the variation between injections is greater than 1.5-fold, take the quantity measured using injection one". The fraction of samples where the ratio between runs had to be >1.5 was 0.5 to apply rule 2. This rule was only applied to 11 proteins in the extended proteome and to 0 proteins in the core proteome. This consistent approach to dealing with large variation in between-run protein measurements was useful in reducing technical variation in the data set, while minimizing reductions in proteome coverage. However, we recognize that there remains increased uncertainty in the measurement of proteins treated in this way, and the issue may be minimized in future studies by increased replicate measurements. Mean values were derived from replicate sample injections except for those cases where rule 1 and rule 2 were applied, and those cases where the protein was quantified in only one sample.

Data Retrieval and Conversion

Data from previous studies of relevant tissues were retrieved from the following sources: the Protein Atlas (www.proteinatlas.org),[45] the HUPO[46,47] plasma reference set, the reference plasma data set from the laboratory of Matthias Mann,[48] sputum proteomes,[2,21,24] proteomes of BAL fluid,[36,37,49−52] exhaled breath condensate,[53,54] pure airway mucus,[55] saliva,[56−61] macrophage proteomes,[62,63] eosinophil proteomes,[64−68] whole neutrophils,[69−72] investigations of neutrophil extracellular traps,[16,73,74] neutrophil microparticles,[75] and neutrophil granules.[76,77] Data were tabulated and identifiers converted to Uniprot format using the Uniprot ID mapping service (http://www.uniprot.org/mapping/) or via DAVID.[78,79] Redundant, discontinued, merged and incomplete entries (e.g., assignments to protein fragments, pseudogenes, or to nonhuman proteomes) were either disregarded or were assigned to Uniprot identifiers.

Statistical Analyses and Informatics

Statistical analyses were performed in R,[80] Inferno RDN,[44] Microsoft Excel and Minitab,[13] using parametric or nonparametric tests as appropriate. Coefficient of variation was calculated for log-normal distributions. Visualization was performed in Origin 9.1 (http://www.originlab.com/91), R, and Inforsense (IDBS). Venn diagrams were generated using Venny (http://bioinfogp.cnb.csic.es/tools/venny/).[81] Tree maps were drawn using Treemap 4.1.2 (http://www.cs.umd.edu/hcil/treemap/).[82] Pathway analysis, functional enrichment analysis and biological inference were performed using Ingenuity Pathway Analysis software (IPA, QIAGEN Redwood City). The IPA analyses were

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performed against a background gene set restricted to Ingenuity Knowledge Base genes from expected sources of sputum proteins, tissues and cell types of the lung or near the lung in *Homo sapiens*. Functional enrichment was calculated via FunRich,[83] and the Secreted Protein Database[84] was used to identify secreted proteins. Protein−protein interactions were explored using String (http://string-db.org/).[85] Ontology was annotated from retrieval via Uniprot,[86] and retrieval and enrichment analysis was performed via GOrilla (http://cbl-gorilla.cs.technion.ac.il/)[87] against the *Homo sapiens* proteome and REVIGO (http://revigo.irb.hr/).[88] The data were clustered by topological data analysis (TDA).[89−93] TDA provides geometric representation of the relationships between patient data and variables in high-dimensional data sets. TDA structures were generated using the Ayasdi Cure application (Ayasdi, Menlo Park, CA) with a norm correlation metric and two MDS lenses (resolution, 20 bins; gain, ×5.0; equalized).

Power calculations were performed using R-package size,[80] assuming two equally sized groups, to achieve 80% power to detect a given fold change (FC), at the FDR-adjusted 5% significance threshold, using a two-sided, two-sample *t* test, assuming that the percentage of true null hypotheses, "p_0", is 95% and 97.5% respectively. Equal variances were assumed for cases and controls. The open source software, variancePartition, was used to identify the drivers of protein measurement variation.[94−96]

RESULTS

Identified Proteins

A total of 4182 proteins were identified in the sputum in ≥1 individual(s): 2354 proteins in ≥2 individual(s), 284 proteins in ≥40% and 73 in ≥90% of individuals (Supplementary Figure S1). High abundance proteins were generally more frequently identified (Figure 1), but many high abundance proteins were also identified at lower frequency. These abundant proteins would be expected to be observed across multiple time points in the same patient, although there may be a proportion that are not replicated because of biological variation or where they are near the limits of detection. With consideration for these effects, we have defined the sputum proteome in two ways, the "core" and "extended" sputum proteome (Supplementary Excel File S1). The 284 proteins identified in ≥40% of participants were defined as the "core sputum proteome" and were used in the statistical analysis. The "core" proteome represents the most commonly detected proteins within the sputum samples. The cutoff was defined at ≥40%, since at this frequency of identification, the frequency vs protein rank curve was close to the point of inflection (Figure 1), where even a slight increase in the frequency of identification "cut off", significantly increased the sparsity of the data set and, hence, the total number of missing values. We also defined an "extended healthy sputum proteome" data set consisting of 1666 proteins identified in ≥3 individuals.

Impact of Gender, Age, Atopy and Granulocyte Counts

No significant differences in proteomes were observed when comparing age, atopic and nonatopic individuals or males and females (Figure S2). Furthermore, using an FDR-adjusted *t* test, only CLIP-associating protein 1, CLASP1_HUMAN, was found to be significantly different (q = 0.04) between males and females. No proteins were significantly different between atopic and nonatopic individuals (q < 0.05). The network shown in Figure S2 is constructed using multidimensional scaling (MDS) lenses (similarity metric) projected onto a TDA network,

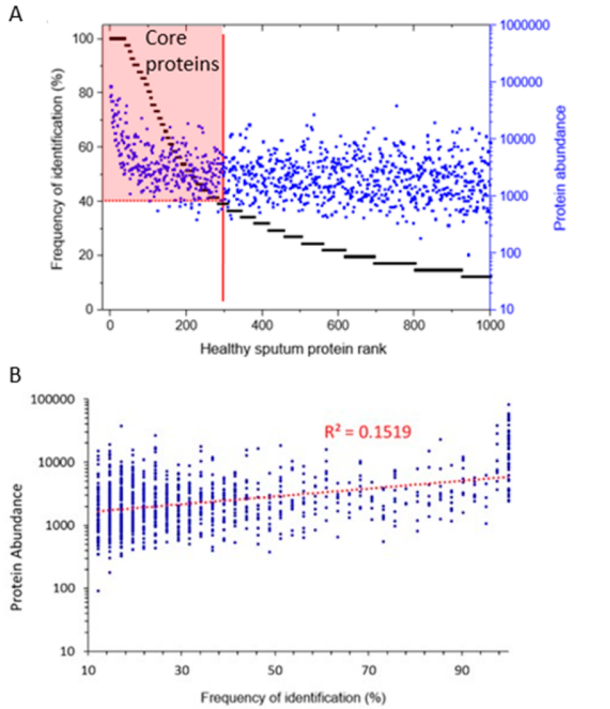

Figure 1. Defining the "core" sputum proteome from the relationship of abundance of identified proteins and frequency of identification across samples. (A) The 40% frequency of detection used as the cutoff for the "core" sputum proteome. The relationship between rank of frequency of detection and the number of proteins identified (healthy sputum protein rank) is approximately linear between 100% and 40%. This is similar to the relationship between protein abundance and rank of frequency of detection. Red lines indicate 40% cut off points for the 284 protein "core sputum proteome" and also illustrate that this level is close to the point of inflection of the curve. The "core sputum proteome" is shaded pink. At this point, increasing the coverage cut off point for analysis significantly increases the sparsity of the data set and hence the total number of missing values. (B) The intensity of protein measurement correlated weakly with the frequency of protein identification.

representing the structure of the proteomic data. This is an advanced technique for clustering data according to similarity and was used to explore the shape of the data for impacts of potential covariates. There was a large range of sputum neutrophil counts, and a small number of individuals had counts (>80%) that would be classified as neutrophilia. Compared to the other participants, these individuals had elevated levels of Neutrophil Defensin (Mann−Whitney; q = 0.02) and borderline results (q = 0.06) for neutrophil-associated proteins: leukocyte elastase inhibitor (Serpin B1), MMP9, and S100A8/9, and RHO protein GDP dissociation inhibitor. There was also a weak, but statistically significant, positive correlation between some of the major granule proteins and neutrophil counts as a % of total inflammatory cells (4 of the top 5 proteins with greatest R^2 correlation scores are shown in Figure 2). The average R^2 for correlation of proteins with neutrophil count was 0.07 and 91% of proteins had an R^2 < 0.2.

Protein Variability, Intensity Adjustment and Measurement Accuracy

Protein measurements between samples were visualized in heatmaps before and after intensity adjustment and after batch effect correction (Figure S3), allowing rapid assessment of fluctuations in instrument performance and systematic variation, e.g., sensitivity and column changes over time and between analytical batches. Effects that can be easily visualized in nonadjusted maps

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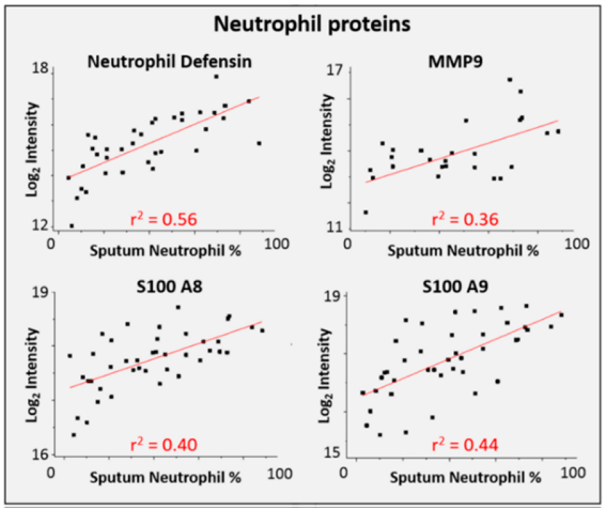

Figure 2. Neutrophil proteins and neutrophil counts. We observed relationships between neutrophil granule proteins and the proportion of neutrophils in sputum. Neutrophil proteins correlated with percentage sputum neutrophil cell counts.

(Figure S3A) were corrected by normalizing the intensity of proteins in each sample to the sum of the measured top3 intensity of the proteins for that sample, up to the level of 10% missing data across all samples; a method which we termed the "top90" method (Figure S3B). The top90 adjustment corrected for protein intensity variation more effectively than normalization to "total intensity"; avoiding effects of rarely measured protein abundances. ComBat was used to adjust for batch to batch variation (Figure S3C).

High sample to sample variability was observed in the sputum proteomes. Pooled sputum sample replicates, which were processed and analyzed at regular intervals throughout the acquisition of healthy sputum data, were compared to the participant sputum samples and the matched healthy serum data set (Figure 4). Compared to individual sputum samples, pooled samples contained higher numbers of proteins with a lower percentage Coefficient of Variation (CV %). In all data sets, the variability in protein measurements increased as the protein frequency of identification decreased. While the level of variability in sputum pools was low, it was even lower in matched serum samples, thus further indicating that the source of variability arises from the sample type rather than the instrumentation.

