Airway inflammation and dysbiosis in antibody deficiency despite the presence of IgG

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Clinical implications statement

Antibody-deficient patients often suffer chronic respiratory symptoms. We demonstrate that, even with normal imaging and despite good levels of IgG in sputum, these patients have significant airway inflammation and dysbiosis.

Keywords: Immunodeficiency, immunoglobulin, sputum, respiratory tract, microbiome, inflammation

Abbreviations: CVID: Common variable immunodeficiency, FEV1: forced expiratory volume in 1 second, IC: immunocompetent, ID: Immunodeficient, IL: interleukin, MMP: matrix-metalloproteinase, PID: Primary antibody deficiency, RT: Respiratory tract, SID: Secondary immune deficiency, XLA: X-linked agammaglobulinaemia

ABSTRACT

Background: Patients with antibody deficiency suffer chronic respiratory symptoms, recurrent exacerbations and progressive airways disease despite systemic replacement of Immunoglobulin G. Little is known about the respiratory tract biology of these patients.

Objective: To measure immunoglobulin levels, inflammatory cytokines and mediators of tissue damage in serum and sputum from patients with antibody deficiency and healthy controls; to analyse the respiratory microbiome in the same cohorts.

Methods: We obtained paired sputum and serum samples from 31 immunocompetent subjects and 67 antibody deficient patients, the latter divided on computed tomography scan appearance into 'abnormal airways' (bronchiectasis or airway thickening) or 'normal airways'. We measured inflammatory cytokines, immunoglobulin levels, neutrophil elastase, matrix-metalloproteinase-9, urea, albumin and total protein levels using standard assays. We employed V3-V4 region 16S sequencing for microbiome analysis.

Results: Immunodeficient patients had markedly reduced Immunoglobulin A in sputum but higher concentrations of Immunoglobulin G compared to healthy controls. Inflammatory cytokines and tissue damage markers were higher in immunodeficient patients, who also exhibited dysbiosis with over-representation of pathogenic taxa and significantly reduced alpha diversity compared to immunocompetent individuals. These differences were seen regardless of airway morphology. Sputum matrix metalloproteinase-9 and elastase correlated inversely with alpha diversity in the antibody deficient group, as did sputum Immunoglobulin G, which correlated positively with several inflammatory markers, even after correction for albumin levels.

65 **Conclusion:** Patients with antibody deficiency, even with normal lung imaging, exhibit
66 inflammation and dysbiosis in their airways despite higher levels of Immunoglobulin G
67 compared to healthy controls.

68 **Capsule summary:** Persistent airway inflammation and dysbiosis despite adequate IgG
69 replacement highlights the need for better therapeutics to maintain lung health in antibody
70 deficient patients.

71

INTRODUCTION

Despite adequate substitution with systemic immunoglobulin G (IgG), recurrent respiratory tract infections and chronic respiratory symptoms constitute a significant disease burden in patients with primary and secondary antibody deficiency [1,2]. Although systemic IgG replacement therapy has significantly reduced mortality and severe bacterial pneumonia in these patient populations, repeated cycles of infection and inflammation drive chronic lung disease leading to bronchiectasis and lung function decline [1,3]. Quality of life in primary immunodeficiency conditions correlates closely with respiratory involvement, with both airflow obstruction and respiratory exacerbation frequency having an important impact [4].

Recent observational studies have documented this residual respiratory disease burden and have investigated putative causes [5]. Besides more obvious risk factors like exposure to young children, low systemic levels of IgG, IgA and IgM were associated with increased infective burden in the airways. However, information on immunoglobulin levels in the respiratory tract itself and associations with both the characteristics of respiratory tract microbiota and clinical disease severity is scarce. In one study, the presence of IgM in serum and sputum in otherwise hypogammaglobulinaemic patients was shown to associate with reduced microbial burden, specifically of *Haemophilus influenzae*, as well as a lower incidence of acute airway infections[6].

In this study, we aimed to understand the respiratory tract biology in a large cohort of patients with primary and secondary antibody deficiency (immunodeficient ID), receiving long-term intravenous or subcutaneous immunoglobulin replacement. The cohort was compared to healthy donors (immunocompetent, IC) with regard to immunoglobulin levels, markers of inflammation and tissue remodeling as well as microbial diversity.