We can describe the variability in the sputum as emerging from heterogeneity in the population, assessed by comparing pool variance to population variance, and technical variability; sample processing and measurement in the mass spectrometer. Such technical variability can be assessed through analysis of pooled reference samples and replicate injections, respectively. Furthermore, we used the open source software, variancePartition, to identify the drivers of protein measurement variation. Heterogeneity in the population drives most variance in the measurement of the top 20 proteins with highest coverage across samples, except for IGHA1_human, Immunoglobulin heavy constant alpha 1, whose variance was most attributable to mass spectrometry running batch. Immunoglobulins are highly conserved proteins, difficult to distinguish; minor changes in the running conditions within the mass spectrometer may result in different identity assignment. Across the top 40 proteins with highest coverage across samples, 75% of variability could be attributed to heterogeneity in the population (Figure 3B).

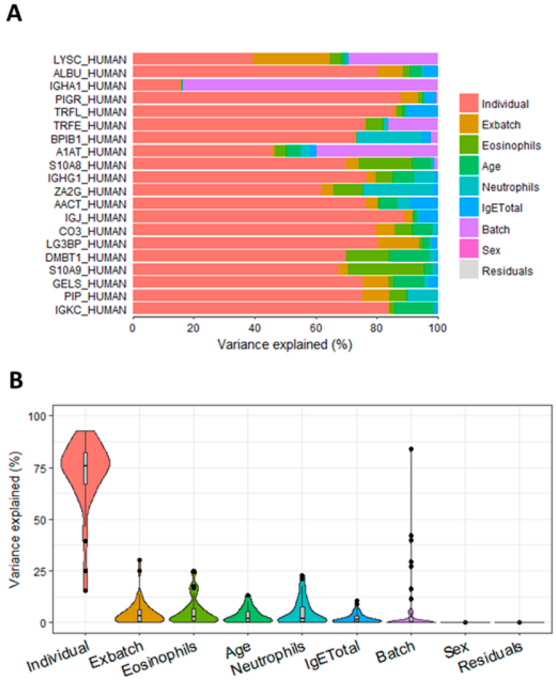

Figure 3. Sources of variability in protein measurement estimated by variancePartition. (A) The fraction of total variation in measurement of the top 20 proteins by highest coverage in samples, attributable to individual, extraction batch, eosinophil cell count, age, neutrophil cell count, total measured serum IgE, mass spectrometry running batch, sex and residuals. (B) Proteome-wide violin plot of the distribution of variance explained by each variable across the top 40 proteins by highest coverage in samples.

Proteins with high interindividual population variance are shown in Supplementary Table S1, which illustrates those proteins that had a high CV % in the healthy population but were relatively stable in the pooled samples and injection replicates. Many of the proteins with individual to individual variation are known to have roles in inflammation (S100A proteins,[97] A1AT[98]), or are likely the result of salivary contamination of samples (e.g., Amylase[99−123]).

One of the features used for assessing measurement error was injection repeatability. We defined a poor injection repeat as any protein in a given sample with >1.5-fold difference in measurement between injections. Such variation occurred in ∼6% of all quantified sputum proteins, and ∼5% of all quantified serum proteins. Variability occurred less frequently in the "core proteome region" of serum where there was only 1.2% variation across duplicate injections. However, in sputum this value increased to 8.1% (1632 of 20 412 individual quantifications) of the identified proteins (∼6% in pools). It should be noted that the majority of these poor replicators in sputum occurred where there was a lower frequency of identification (higher rank in Supplementary Tables S2 and S3).

Proteins that showed variability due to sample handling were identified by their high measurement stability in replicate injections but high variability across the pools (Supplementary Table S2). We observed a number of proteins with poor repeatability of quantification.

Some proteins are difficult to measure with good repeatability by mass spectrometry. These poor MS quantifiers showed high CV % in pooled samples and poor replication of quantification across injections. This variability in measurement is likely due to the behavior of their peptides in HDMS^{E} or errors in database

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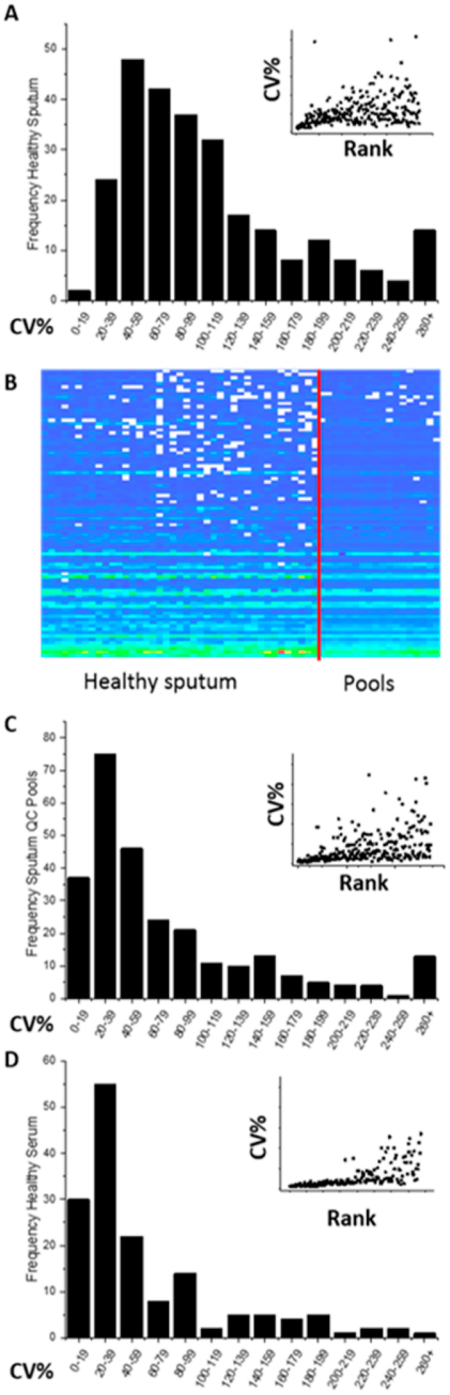

Figure 4. Variability in protein abundance measurements across samples. Frequency histograms represent: on the *x* axis CV % in increments of bin size 20, and inset scatter plots show CV % vs protein rank (proteins were ranked by order of abundance and frequency of identification across samples). Heatmap (B) illustrates Healthy sputum is highly variable from participant to participant compared to pools. Plots (A) and (C) show variability in protein abundance across healthy sputum samples and pooled samples, respectively, with the pools showing least variability across the different samples. Inset graphs illustrate the variability increase as coverage and abundance decreases. The variability seen in sputum is likely due to sample heterogeneity, and this is contrasted to serum sample measurements in plots (D), which illustrate the relative homogeneity of that fluid across study participants.

searching and quantification (e.g., due to homologues or protein to protein ambiguity) (Supplementary Table S3).

The variability in quantitation was nonuniform throughout the data set; i.e., it varied on a protein to protein basis. This observation in the samples from healthy participants, which would be used as a control group for comparison with samples from participants with disease, has far reaching implications. This is particularly so in terms of experimental design and statistical power for biomarker discovery using unbiased sampling techniques. In order to explore this phenomenon further, we performed a literature search and generated a database of potential respiratory biomarkers (Supplementary Excel File S2). The database was used to identify highly cited respiratory proteins associated with disease, and then, in a posthoc manner and using the data on variability gathered from our experiments, we performed an in silico exploratory evaluation of sample size requirements for MS-based biomarker studies for these biomarkers.

Biomarker proteins identified in more than three published studies and also identified in our study were chosen for more detailed analysis. A subset of these proteins are presented in Figure 5. Low variability in the pooled samples, but high

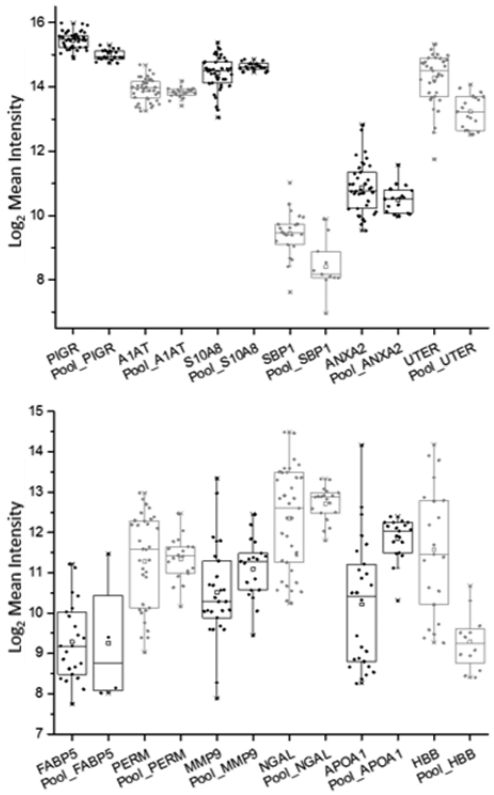

Figure 5. Distribution and variation in measurement of potential inflammatory biomarkers in the healthy population compared to pooled controls. Proteins showed varying levels of spread indicating that the sample size required for statistical power will vary significantly per analyte, with the top graph showing those proteins with lower variability and the bottom, those with higher variability. Note that the pooled samples were taken from asthmatics and nonasthmatics, and as such the means of a number of these proteins will be higher or lower in the pools depending on each protein's role in inflammatory disease. Therefore, pool samples can be used to contrast not only the measurement error but also any potential subclinical inflammatory effects in healthy participants.

variability in the population, indicated that the majority of variability arose from interindividual variation, while diverse measurements in both the pools and the population indicated that there was a likely influence of experimental variation to consider for that protein.

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Twenty-four of the proteins in the biomarker database were observed in ≥20 healthy participants in our study, which allowed a series of sample size calculations to be performed. The calculations quantified the relationship between variability of the samples, given by the standard deviation of the measurements, and the sample size required to achieve 80% power (Table 2 and Supplementary Figure S4). The sample size required for the given statistical power varied between proteins because of a combination of experimental and biological variation. This highlights that while statistical differences for some biomarkers can be reliably identified from sample sizes that are routinely used in proteomic analysis, others require very large sample sizes in order to confidently identify an effect. It is noted that in studies where patient allocation is unbalanced (e.g., 1:2 or 1:3 cases to controls), the total necessary sample size required is greater. A 1:1 allocation provides the most efficient design.

Table 2. Sample Size Per Group, To Detect 1.5- or 2-Fold Differences with 80% Power

		1.5-fold change		2-fold change	
protein	SD ($\log_2(x)$)	P0 95%	P0 97.5%	P0 95%	P0 97.5%
PIGR	0.264	9	10	5	6
BPIB1	0.303	11	12	6	6
A1AT	0.349	14	15	7	7
LYSC	0.352	14	15	7	7
ACTB	0.379	16	17	7	8
TRFL	0.478	23	26	10	11
S10A8	0.525	27	30	11	13
S10A9	0.609	36	40	14	16
CO3	0.631	38	43	15	17
SBP1	0.711	48	53	18	20
VTDB	0.714	48	53	18	20
CFAH	0.743	52	58	20	22
ANXA2	0.776	56	63	21	24
PRDX1	0.804	60	67	23	25
UTER	0.825	63	70	24	26
PEDF	0.879	72	80	26	29
B2MG	0.882	72	80	27	30
FABP5	0.956	84	94	31	34
PERM	1.166	124	137	44	49
DEF1	1.168	124	138	44	49
MMP9	1.201	131	146	47	52
NGAL	1.307	155	172	55	61
APOA1	1.588	227	252	80	88
HBB	1.609	233	259	82	91

Salivary Contamination

We compared the abundance of proteins reported to be salivary proteins against the squamous cell counts from the study participants (Supplementary Figure S5). Although salivary proteins tended to be high when the percentages of squamous cell counts were high, this was not consistent, and many participants had low squamous counts but high levels of salivary proteins.