METHODS

Patients and Clinical data

Patients were recruited between September 2017 and March 2019 when they attended the department for routine clinic visits or immunoglobulin infusions. Patients were eligible for the study if they were aged ≥18 years and had any diagnosis of antibody deficiency made by a consultant immunologist. Data were collected on immunodeficiency diagnosis, the route and dose of immunoglobulin replacement therapy, the most recent serum IgG trough level and C-reactive protein concentration, and the use of prophylactic antibiotics and inhaled corticosteroids. Patients were asked to report respiratory infection frequency over the past year and their smoking habits. From the lung function tests performed closest to the time of sampling, we collected percentage predicted forced expiratory volume in 1 second (FEV1%). We also classified patients on the basis of their most recent computed tomography (CT) scan as having 'abnormal airways' or 'normal airways' (presence or absence of bronchiectasis or widespread airway thickening as diagnosed by a consultant radiologist).

At the RFH Immunology Department, patients on immunoglobulin replacement therapy are usually targeted to a minimum IgG trough level of 8 g/L, or 10 g/L in patients with X-linked agammaglobulinaemia (XLA).

Sputum Collection and Processing

While there are readily available methods for quantifying antibody isotypes in serum of individuals, the sampling of the respiratory space is not well standardized. Here we focused on spontaneous and induced sputum from immunodeficient patients (ID) and immunocompetent controls (IC) respectively, as opposed to bronchoalveolar lavage, saliva collection or airway brushing. Sputum sampling as defined in the European Respiratory Society guidelines[7] was practical to obtain suitable samples representative of the epithelial lining fluid in the conducting airways. Sputum samples reflect lower airway inflammation;

although viable cell counts may be lower in spontaneous compared to induced sputum, the concentrations of inflammatory markers such as Interleukin (IL)-8 have been reported as similar [8–10].

Spontaneous sputum from patients with antibody deficiencies was obtained during clinic visits and processed immediately at the Royal Free Hospital in London. Separation of soluble content from dense portions was achieved by centrifugation at 18'000g for 1h at 4°C. 500mcl RNALater (Sigma-Aldrich) was added to the pellet and it was frozen at -70°C for microbiome sequencing. The supernatant was stored at -70°C for protein quantification. Sputum for the healthy control cohort was collected in the Ukraine by a biosample management company (BioPartners Inc., Woodland Hills, CA, USA) according to a standardized induced sputum sampling protocol involving saline inhalation[8]. Frozen samples were shipped to CSL, where samples were thawed and mixed with complete protease inhibitors (Roche). Separation of soluble content from dense portions was achieved by centrifugation at 18'000g for 1h at 4°C. The pellet was snap frozen in liquid nitrogen and kept at -70°C for microbiome sequencing. The supernatant was stored at -70°C for protein quantification.

Serum Collection

Paired blood samples were collected at the same time point via venipuncture and serum was obtained by centrifugation according to standard protocols. Samples were not necessarily taken at the time of IgG trough. Serum samples were stored at -70°C until analysis.

Immuno-based assays

Immunoglobulin (Ig) and albumin concentrations (Abcam) in sputum as well as IL-8, MMP-9 (HS ELISA, R&D Systems), specific IgG against *Haemophilus Influenza*e type B (HiB) and *Streptococcus pneumoniae* (PCP: Pneumococcal capsular polysaccharide) (BL International) and IgG subclass levels (Invitrogen) in sputum supernatant as well as serum were quantified using commercial ELISA kits. A Cytokine Multiplex assay was used to simultaneously detect

the levels of cytokines (IL-1β, IL-5, IL-6, IL-13, TNFα) in sputum supernatant and serum (HS Cytokine premixed panel B, R&D Systems). Assay limits of quantification of the respective analytes are shown in Supplementary Table 1. The formula for albumin correction was calculated as the ratio of sputum analyte value/sputum albumin.

Nephelometry

Immunoglobulins G, A, M and albumin in serum were assessed by nephelometry using commercially available tests (Beckman Coulter).

Colorimetric quantification of total protein and urea

Total protein in sputum was measured based on the Bradford dye-binding method (Bio-Rad Laboratories). Urea was quantified in sputum and serum using a commercial urea assay kit (Abcam). Urea correction factor was calculated as the ratio of serum urea/sputum urea and was used to multiply the sputum analyte values.