Tissue and Cell Origins of the Sputum Proteome

Proteins found in this study have been previously observed in studies of human sputum proteomes. Comparison with studies of induced sputum by Gharib et al.,[24] Nicholas et al.,[2] and Titz et al.[21] (Figure 6A,B) showed an extensive overlap of measured proteins. Here, in the extended proteome, we found 63% of the 232 proteins identified by Gharib et al.,[24] 81% of the 171 the proteins identified by Nicholas et al.,[2] but only 31% of the 2178 proteins found in the study by Titz et al.,[21] reflecting the heterogeneity in sputum proteomes. Overlap of proteins found between two studies particularly with different methodologies increases confidence of the presence of this protein in sputum. Overlap of proteins between more than two studies further supports the identifications. The extended proteome in the current study showed the highest number of proteins shared with other studies, representing an improvement in protein identification, but also added confidence to the proteins identified in few samples by HDMS^E.

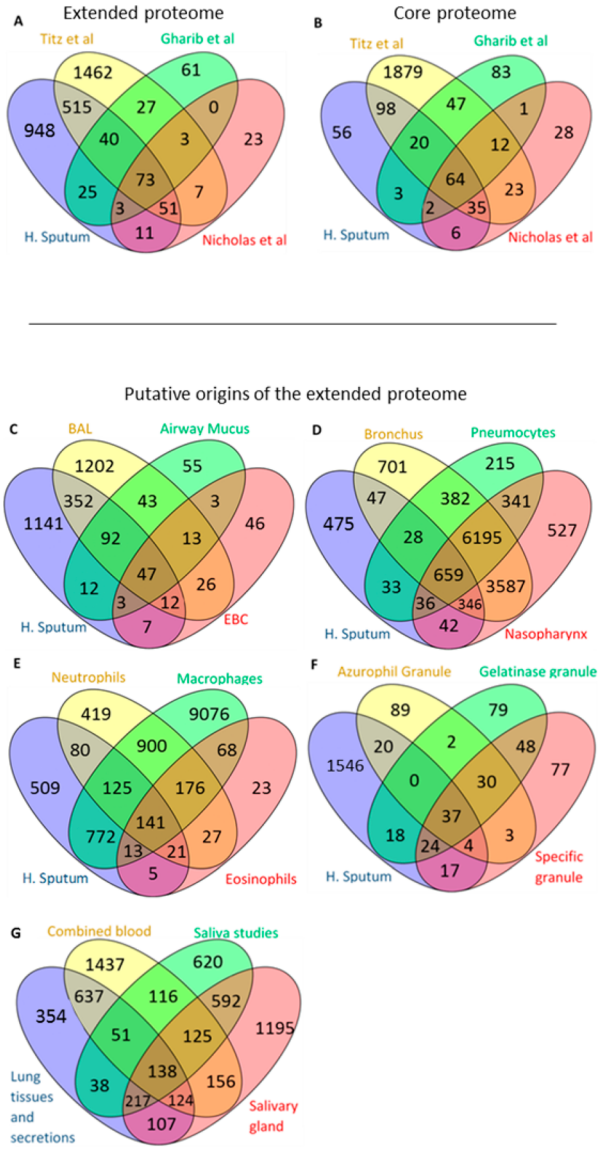

Figure 6. Comparison of coverage of U-BIOPRED sputum with other studies. Including other sputum studies (A,B), other proteomic studies of airway fluids and secretions (C), and respiratory tissues found in the protein atlas normal expression database (D). U-BIOPRED (Healthy) sputum proteome was compared to known proteomes of granulocytes and macrophages (E), and to the proteomes of neutrophil granules (F). Comparisons were also made with Saliva and Blood (G).

The overlap of proteins found between the extended sputum proteome and other tissues and cells indicates potential origins of proteins identified in this study of the sputum proteome. Sputum is a complex biofluid, consisting of proteins of multiple origins and can therefore reflect a complex biological picture. Despite sampling different airway compartments, similar patterns of

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proteome coverage were also observed when comparing airway mucus,[55] exhaled breath condensate (EBC)[53,54] and studies analyzing bronchial alveolar lavage (BAL)[36,37,49−52] (Figure 6C). However, the number of proteins identified here were only of similar magnitude to those in the BAL study. This may be partially due to improvements in sensitivity of protein measurement. Approximately 20% of proteins identified in the BAL study were also identified in the current study, which likely reflects an overlap in the high confidence detection of high abundance proteins, and a variability between studies in sampling of less abundant proteins. Results also showed extensive overlap with proteins identified from respiratory tissue analyses in the Protein Atlas[45] (Figure 6D). There was also overlap in the identifications of measured proteins to those of proteomes measured for eosinophils, macrophages and granulocyte (Figure 6E) and neutrophil granules (Figure 6F). Identification of proteins from all major tissues and cell types of the airways highlights not only the complexity of sputum as a clinical fluid, but also its utility for accessing lung biology. Such results were also reflected in our core sputum proteome, where extensive overlap was observed across a variety of tissues and/or biofluids (Supplementary Figure S6). The interpretation of these results is limited due to the differences in the sensitivity and variability of the protein measurement techniques. In most cases, the proteins identified in the studies are not tissue or cell specific, for example, the proteome of the compared macrophage data set covers half of the genome, which suggests that most of these proteins are not macrophage specific. With increasing sensitivity in protein measurement techniques, there is a corresponding increase in the proportion of low abundance proteins; therefore, overlap of proteins detected in studies reporting fewer proteins may be more useful in finding the biological origins of the proteins. In studies where fewer proteins are reported, these likely reflect high abundance, easy to detect proteins and do not include low abundance proteins that are harder to detect.

To further understand the protein composition of sputum, we also investigated possible systemic or nonrespiratory origins of our identified proteins through comparison to other relevant biofluids. We identified ∼50% overlap with proteomes of sputum, the upper airway, saliva and blood, similar to that reported previously[2,61] (Figure 6G).

Functional Analysis

Subcellular localization of proteins was analyzed using Ingenuity Pathways Analysis (IPA) (Figure 7). 27% of the proteins in the core sputum proteome were predicted to be extracellular or secreted, with a further 8% predicted to be integral membrane or cell surface proteins. 50% of identities were predicted to be cytoplasmic proteins.

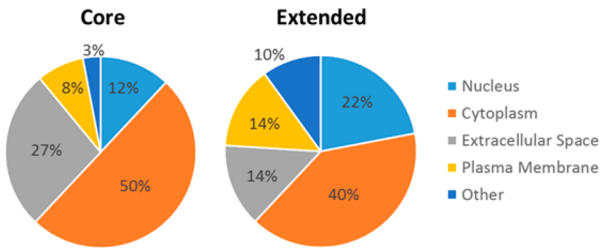

Figure 7. Subcellular localization prediction results from IPA analyses of the proteins from the core and extended sputum proteome against a background gene set restricted to Ingenuity Knowledge Base genes from expected sources of sputum proteins.

The LXR/RXR pathway refers to liver X receptor (LXR), which is activated by oxysterol ligands to bind retinoid X receptors (RXRs). Resultant LXR−RXR heterodimers bind LXR response elements and regulate expression of genes involved in inflammation, metabolism and cholesterol metabolism. The FXR/RXR pathway refers to the bile-acid concentration-mediated farnesoid X receptor (FXR) and RXR regulation of lipid metabolism. Integrin linked kinase (ILK) signaling refers to the ILK-mediated control of cytoskeleton remodelling. eNOS Signaling refers to the mechanism of nitric oxide (NO) production by the endothelial NO Synthase (eNOS).

Enrichment and pathway analyses for the core sputum proteome and extended sputum proteome were performed using Funrich, Go-Rilla and IPA. As gene ontology mapping presents a high-level of redundant terms, we utilized REVIGO to collapse and summarize like terms as treemaps. The results of these GO enrichment analyses for the core sputum proteome are shown in Supplementary Figure S7, A for cellular components, B for molecular function, and C for biological processes. Analyses of the extended sputum proteome were also performed and shown in Figure S7D,E. The size of each individual square represented in each treemap is proportional to the $-\log_{10} p$ value for the enrichment of that category, measured by Fisher's exact test. The ontologies are grouped by related terms and defined by color, providing a landscape overview of induced sputum. Enrichment was observed for vesicle related components; GORilla $q = 1.92 \times 10^{-7}$, with 111 proteins identified with the GO term "vesicle"; however, proteins have multiple GO terms and these results only suggest an enrichment of proteins originating from vesicles. Many granulocyte functions involve vesicle formation, such as the release of extracellular vesicles, some with antibacterial effect, released during spontaneous death of neutrophils.[131] Enrichment was also observed for extracellular proteins, (immune) receptor and antigen binding functions and dominated by processes involved in homeostasis, and mucosal and innate immunity. Further analysis of enriched biological pathways showed that the top IPA canonical pathways are thematically similar (Table 3), with cell migration and tissue organization (integrin linked kinase signaling, actin cytoskeleton signaling, leukocyte extravasation signaling), innate immunity (acute phase response, complement) and regulation of cytoskeleton, extracellular matrix (ECM) remodelling and inflammation (e.g., FXR/RXR (farnesoid X receptor/retinoid X receptor) or LXR (liver X receptor)/RXR; RhoA, Ras homologue A; RhoGDI, Rho GDP-dissociation inhibitor) being the most enriched pathways, and, to a lesser extent, those involved in energy metabolism (e.g., glycolysis). Similar trends were observed when exploring the association of proteins with specific functions and diseases, with inflammatory responses and immune cell migration dominating the functional enrichment categories (Table 4). These enriched pathways and functions were also conserved in the extended sputum proteome data set (Supplementary Tables S4 and S5).

Investigating protein interaction networks (http://string-db.org) in our core sputum proteome data set (Supplementary Figure S8), we observed a number of small groups of interacting protein partners and significantly one large interaction network, highlighting the large number of functional relationships that are experimentally accessible in the core sputum proteome.

■ DISCUSSION

This study provides the first large-scale analysis of sputum using MS^E, a data-independent proteomics approach. To our knowledge, it provides the most comprehensive description to date of

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Table 3. Top 20 Enriched Canonical Pathways in the Core Sputum Proteome Set

ingenuity canonical pathways	enrichment p value	total number matched proteins
LXR/RXR Activation	3.16×10^{-12}	17
Glycolysis I	2.00×10^{-11}	9
Acute Phase Response Signaling	6.31×10^{-11}	17
FXR/RXR Activation	5.01×10^{-10}	15
Gluconeogenesis I	7.76×10^{-10}	8
Actin Cytoskeleton Signaling	2.69×10^{-07}	15
ILK Signaling	2.00×10^{-06}	13
Primary Immunodeficiency Signaling	3.89×10^{-06}	7
Pyruvate Fermentation to Lactate	1.45×10^{-05}	3
Complement System	1.70×10^{-05}	6
RhoGDI Signaling	2.95×10^{-05}	11
Hematopoiesis from Pluripotent Stem Cells	3.16×10^{-05}	6
Leukocyte Extravasation Signaling	3.89×10^{-05}	12
RhoA Signaling	3.89×10^{-05}	9
Clathrin-mediated Endocytosis Signaling	5.50×10^{-05}	11
Glucocorticoid Receptor Signaling	7.08×10^{-05}	14
Cell Cycle: G2/M DNA Damage Checkpoint Regulation	8.13×10^{-05}	6
Epithelial Adherens Junction Signaling	1.69×10^{-04}	9
Coagulation System	1.90×10^{-04}	5
eNOS Signaling	1.99×10^{-04}	9

airway lining fluid proteins and, thus, significantly advances on the sputum proteome we have previously reported.[2] The study has identified 284 proteins in the core healthy sputum proteome that are reliably and repeatedly measured and 1666 proteins in the extended proteome, additionally detailing less repeatedly measured proteins that are usually also less abundant. We have assessed the biological relevance of the proteome, particularly in the context of enrichment against the wider human proteome and have highlighted that secreted proteins and vesicular components are highly enriched in this biofluid.

Proteomic studies of sputum to date have been small but, nevertheless, useful. Our previous study[2] identified 191 proteins in the sputum of a single individual using 2-dimensional gel electrophoresis and mass spectrometry, which is biased toward high abundance proteins and not amenable to high throughput analysis. Comparison of our results with sputum studies by other authors shows excellent overlap in detected proteins, but also a number of proteins unique to each study. Gharib et al., applied shotgun mass spectrometry to assess 5 healthy and 10 asthmatic participants,[24,25] and identified 254 proteins in all participants' sputum. Using a nonparametric test developed by the authors, called the spectral index,[26] they found 17 proteins whose concentrations were significantly different between asthma and health, including serpin peptidase inhibitor (SRPINA1) and secretoglobin (SCGB1A1, also known as Clara cell 10-kD protein) that were increased and decreased in concentration, respectively in asthma. Titz et al.,[21] conducted an impressive study applying LC−MS/MS of tryptic peptides labeled with Tandem Mass Tags (TMT) to sputum samples of 216 participants equally composed of healthy nonsmokers, healthy never smokers, COPD patients and current smokers. Relative quantification was achieved by sequentially measuring the proteome of demographically matched samples (1 from each cohort per run) against a pooled reference sample. They analyzed differential protein expression only in proteins detected in at least 2/3 of samples per study group and reported proteins differentially abundant between groups but did not report the total proteomes. This approach pointed to 15 proteins differentially abundant when comparing patients with COPD and current smokers and many more when compared with nonsmokers.

Differences in reported proteins between studies can be explained by differences between individual phenotypes; biological variability. Differences are also attributable to differences in sample preparation and analysis methods. Likewise, where there are similarities in techniques used, there are large overlaps in proteins reported. Highlighting this paradigm, mucins 5A and 5C and the IgGFc-binding protein, which are all major components of airway mucus,[132−134] were not identified in our study, likely due to postdigestion filtration of samples to remove large indigestible substances, e.g., mucopolysaccharides, thereby removing these proteins from the analysis.

Protein Detection and Quality Control

The study design and attention to quality control has allowed us to investigate numerous experimental and measurement effects in our data set: technical reproducibility in measurement of each protein, interindividual variability of each protein and identification of salivary contamination of sputum samples. The semi-stochastic nature of peptide sampling in MS-based proteomic approaches, even when using data independent methods, often results in highly abundant proteins being measured more reproducibly, with proteins of lower abundance being identified less frequently, leading to increased sparsity within a data set. This is most evident in larger studies, where the proportion of proteins identified across all samples is lower than in smaller studies. These effects were visible in both the serum and sputum data sets in the current study, and as such, influenced our approach to developing our "Top-90 intensity normalization" strategy. In a data matrix ranked by protein intensity and frequency of identification, the low frequency region was seen to be more variable between samples, both in the number of protein identities per sample and their intensity measurement. Such variation can influence normalization strategies based upon total MS intensity. We have found that using the region with the greatest coverage for adjusting samples, the top90 method, is an effective method of normalization for protein load and intensity.

Comparison of variability across injection replicates and pooled QC samples within the healthy sputum proteome data set allowed the precision of measurement for specific proteins to be assessed. Notably, measurements of some proteins were reproducible across samples, whereas other proteins showed poor replicability across sample replicates and pooled QC samples, indicating that these proteins are intrinsically recalcitrant to precise measurement by HDMS^{E}. As expected, there was a trend for proteins with lower intensity to show higher variability since they are generally represented by fewer peptides and are more prone to interference due to noise. In this study we chose to define a core proteome using proteins detected in ≥40% of samples representing proteins reproducibly measured. However, we recognize that the proteome presented here will require verification in future studies.

By systematically examining the variability in pools, samples and replicate injections, we were able to identify potential sources of variation as either: sample heterogeneity, sample processing effects, and MS measurement or post processing effects. Proteins that display high CV of measurement across pools, but good replication between injections, are likely to be poor quantifiers due to sample preparation and are likely to be unstable or variably modified. The majority of these are at the lower end of

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Table 4. Top 20 Enriched Functions and Diseases Found by IPA of the Core Sputum Proteome

disease and function category hierarchy	specific annotation	enrichment p value	number of matched proteins
Infectious Diseases, Inflammatory Disease, Respiratory Disease	Severe acute respiratory syndrome	1.92×10^{-09}	14
Infectious Diseases	Viral infection	4.17×10^{-09}	18
Inflammatory Response	Inflammatory response	4.40×10^{-08}	18
Connective Tissue Disorders, Inflammatory Disease, Skeletal and Muscular Disorders	Rheumatic Disease	9.30×10^{-08}	19
Connective Tissue Disorders, Inflammatory Disease, Skeletal and Muscular Disorders	Arthritis	1.30×10^{-07}	18
Cellular Movement, Immune Cell Trafficking	Leukocyte migration	2.22×10^{-07}	20
Cellular Movement, Hematological System Development and Function, Immune Cell Trafficking, Inflammatory Response	Cell movement of phagocytes	2.87×10^{-07}	16
Immunological Disease	Systemic autoimmune syndrome	3.05×10^{-07}	16
Inflammatory Disease	Chronic inflammatory disorder	5.92×10^{-07}	16
Cellular Movement, Hematological System Development and Function, Immune Cell Trafficking	Cell movement of leukocytes	6.79×10^{-07}	19
Connective Tissue Disorders, Immunological Disease, Inflammatory Disease, Skeletal and Muscular Disorders	Rheumatoid arthritis	1.02×10^{-06}	15
Cellular Movement, Hematological System Development and Function, Immune Cell Trafficking	Cell movement of myeloid cells	1.73×10^{-06}	15
Cell-to-Cell Signaling and Interaction, Hematological System Development and Function, Immune Cell Trafficking	Adhesion of immune cells	3.58×10^{-06}	13
Cellular Movement, Hematological System Development and Function, Immune Cell Trafficking, Inflammatory Response	Chemotaxis of phagocytes	5.26×10^{-06}	12
Cellular Movement, Hematological System Development and Function, Immune Cell Trafficking, Inflammatory Response	Chemotaxis of leukocytes	6.22×10^{-06}	14
Cellular Movement, Hematological System Development and Function, Immune Cell Trafficking, Inflammatory Response	Chemotaxis of myeloid cells	8.82×10^{-06}	12
Cellular Movement, Hematological System Development and Function, Immune Cell Trafficking	Cell movement of granulocytes	1.06×10^{-05}	11
Cellular Assembly and Organization	Formation of rosettes	1.45×10^{-05}	3
Organismal Survival	Organismal death	1.86×10^{-05}	7
Cellular Movement, Hematological System Development and Function, Immune Cell Trafficking, Inflammatory Response	Cell movement of neutrophils	2.08×10^{-05}	9

the coverage spectrum. Many of the variable proteins were also identified as membrane or centriolar proteins, which often contain hydrophobic protein regions that can affect protein digestion or peptide extraction efficiencies, thereby influencing the MS measurement. In a similar fashion, those proteins that were stable in the pools, but were variable in injection replicates, are likely to be poor MS quantifiers or liable to postprocessing errors. Many of these have potential homologues which could interfere with their in silico quantitation. The results of our QC analysis suggest that variation of proteins in pooled QC samples and sample replicate measurements need to be routinely assessed across sample populations for individual proteins and for different biofluids. This will be critical in developing targeted biomarker assays and designing large discovery projects.

Protein Measurement and Concentration Variability and Effect on Statistical Power

Determination of normal/healthy levels of proteins is essential for the identification of potential disease biomarkers. It is, therefore, crucial to understand both the technical and biological variation affecting measurement of this baseline proteome. In the current study, the measurements of a number of "core sputum proteome" proteins were highly stable in repeat analyses of pooled sputum samples, pointing to the robustness of the MS analysis, but interindividual variability was significant (Supplementary Table S1), in stark contrast to the low interindividual variability in serum from the same individuals.

Upon the basis of respiratory protein biomarkers previously identified from other studies and the variability of the proteins observed in our healthy population study, we calculated the sample size required to observe a specific effect against that baseline in the context of a disease setting. By parallel reference to our pooled sample measurements, we inferred how much of the variance can be attributed to processing and/or technical measurement and how much is a consequence of interindividual heterogeneity. The greater the variability in the measurement, the greater the sample size required to achieve 80% power (Table 2). For example, quantitation of modest (1.5-fold) differences for a highly variable protein, such as hemoglobin B (HBB), requires >500 patients. By contrast, differential expression of proteins such as polymeric immunoglobulin receptor may be quantifiable with as few as 20 patients. In the case of HBB, high sample-to-sample variation was observed, although variation of HBB across the pooled samples was low. As highlighted above, saliva is often a contaminant in healthy individuals who have difficulty producing an adequate sputum sample. Since saliva is susceptible to contamination with blood as a result of gum disease,[129,130] HBB may be a consequence of oral-derived blood contaminating the sputum. The other highly variable proteins, neutrophil gelatinase associated lipocalin, MMP9, and neutrophil defensin are all known to be involved in inflammation, and several of these seen in the current study correlated with sputum neutrophil numbers.

Protein biomarkers have been reported in previous respiratory studies, however, high variability in protein concentrations, in combination with small sample size[30] can result in false positive biomarker identifications. Additionally, as we have shown, the protein to protein differences in variability means that predicting sample size to sufficiently power biomarker investigations is difficult to achieve without extensive prior investigation.