Neutrophil elastase

Supernatant obtained from fresh sputum samples were used for quantification of neutrophil elastase via Protease Tag immunoassay (ProAxsis).

DNA isolation

Metagenomic DNA was extracted from approximately 250 uL sputum using DNeasy PowerLyzer PowerSoil kit following the instructions provided with an additional heat incubation at 70 °C for 10 minutes during lysis.

Microbiome Sequencing

The V3-V4 hypervariable region of 16S rRNA gene was amplified via a single-step PCR approach using universal 341F (5`- CCTACGGGNGGCWGCAG-3`) and 805R (5`-GACTACHVGGGTATCTAATCC-3`) primers fused with Nextera XT index and MiSeq adapter sequences (Sigma-Aldrich). To generate target region amplicons from approximately

5-10 ng metagenomic DNA, PCR was carried out as follows: initial denaturation at 95 °C for 5 minutes, then 30 cycles of denaturation at 95 °C for 30 sec, annealing at 58 °C for 40 seconds and extension at 72°C for 60 seconds plus a final extension at 72 °C for 10 minutes. The amplicons were cleaned using the high stringency Agencourt AMPure XP, and were quantified by Qubit™ dsDNA HS Assay Kit and pooled at equimolar concentrations to generate a 5 nM library. Subsequently, a 4 pM library containing 10% 12 pM PhiX (Illumina Inc. USA) was prepared and loaded according to Illumina`s instructions for MiSeq 2x250 bp paired-end sequencing run (Illumina Inc., v2 kit). Two sequencing runs were performed for a total of 95 samples.

Statistical Analysis

Individuals were grouped according to clinical diagnoses and the presence or absence of abnormal airways. The biomarker comparisons between groups immunocompetent (IC), immunodeficient with normal airways (ID normal) and immunodeficient with abnormal airways (ID abnormal) were performed using non-parametric Kruskal-Wallis, or Wilcoxon-Mann-Whitney (when only two groups were available) tests without overall adjustment for multiple testing. For pairwise post-hoc comparison after performing Kruskal-Wallis tests, we used pairwise Dunn test with Holm adjustment for multiple testing. For investigation of correlates of normal / abnormal airways in immunodeficient patients, variable selection was carried out by elastic net with logistic link [11]. The computations were run in R 3.5.3 under Windows 10.

Microbiome sequencing data was analysed using ‘Quantitative insights into microbial ecology 2’ (QIIME2 version 2021.2, https://qiime2.org/) [12]. For each sequencing run, raw sequences were de-multiplexed, and de-noised using the DADA2 algorithm with default parameters to create amplicon sequence variants (ASVs). After pre-processing, two data set were merged, and the resulting data set had a mean sequencing depth of 45,196 with a range of 15,769 –

77,284 sequences. Taxonomic classification of the ASVs was completed using the HOMD extended database. Taxonomy bar plots were generated using the 20 most abundant genera across all samples. For alpha diversity analysis, the merged data set was collapsed at species level to reduce variation arising from sequencing runs. Two alpha diversity metrics, observed species (number of unique species) and Shannon index, was calculated. Wilcoxon-Mann-Whitney test was used to compare the alpha diversity metrics between IC, ID normal ID abnormal groups.

To investigate associations between the sputum microbiome and host proteins, Spearman correlations were calculated.

Ethics

All patients provided written informed consent for the collection of samples and other data under a protocol approved by the NHS Research Ethics Committee (Reference 04/Q0501/119). Immunocompetent control subjects provided written informed consent for participation in bio-specimens collection study approved by Ethics Commission at Kyiv Municipal Blood Center (Protocol No. 20001501/17).

RESULTS

Study population

Table 1 details patient demographics and clinical details. To gain information about lung disease progression, ID patients were divided into subgroups according to their airway morphology as determined by their most recent CT scan. More patients in the abnormal airways group were receiving prophylactic antibiotics.

As sputum from immunocompetent donors was induced, we measured urea in serum and sputum, which should diffuse freely between compartments, to identify any dilution effect of exogenous fluid addition [9,13]. There was no significant difference in urea concentration between the induced (IC controls) and spontaneous (ID patients) sputum samples and correcting for the ratio of serum:sputum urea did not change key findings (data not shown). Total protein and albumin levels in IC sputum were lower than in ID sputum, which may represent a true biological difference related to airways disease (Supplementary Table 2). We therefore present unmodified sputum results but correct for albumin as a non-specific marker of protein leakage from the systemic circulation into the respiratory tract where applicable.

Patients with antibody deficiency have reduced airway IgA but increased airway IgG concentrations compared to healthy controls

Control subjects had normal IgA concentrations in serum (median=2.1 g/L) with median sputum levels of 0.25 g/L, whereas ID patients showed significantly reduced IgA in both serum and sputum (Figure 1, Supplementary Table 2). Similarly, IgM concentration in the serum of IC was within the normal range, median at 1.15 g/L whereas a distinct reduction of serum IgM was observed in ID patient groups. IgM concentrations in sputum did not differ and were very low across all groups.

IgG serum concentrations for healthy controls were in the normal range (median 10.8 g/L; normal range 7.0 – 16.0 g/L). Due to subcutaneous or intravenous replacement therapy, serum

IgG concentrations in ID patients were also normal at the time of sampling. Notably, ID subjects with abnormal airways had significantly increased serum IgG (median 12.6 g/L) in comparison to IC controls. There was no significant difference in trough IgG between ID subjects with normal airways versus ID with abnormal airway morphology, median IgG trough levels were 9.07g/L and 9.70g/L, respectively (Supplementary Figure 1). IgG concentration was considerably lower in sputum than in serum and for the IC controls the median was 0.029g/L. However, ID patients had significantly higher IgG concentration in sputum at 0.215g/L and 0.178g/L in the normal and abnormal airway groups, respectively (6 to 7-fold increase compared to IC).

To assess the contribution of local IgG synthesis versus serum leak, linear regression analysis was performed (Supplementary Figure 2). IgA and IgM levels in sputum associated with the respective serum levels in ID subjects (IgA: r^2=0.43, p<0.0001, IgM: r^2=0.49, p<0.0001), while no association was observed in IC controls (IgA: r^2=0.1, p=0.08, IgM r^2=0.11, p=0.064). No association was apparent between sputum and serum IgG in both groups.

We also investigated IgG subclass distribution in a subset of IC and ID samples. The distribution of the IgG subclasses in sputum and serum was very similar between compartments and was as expected in terms of relative abundance. No statistical differences were found between samples from IC and ID subjects (Supplementary Figure 3), but we had less power to detect differences here due to the small number of patients and division of IgG into subclasses.

The sputum of patients with antibody deficiency demonstrates marked increases in markers of inflammation and airway damage compared to healthy controls

To characterize the extent of inflammation in the respiratory tract we quantified inflammatory cytokines and proteolytic markers in sputum and serum. In all groups, absolute cytokine

concentrations were considerably higher in sputum compared to serum. Inflammatory cytokines were increased in serum of ID patients compared to IC controls irrespective of the airway morphology (Figure 2, Supplementary Table 2). The release of inflammatory cytokines (IL-1β, IL-6, IL-8, TNFα) by resident cells is crucial in the response to respiratory infections but also implicated in the pathogenesis of airways disease. ID patients showed significantly elevated pro-inflammatory markers in sputum in comparison to IC. The only significant difference between the ID groups was in IL-8 (higher in those with abnormal airways). However, the median concentrations of all markers tended to be higher in those with abnormal airways and this group demonstrated more marked differences compared to healthy controls. Interestingly, IL-6 levels in IC sputum differed significantly according to smoking status in sputum, but not in serum (Supplementary Figure 4). Measured Th2 cytokines included IL13 and IL5; sputum and serum levels of both cytokines were close to the detection limits of the assay and may not be as statistically reliable as the other markers. Nevertheless, similar trends towards higher levels in ID patients were observed.

Median C-reactive protein (CRP) levels in patient serum were 4 mg/L (1.0-38.5 mg/L) for ID with normal airways and 5 mg/L (0.9-26 mg/L) for ID with abnormal airways (Supplementary Figure 5). No statistical difference was seen between the two groups. CRP levels in IC controls were not available.