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These problems are likely strong contributors to the poor record of translating many biomarkers to the clinic.[27,28]

Confounding Factors in Sputum Analysis

Previous studies have shown intermediate levels of inflammation and even tissue remodelling in healthy atopic individuals relative to those with a clear diagnosis of asthma (Djukanovic ERJ). However, in the current study, there were no significant differences between atopic and nonatopic participants, possibly because their allergic status was too mild or the allergic processes in the airways were not active at the time of sampling.

A small number of individuals displayed neutrophilia (>80% neutrophils) which was reflected by observed differences in neutrophil proteins measured in these study participants. Neutrophilic inflammation is often a hallmark of infection, disease exacerbations, or severity of chronic airway diseases like asthma and COPD,[124,125] but is often confounded by smoking status and steroid treatment, influencing neutrophil half-life, activity and migration.[126−128] A number of neutrophil proteins correlated with neutrophil cell counts (Figure 2); neutrophil defensin, S100A8, S100A9 and MMP9. Concentrations of the major neutrophil granule protein, myeloperoxidase only weakly correlated with sputum % neutrophil cell counts. Although neutrophil cell counts correlate specifically with these neutrophil derived proteins, we do not observe a large effect across the rest of the core or extended proteome as shown in Figure 3. As such, these proteins are useful biomarkers of neutrophilia while not compromising the potential identification of other disease signatures.

Salivary contamination is an inevitable confounding factor in sputum analyses, particularly in healthy individuals and other participants where sputum induction is less successful. We compared squamous cell counts with the expression of proteins found in saliva in previous studies and found mixed correlations. Squamous cell count does not perfectly reflect salivary contamination of sputum samples, however, the salivary proteins studied in these comparisons may additionally originate from other tissues, not just saliva. Further investigation is required to determine better markers of salivary contamination in sputum.

Origins and Biological Context of the Sputum Proteome

We investigated the predicted subcellular localization of the proteins from the core and extended sputum proteomes (Figure 7 and S7). A large portion of the proteins measured in the sputum proteome were reported to be cytoplasmic by GOrilla analysis. Since our analysis is of the supernatant from induced sputum, where cells and cell debris are removed prior to analysis, this was expected. In addition, the supernatant would also include granulocytes, which contain granules of cytoplasmic proteins for release into the sputum.

When comparing the sputum proteome in the current study with those from studies of airway tissue and fluid samples, significant numbers of identified proteins were seen to overlap. The number of proteins for which a potential cell or tissue origin could not be assigned was low. Significant numbers of the identified proteins likely have origins in other tissues of the body: for example, acute phase proteins are produced largely in the liver and enter the lungs via capillaries together with other plasma proteins. Functional analysis of the healthy sputum proteome identified proteins associated with innate immune defense, inflammatory responses via complement and acute phase proteins, phagocytic cells (macrophages and neutrophils) and, to a lesser extent, eosinophils.

A number of signaling pathways were enriched in sputum, including LXR, a member of the nuclear receptor family of transcription factors that are closely related to nuclear receptors, such as the peroxisome proliferator-activated receptors (PPARs), FXR, also known as bile acid receptor, RXR, that is activated by retinoic acid. FXR/RXR are known to be important in macrophage lipid metabolism and inflammation.[133,135,136] Such regulatory pathways have complex roles in inflammatory biology: FXR inhibits inflammation,[137] while LXR agonists have been shown to increase airway reactivity and smooth muscle growth in an asthma model.[138] LXR has also been implicated in counter-regulation of toll like receptor induced inflammatory responses,[139,140−142] which are involved in inflammatory respiratory diseases.[146,147] We also observed an enrichment of the RhoA and RhoGDI signaling pathways, known to be involved in hyper-responsiveness in asthma,[143,144] and superoxide generation in macrophages,[145] respectively.

An interesting observation was the significant enrichment of vesicles and vesicular components in the sputum (Figure S7). These small membraned particles, either described as exosomes (nanovesicles) or ectosomes (microparticles), are released by multiple cell types, including immune cells, and have been reported in sputum and lung secretions.[146] Secretory vesicles have recently become an area of interest for their potential pro and anti-inflammatory functions.[146] In response to nonspecific complement mediated inflammation neutrophils produce ectosomes that are coated in and loaded with proteins often associated with granules.[148] In addition to neutrophil derived vesicles, antigen-loaded exosomes from mast cells, dendritic cells, epithelial cells and T lymphocytes have been highlighted as being potentially important for allergy,[149] and eosinophils produce cytokine containing vesicles, e.g., degranulation, that may also be important for asthma (reviewed in Spencer et al.[150]).

Secretory vesicles contain high levels of cytoplasmic proteins.[77] For example, exosomes isolated in the BAL of asthmatics are enriched for inflammatory leukotriene production[149] and may help explain the large numbers of cytosolic proteins we have identified in sputum. In addition to the protein loading capacity of these vesicles, functions involving the transport of nucleic acids have also been identified. For example, microRNA-loaded vesicles have been highlighted as potentially important in asthma and inflammatory lung disease signaling,[151] and have been shown to be different between BAL samples of healthy and asthmatic individuals.[152]

■ CONCLUSIONS

The mapping of the healthy human sputum proteome in the current study has considered experimental and technical variability, population variance driven by daily exposure of the lung to the external environment, and sample complexity due to multiple potential protein origins, effects of cellular composition and potential for contribution of vesicular components. Functionally, homeostasis and defense mechanisms dominate the measured sputum proteome. The specific experimental and technical variability of the applied methodology must not be underestimated, and there are implications for minimum sample sizes required for determining differential abundance between groups with statistical power. The comprehensive approach we have used for this analysis of the healthy sputum proteome provides a good comparator data set for proteomes from patients suffering from inflammatory lung disease.

■ ASSOCIATED CONTENT

Supporting Information

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Figures S1−S8, Tables S1−S5 (PDF)
Proteins of the core and extended sputum proteomes (XLSX)
Database of potential respiratory biomarkers (XLSX)

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Notes

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■ ABBREVIATIONS

U-BIOPRED, unbiased biomarkers predictive of respiratory disease outcomes; HDMSE, high definition mass spectrometry; COPD, chronic obstructive pulmonary disease; CE, collision energy; BAL, bronchoalveolar lavage

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Supporting Information

Large-scale label-free quantitative mapping of the sputum proteome

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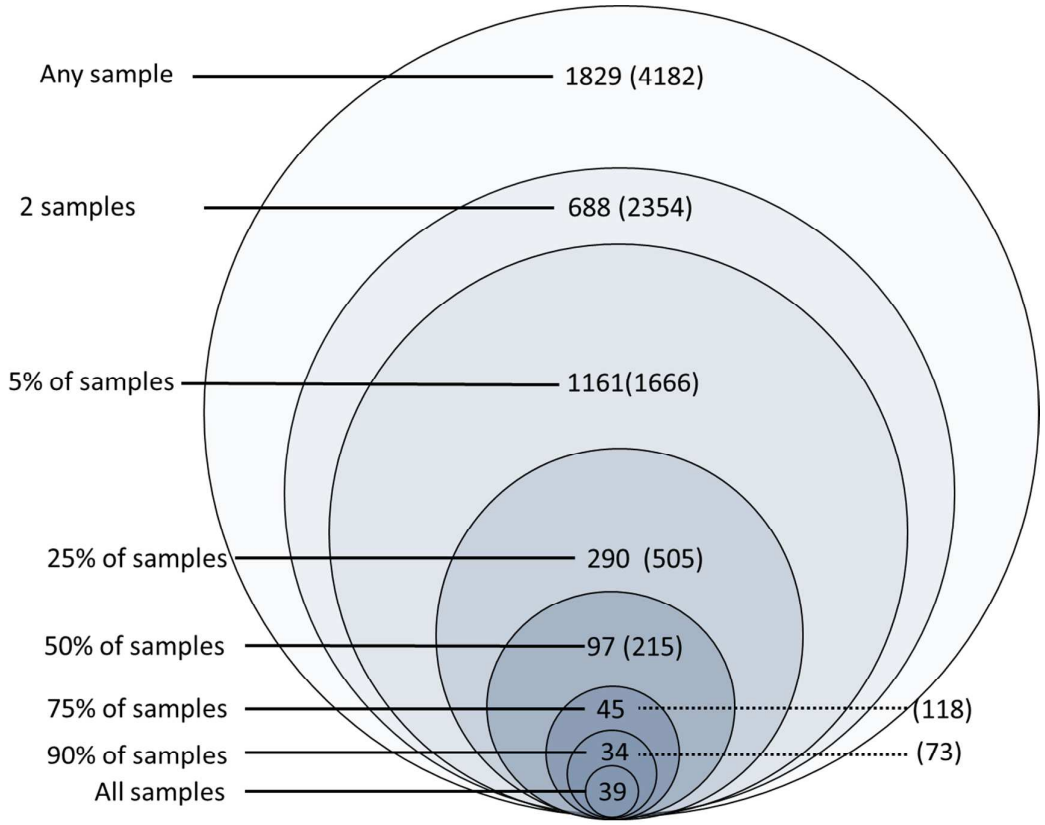

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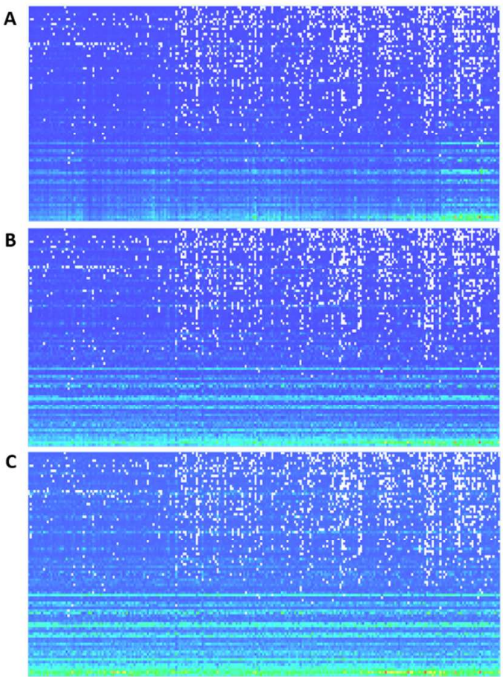