Recurrent infections and activation of inflammatory cells in the lung lead to airway remodeling and progressive damage of the lung tissue, in part mediated by matrix metalloproteinases[14]. MMP-9 (pro- and active enzyme) concentration was highly increased in sputum of ID patients (Figure 3A). Serum MMP-9 levels were much lower, but also markedly increased in both ID groups and significantly higher in ID patients with abnormal airway morphology in comparison to normal airways. Neutrophil elastase is another marker for tissue damage driven by infiltrating neutrophils and highly implicated in the development of

bronchiectasis. This was quantified in ID sputum only. The median elastase concentration in the sputum was 136 ng/mL (ID with normal airways) and 172 ng/mL (ID with abnormal airways) (Figure 3B). Despite the large variability, elastase concentration was significantly higher in ID patients with abnormal airways.

High abundance of proteases such as MMP-9 and elastase in sputum potentially leads to non-specific protein digestion. IgG fragmentation patterns in sputum samples were visually assessed by immunoblotting: the results indicate that in some sputum samples minor fragmentation might occur, but the major proportion of the IgG is structurally intact (Supplementary Figure 6).

Sputum IgG concentration correlates with markers of inflammation and tissue damage

We next investigated for correlations between the measured concentrations of immunoglobulins and inflammatory markers. The analysis was performed within and between compartments for the IC and the ID subjects separately (Supplementary Figure 7). Only weak relationships were observed between sputum and serum, indicating compartmentalization of inflammation. However, there were several positive correlations between sputum and serum immunoglobulins that were assessed by linear regression in more detail to study the contribution of passive ‘leakage’ from serum (see above and Supplementary figure 2).

A cluster of positive correlations was found between proinflammatory cytokines in ID serum but not in IC controls. In sputum, inflammatory cytokines generally correlated positively, especially in the IC group (although we note that many of these levels were very low). Of note, IgG in sputum also correlated positively with inflammatory markers in both groups. However, when sputum IgG was normalized with sputum albumin to correct for non-specific protein leak, the correlations were lost in IC and weaker in ID subjects. Nevertheless, IgG

corrected for albumin still showed significant positive correlation with IL-1β, IL-8, TNFα, MMP-9 and neutrophil elastase in ID patients. In the IC group, IgA and IgM also correlated positively with inflammatory markers but no similar relationship was seen in ID patients. Sputum elastase in ID patients also correlated positively with multiple other sputum inflammatory markers, which is reflective of respiratory tract neutrophilia as a driver of chronic lung disease.

Lung function of ID patients as assessed by % predicted FEV1 negatively and significantly correlated with total protein, IgG, IgG/albumin and elastase in sputum.

Concentrations of specific antibodies against respiratory pathogens correlate with the total IgG in the ID sputum

Patients suffering from antibody deficiency have very limited capability to produce specific antibodies against important respiratory pathogens. We measured specific IgG against *H. influenzae B* (HiB) and *S. pneumoniae* (pneumococcal capsular polysaccharide (PCP)) in serum and sputum (Figure 4). In serum, anti-PCP IgG was increased in ID subjects with abnormal airways in comparison to IC controls. No difference between the groups was observed for anti-HiB IgG. In sputum, total IgG levels were low and specific IgG was detectable only when a threshold of total IgG around 25-50 µg/ml was reached. Specific sputum IgG levels were higher in ID abnormal compared to IC controls, consistent with the higher total sputum IgG in the ID patients with abnormal airways. There was no significant difference in specific antibody concentration between the IC group and the patients with normal airways, and the overall magnitude of difference appeared lower than for total IgG. However, many values were at the limit of detection.

Immunodeficient patients demonstrate greater prevalence of pathogenic bacterial species and have reduced microbial alpha diversity, which correlates negatively with markers of tissue destruction and sputum IgG concentration

We proceeded to analyse the sputum microbiome via 16S sequencing. As demonstrated in Figure 5A (individual taxa bar plots) and Figure 5B (summary bar plots), many immunodeficient patients exhibited a marked expansion of *Haemophilus* spp. compared to immunocompetent controls. In other patients, *Streptococcus* spp. or other pathogens (eg *Pseudomonas*) were noticeably enriched. As most of the healthy samples were obtained in the Ukraine, we included one sample from a non-PID asthmatic patient in the UK for comparability reasons. The microbiome in that sample was similar to the other immunocompetent donors (Figure 5A).