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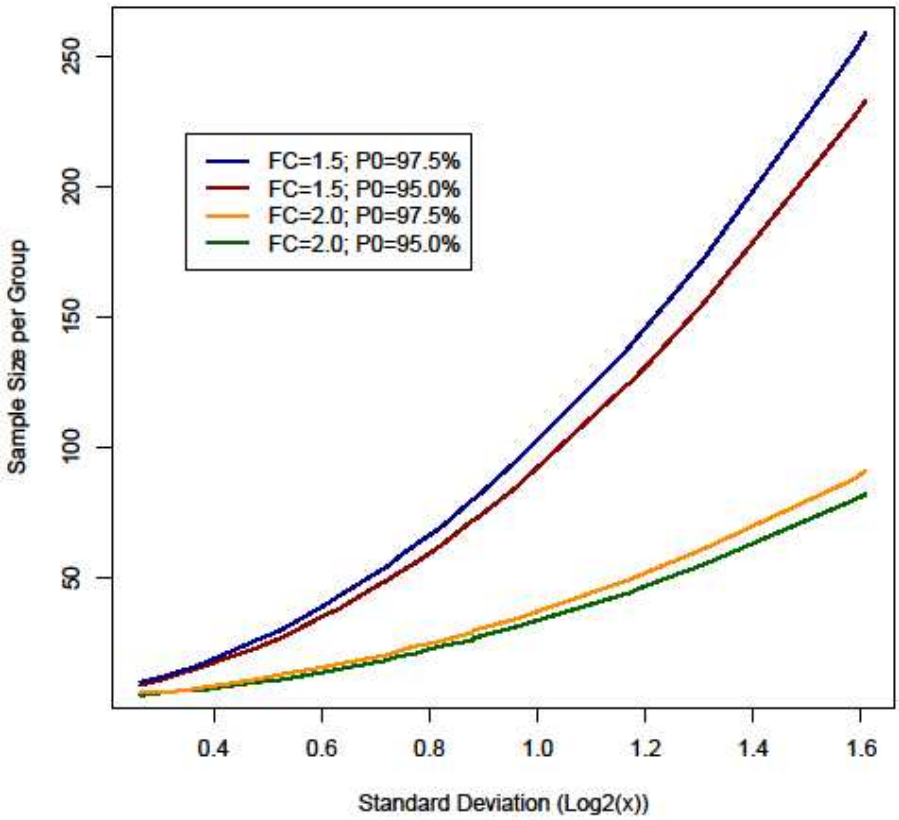

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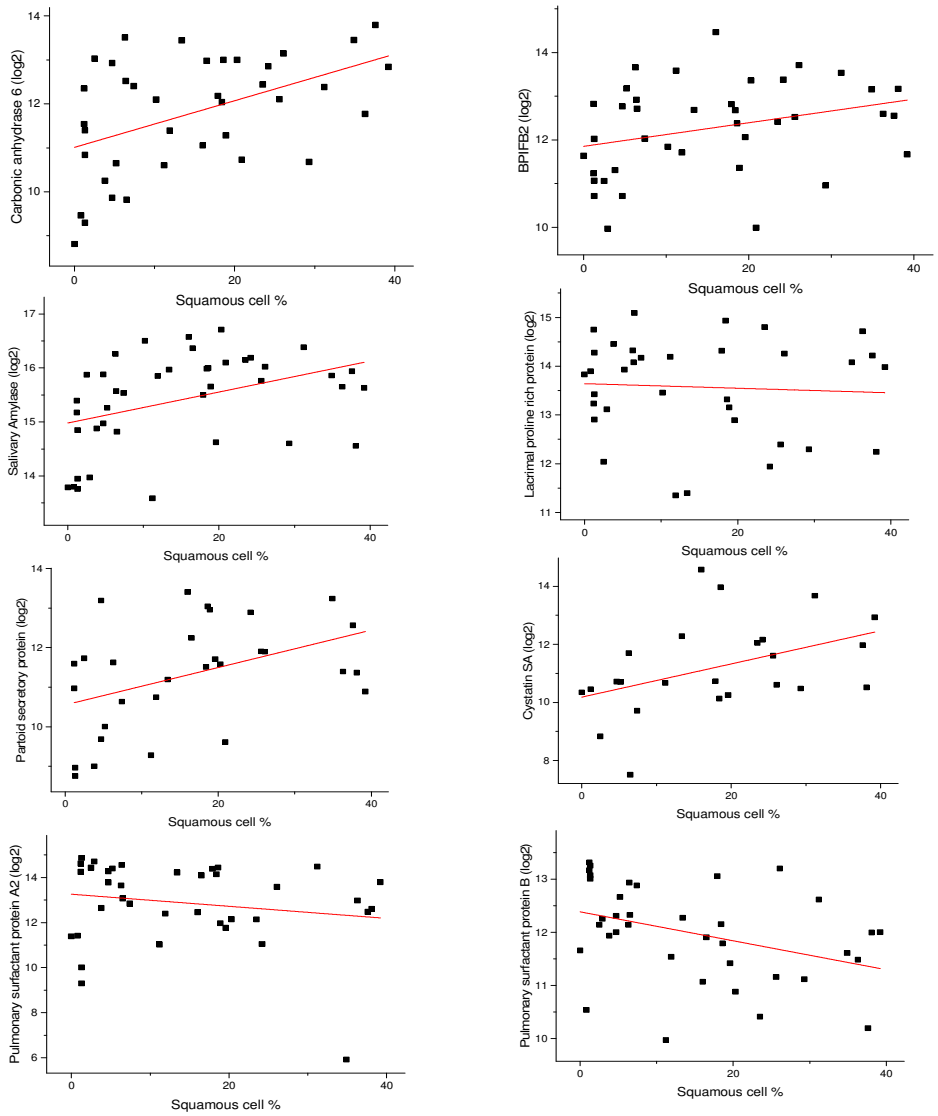

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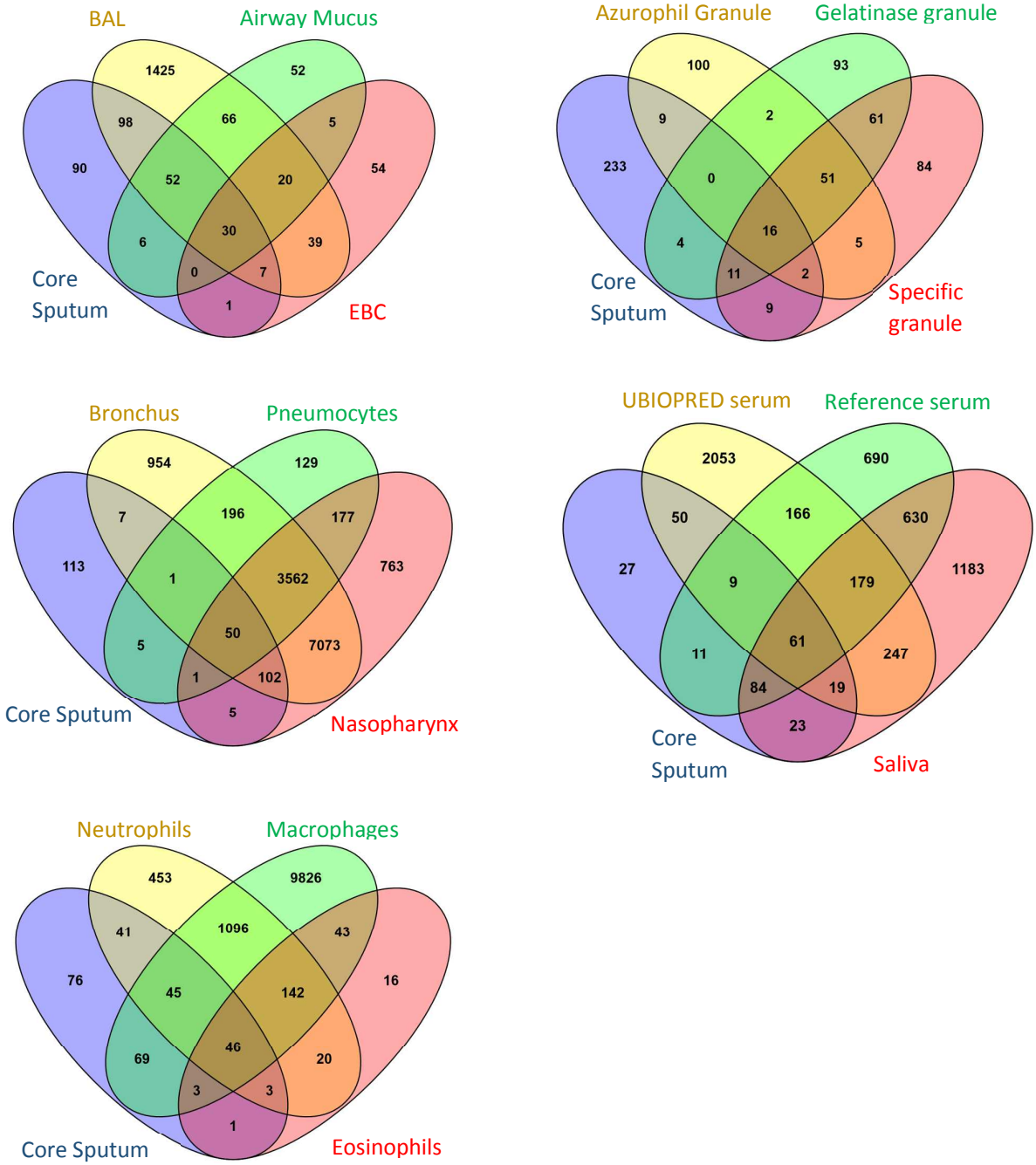

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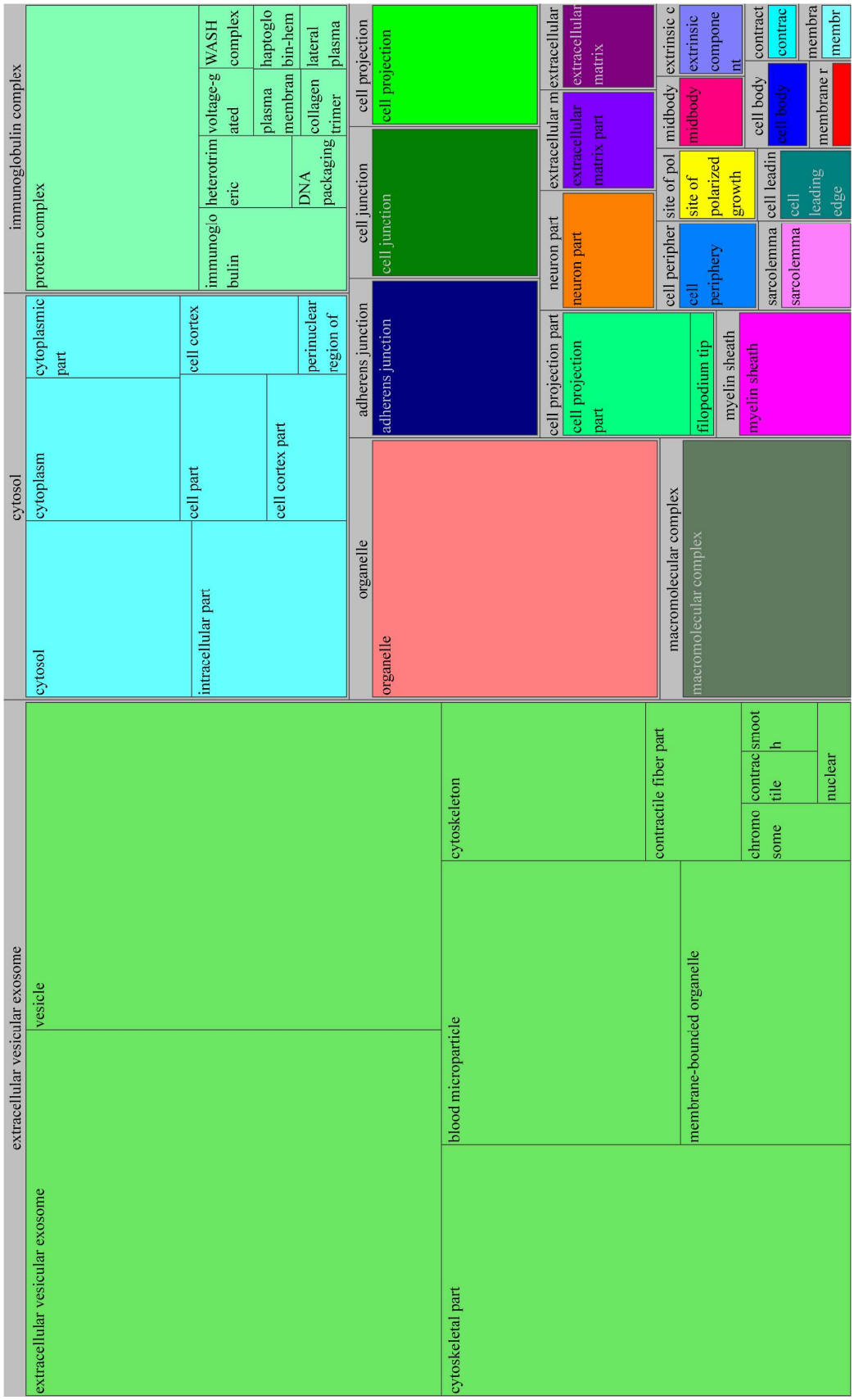
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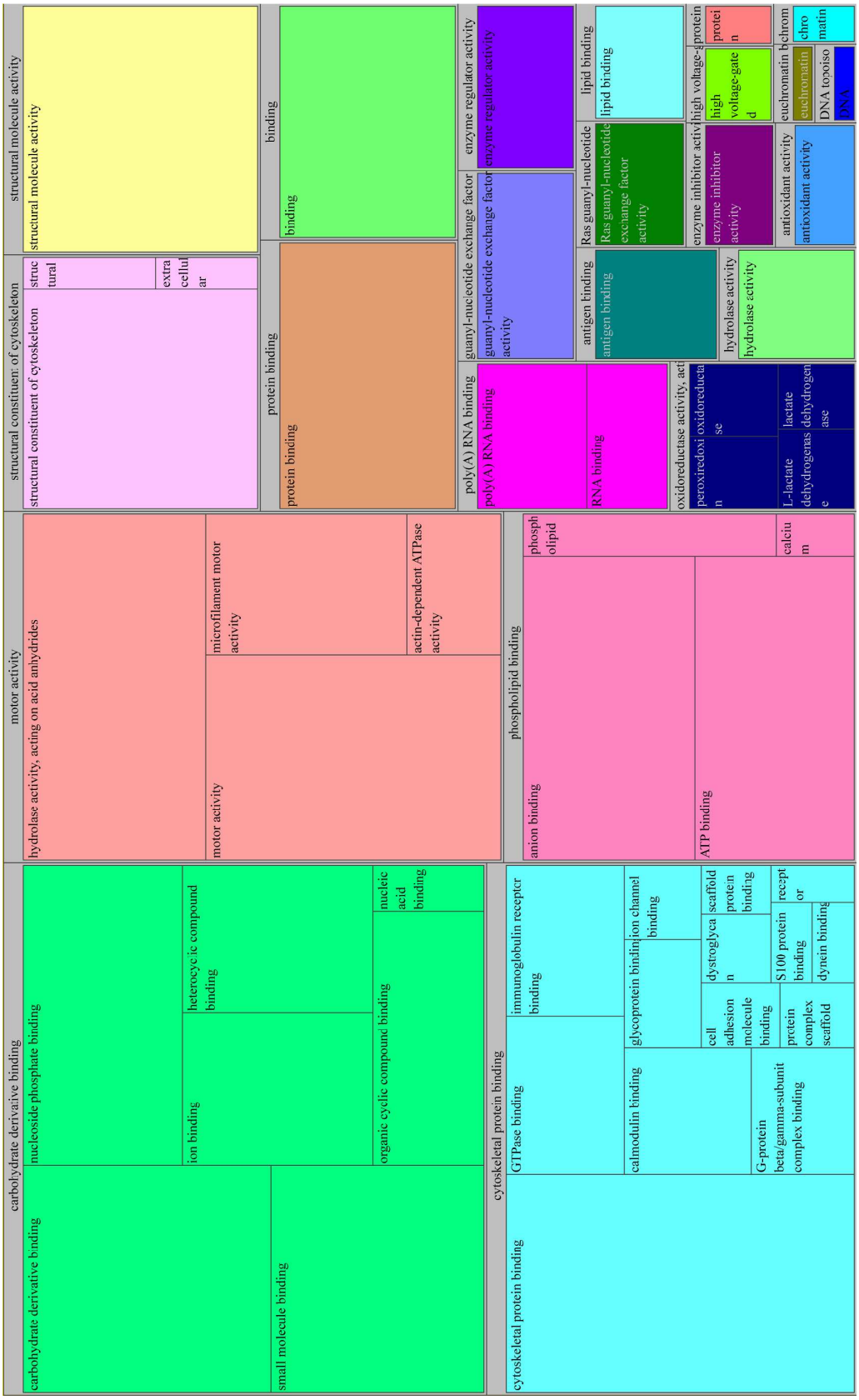
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glycoprotein binding
ion channel binding
G-protein beta/gamma-subunit complex binding
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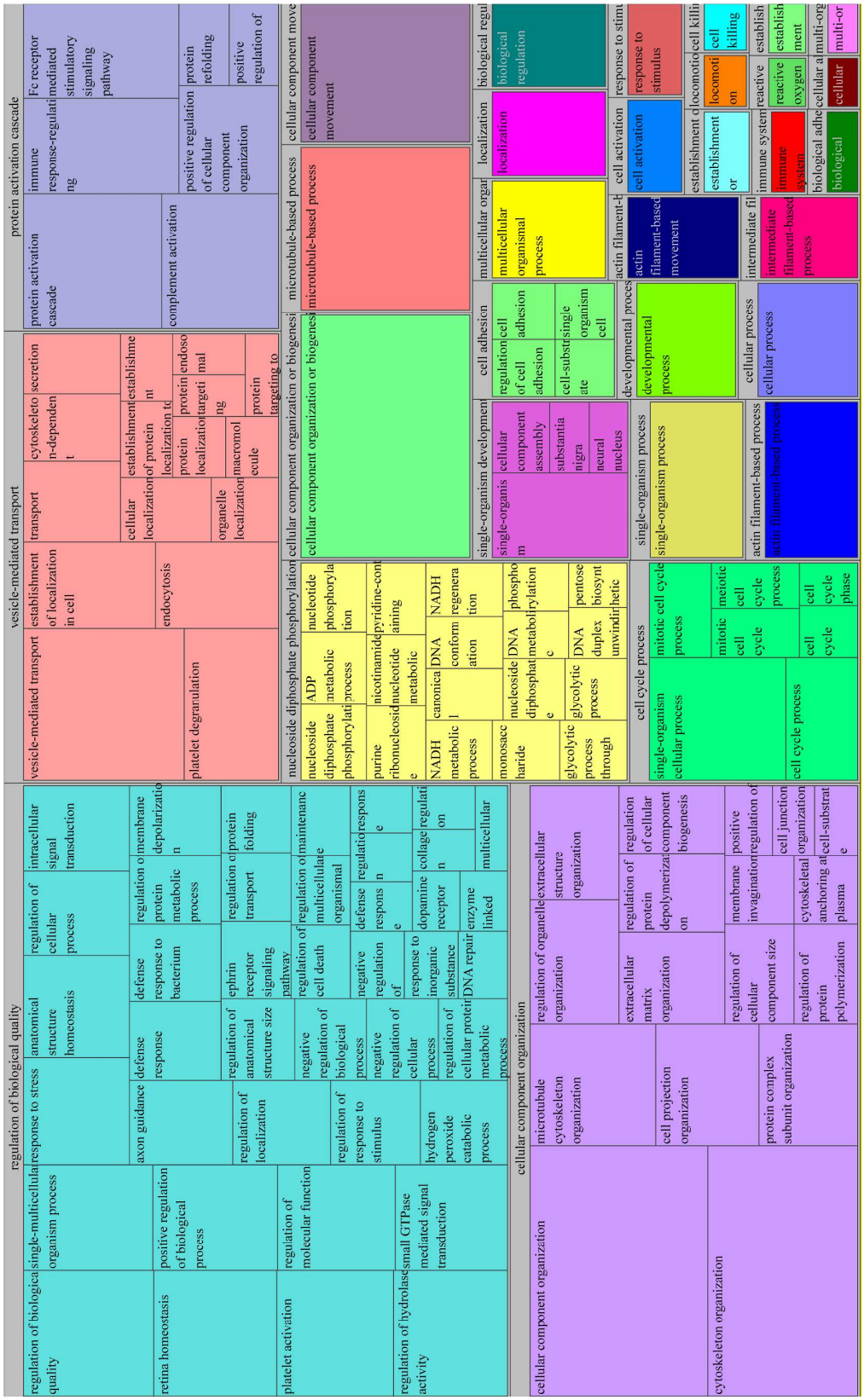

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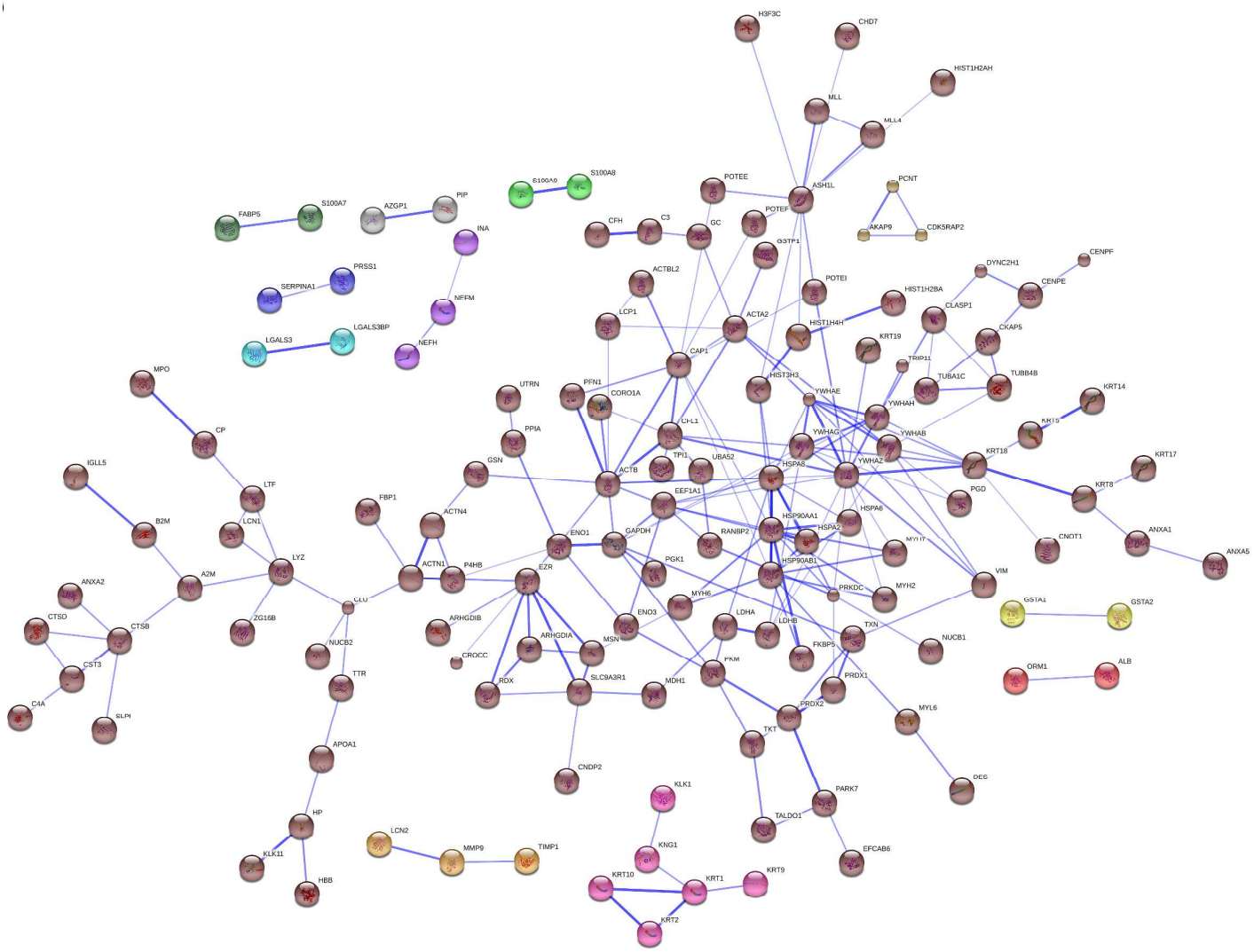