As anticipated from the bar plots, alpha diversity was significantly lower in ID patients than the IC cohort ($p<0.001$ for comparison of number of species or Shannon index; Figure 6A). Interestingly, there were no clear differences between the ID patients with normal or abnormal airways. As an interplay between dysbiosis and inflammatory or destructive airways disease is suspected, we correlated the alpha diversity measures with MMP-9 concentration. As seen in Figure 6B, there was a strong negative correlation of MMP-9 levels with number of observed species ($r= -0.32$, $p=0.013$) and Shannon index ($r= -0.47$, $p=0.0002$). There was also a negative correlation of elastase with number of observed species ($r= -0.32$, $p=0.02$; data not shown). Given the apparent correlation we had demonstrated between sputum IgG concentration and markers of inflammation, we also investigated the relationship between IgG and alpha diversity. Again, we observed a significant negative correlation ($r= -0.26$, $p=0.04$ for number of species and $r= -0.44$, $p=0.0004$ for Shannon index; Figure 6B).

There was no significant difference in alpha diversity measures according to the use of inhaled corticosteroids or antibiotics.

Immunoglobulins, elastase, albumin and MMP-9 may be suitable markers for prediction of abnormal airways status in immunodeficient subjects.

While multiple analytes were significantly different between the ID normal and ID abnormal patients, it remains unclear whether any biomarkers are able to accurately predict airway inflammation. We therefore searched for a combination of biomarkers separating as far as possible the ID patients with normal airways from those with abnormal airway morphology. First, we performed variable selection by fitting a sparse logistic regression by elastic net with airways status as the dependent variable and all available biomarkers as covariates; second, we fitted a multivariable logistic regression with airways status as the dependent variable and, as explanatory variables, the 6 most important analytes selected by elastic net (serum IgG and MMP-9, sputum IgM, albumin, IgG and elastase). Even though we did not have a separate cohort for validation, the model revealed a promising linear combination separating ID normal from ID abnormal (Figure 7, estimated coefficients of the combination in Supplementary Table 3).

DISCUSSION

To our knowledge, we have here conducted the most detailed study to date on the biology of the respiratory tract of immunodeficient patients.

Lung mucosal immunity is primarily mediated by secretory IgA and to a lesser extent IgM [15]. Therefore, it was not surprising to find IgA as the most abundant immunoglobulin in the sputum of healthy controls. IgG is the most abundant Ig in serum, and our study demonstrated that IgG reaches the respiratory tract and can be measured in sputum samples not only of IC subjects, but also of ID patients on IgG replacement. These patients with antibody deficiency conditions have significantly lower IgA but actually have conspicuously higher concentrations of IgG in the airways compared to healthy individuals.

It is not well understood how systemic IgG reaches the airways [16,17]. No obvious correlation between serum and sputum IgG was seen in our study. However, we demonstrated that specific IgG against Hib and PCP, which cannot be generated by antibody-deficient patients, could be found in the respiratory tract of people receiving systemic IgG when total sputum IgG reaches a certain threshold; in fact, the concentrations of these specific antibodies tended to be higher than in immunocompetent individuals (consistent with the higher total IgG). There was also a strong positive correlation between sputum IgG and albumin supporting a predominant mechanism of passive transfer, although we hypothesise that the degree – and potentially pattern – of IgG leakage may be associated with local inflammation. In contrast, IgA and IgM levels were correlated between sputum and serum in ID patients, suggesting a simple mechanism of passive transfer. The same relationship was not seen in healthy controls who are presumably capable of local IgA and IgM synthesis.

Patients with antibody deficiency had significantly higher concentrations of inflammatory cytokines compared to healthy individuals in both serum and sputum, especially in those with abnormal airways. However, we did not observe clear correlations between the serum and

sputum levels which suggests compartmentalisation of inflammation. There was evidence of localized inflammation due to smoking in the IC cohort, indicated by elevated levels of the pro-inflammatory cytokine IL-6 in sputum of IC smokers.