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Supplementary Table S1. Proteins with high population variance compared to measurement variance

UNIPROT ID	Protein name	pool CV %	Healthy Sputome CV %
S10A8_HUMAN	Protein S100A8 (Calgranulin-A)	7.978	365.521
HPT_HUMAN	Haptoglobin	9.576	651.593
AACT_HUMAN	Alpha antichymotrypsin	10.276	331.670
AMY1_HUMAN	Salivary Amylase	10.787	525.235
FETUA_HUMAN	Alpha-2-HS-glycoprotein	11.355	384.569
S10A9_HUMAN	Protein S100A9 (Calgranulin-B)	12.240	256.063
ILEU_HUMAN	Leukaocyte elastase inhibitor (SERPINB1)	14.431	338.942
CYTS_HUMAN	Cystatin-S	16.125	536.631
IGHM_HUMAN	Ig mu chain C region	16.305	404.459
PLSL_HUMAN	Plastin-2	16.830	206.644

These proteins are likely biologically relevant. Many of these are listed in the biomarker summary database. Supplementary excel file 1

Supplementary Table S2. Proteins with poor repeatability due to sample processing

Protein	Rank	Sample Coverage %	pool CV%	% poorly replicated pool	% poorly replicated sputome	Homologues?	Comments
A1ATR_HUMAN	241	43.6	425.12	**0.00**	12.84	No proteins with homolgous regions in search database	
SKT_HUMAN	234	44.8	266.94	**0.00**	11.61	No proteins with homolgous regions in search database	
TRRAP_HUMAN	246	42.8	241.75	**0.00**	13.08	No proteins with homolgous regions in search database	Nuclear
K1671_HUMAN	255	41.2	222.14	**0.00**	14.56	No proteins with homolgous regions in search database	
CNTLN_HUMAN	249	41.6	213.01	**0.00**	11.54	No proteins with homolgous regions in search database	Centrosome, centriole
MYH11_HUMAN	272	40	200.40	**0.00**	**0.00**	Multiple Myosin family members	
BASP1_HUMAN	124	67.2	196.75	5.26	1.79	No proteins with homolgous regions in search database	membrane protein
1433G_HUMAN	237	44	190.22	**0.00**	10.91	Similar to other 14-3-3 proteins but homology with 14-3-3F	
LDHB_HUMAN	87	80.8	180.97	9.52	4.46	Homologous regions to LDHA see below	
K1C18_HUMAN	252	41.2	169.52	17.65	10.68	Some proteins with high identity but minimal-no tryptic homology	
HPTR_HUMAN	260	40.8	163.28	**0.00**	10.78	Homologous regions with haptoglobin	
TRNK1_HUMAN	266	40.4	158.58	**0.00**	10.89	No proteins with homolgous regions in search database	
1433F_HUMAN	254	41.2	154.77	**0.00**	0.97	Similar to other 14-3-3 proteins but homology with 14-3-3E	
K1C10_HUMAN	89	79.2	145.96	4.55	3.54	Similar to other keratins. Some tryptic regions of homolgy	
LDH6A_HUMAN	171	56	126.66	**0.00**	12.14	Homologous regions to LDHA and B proteins see above	

Proteins that display high CV of measurement across pools but good replication between injections are likely to be poor quantifiers due to sample preparation and are likely to be unstable or variably modified. Many of these have potential homologues which could interfere with their quantitation. The majority of these are at the lower end of the coverage spectrum.

Supplementary Table S3. Poor quantifying proteins in the mass spectrometer

Protein	Rank	Sample Coverage %	pool CV%	% poorly replicated pool	% poorly replicated sputome	Homologues?	Comments
POTEE_HUMAN	40	95.6	184.42	50.00	41.00	Multiple POTE family members	
GOGB1_HUMAN	132	64	140.09	18.75	33.75	No proteins with homolgous regions in search database	Membrane protein
PCNT_HUMAN	145	60.8	73.69	17.65	32.89	No proteins with homolgous regions in search database	Centrosome, centrioles, cilia
MYH6_HUMAN	189	51.6	146.93	42.86	32.56	Multiple Myosin family members	
HS90B_HUMAN	97	76.8	129.94	50.00	32.29	Multiple proteins with homologous regions	
MYH7_HUMAN	169	56.4	571.91	18.75	31.21	Multiple Myosin family members	
MYH7B_HUMAN	209	48.8	130.44	21.43	29.51	Multiple Myosin family members	
GOGA4_HUMAN	120	68	49.90	33.33	29.41	No proteins with homolgous regions in search database	Membrane associated
MYH4_HUMAN	232	45.2	135.92	15.38	28.32	Multiple Myosin family members	
TMPSD_HUMAN	170	56	149.17	20.00	27.86	No proteins with homolgous regions in search database	Membrane protein
CE290_HUMAN	146	60.8	445.27	26.67	27.63	No proteins with homolgous regions in search database	Centrosome, cilia
MYH3_HUMAN	222	47.2	128.56	20.00	27.12	Multiple Myosin family members	
ACTN3_HUMAN	263	40.4	402.26	53.33	26.73	Multiple actinin family members	
CK5P2_HUMAN	155	59.2	270.85	17.65	26.35	No proteins with homolgous regions in search database	Centrosome, centrioles
H90B2_HUMAN	175	55.2	979.89	20.00	25.36	Multiple proteins with homologous regions	

Proteins that display high CV of measurement across pools and high levels of poor replication between injections are likely to be poor quantifiers in the MS instrument. Many of these are from hydrophobic compartments or have potential homologues which could interfere with their quantitation. The majority of these are at the lower end of the coverage spectrum.

Supplementary Table S4. Top Canonical pathways enriched in the extended Healthy sputum proteome.

Ingenuity Canonical Pathways	Enrichment p-value)
Actin Cytoskeleton Signaling	2.51189E-22
Epithelial Adherens Junction Signaling	3.16228E-19
Remodeling of Epithelial Adherens Junctions	1.99526E-14
Signaling by Rho Family GTPases	7.94328E-14
RhoGDI Signaling	3.16228E-13
Cellular Effects of Sildenafil (Viagra)	1.25893E-12
RhoA Signaling	1.38038E-10
ILK Signaling	2.23872E-10
LXR/RXR Activation	2.69153E-10
Germ Cell-Sertoli Cell Junction Signaling	5.49541E-10
Ephrin B Signaling	2.29087E-09
Glycolysis I	8.91251E-09
14-3-3-mediated Signaling	4.16869E-08
Regulation of Actin-based Motility by Rho	4.7863E-08
Sertoli Cell-Sertoli Cell Junction Signaling	9.54993E-08
Gap Junction Signaling	1.20226E-07
FXR/RXR Activation	1.23027E-07
Breast Cancer Regulation by Stathmin1	2.75423E-07
Thrombin Signaling	3.16228E-07
Virus Entry via Endocytic Pathways	4.36516E-07

Supplementary Table S5. Top function and disease representatives in the extended healthy sputum proteome.

Diseases and Functions Category heirarchy	Diseases or Functions Annotation	Enrichment p-Value
Infectious Diseases, Inflammatory Disease, Respiratory Disease	Severe acute respiratory syndrome	1.02E-06
Cellular Movement, Immune Cell Trafficking	Leukocyte migration	2.43E-05
Infectious Diseases	Viral Infection	3.76E-05
Cellular Movement, Hematological System Development and Function, Immune Cell Trafficking	Cell movement of leukocytes	4.84E-05
Cell-To-Cell Signaling and Interaction	Response of granulocytes	1.30E-04
Inflammatory Response	Inflammatory response	1.94E-04
Cell-To-Cell Signaling and Interaction, Cellular Compromise, Cellular Function and Maintenance, Inflammatory Response	Respiratory burst of granulocytes	5.22E-04
Free Radical Scavenging	Generation of reactive oxygen species	7.85E-04

Cell-To-Cell Signaling and Interaction, Hematological System Development and Function, Inflammatory Response	Immune response of neutrophils	9.60E-04
Cell-To-Cell Signaling and Interaction, Cellular Compromise, Cellular Function and Maintenance, Hematological System Development and Function, Inflammatory Response	Respiratory burst of neutrophils	1.04E-03
Cell-To-Cell Signaling and Interaction, Hematological System Development and Function, Immune Cell Trafficking	Binding of antigen presenting cells	1.14E-03
Cell-To-Cell Signaling and Interaction, Hematological System Development and Function, Immune Cell Trafficking	Adhesion of granulocytes	1.16E-03
Organismal Survival	Organismal death	1.16E-03
Cell-To-Cell Signaling and Interaction, Hematological System Development and Function, Immune Cell Trafficking, Inflammatory Response	Binding of macrophages	1.21E-03
Cellular Movement, Hematological System Development and Function, Immune Cell Trafficking, Inflammatory Response	Chemotaxis of leukocytes	1.81E-03
Cellular Movement	Chemotaxis of cells	1.92E-03
Cell-To-Cell Signaling and Interaction, Cellular Growth and Proliferation, Hematological System Development and Function	Stimulation of leukocyte cell lines	2.13E-03
Cellular Movement, Hematological System Development and Function, Immune Cell Trafficking, Inflammatory Response	Chemotaxis of natural killer cells	2.35E-03
Cell-To-Cell Signaling and Interaction, Hematological System Development and Function, Immune Cell Trafficking	Adhesion of immune cells	2.45E-03
Cellular Assembly and Organization	Formation of rosettes	3.05E-03