We also measured higher levels of MMP-9 in the sputum of ID patients, consistent with a tissue damage phenotype. MMP-9 in serum of ID patients with abnormal airways was higher than in ID with normal airways, which may reflect a greater disease severity affecting not only the lung, but also the systemic circulation. Although we could not measure neutrophil elastase in the IC cohort, we did also observe higher elastase levels in patients with abnormal airways, in line with the putative role of this enzyme in the development of bronchiectasis [18].

Collectively, these results indicate a state of chronic inflammation in the airways of patients with immune deficiency, which helps to explain the persistent respiratory symptoms which compromise quality of life [4]. It is interesting to note that this is seen even in the absence of demonstrable abnormalities on cross-sectional imaging, albeit to a somewhat lesser degree. However, all immunodeficient patients in this study were able to spontaneously produce sputum which might imply either acute symptoms or a cohort with risk of progression to structural lung disease: for example, we note that MMP-9 levels were similar in patients with or without radiological lung disease. We would therefore recommend close monitoring of patients with chronic sputum production who do not currently have evidence of airways disease. Studying induced sputum from ID patients who cannot spontaneously expectorate would be interesting in future work.

Intriguingly, the concentration of IgG in the sputum correlated positively with several inflammatory markers. In large part these relationships disappeared after correction for albumin, suggesting that a passive leak of protein is the major explanation and is presumably increased in the context of inflammation. However, there were still significant correlations of IgG (after correction for albumin) with IL-1β, IL-8, TNFα and MMP-9 in ID patients. This

421 may indicate some mechanism of either enhanced recruitment or retention of IgG in the
422 context of inflammation. We considered whether patients with worse symptoms may be
423 treated with more immunoglobulin by the clinical teams, but there were no clear relationships
424 between inflammatory markers and serum IgG concentration or IgG trough.

425 Unlike in the immunodeficient cohort, we observed positive correlations of inflammatory
426 markers with IgA and IgM in immunocompetent individuals: this presumably indicates an
427 appropriate local response to inflammation without leakage of systemic proteins.

428 We also assessed the airway microbiome in the study participants. Many patients with
429 immunodeficiency exhibited marked dominance of pathogenic organisms, especially
430 *Haemophilus* spp., in comparison to immunocompetent individuals. Correspondingly, the
431 immunodeficient group also demonstrated far lower alpha diversity measures (indicating
432 lower richness and evenness of the bacterial species). The alpha diversity metrics correlated
433 negatively with MMP-9 and neutrophil elastase suggesting that dysbiosis is directly
434 associated with host damage in inflammatory airway disease [19]. As with the inflammatory
435 markers, alpha diversity also correlated negatively with sputum IgG concentration. Again, this
436 suggests that sputum IgG paradoxically associates with airway pathology, presumably due to
437 greater protein leak in the context of inflammation and dysbiosis.

438 These findings are underlined by the negative correlation of lung function (FEV1% predicted)
439 with IgG, total protein and elastase.

440 A previous study[20] identified IgA concentration as a key driver of oropharyngeal microbiome
441 (although intriguingly alpha diversity was actually increased in the absence of IgA, unlike
442 observations in the gut)[21]. In our study, using sputum rather than oropharyngeal samples, we
443 did not replicate this result and could not differentiate samples on the basis of IgA
444 concentration.

Instead, IgG (and IgM) concentration in sputum was among the variables which helped to differentiate immunodeficient patients with normal airways from those with abnormal airways, with higher levels correlating with an increased risk of radiological airway pathology. As expected, sputum elastase and MMP-9 also helped to differentiate the groups, while lower serum IgG and sputum albumin were the final analytes in our model. If this is replicated in other cohorts, these could be useful biomarkers to predict the progression of lung disease.

Our study does have limitations. Most significantly, the healthy controls and immunodeficient patients were recruited from different sites and sputum was collected via different techniques. However, we extensively investigated the potential confounding impact of sputum induction and did not confirm any significant dilutional effect. Furthermore, the key findings remained robust even after correcting for the serum:sputum urea ratio. We do not believe there is any reason to expect that levels of immunoglobulins and inflammatory markers would be different according to country of origin of donors. Taxa identified via 16S sequencing were consistent with organisms expected in the airways, and although gastrointestinal microbiome may vary according to geographical area, the respiratory microbiome appears to be largely similar worldwide in health [22] and with airways disease [23]. Even if there were minor geographical differences, alpha diversity and the presence of pathogens should not be affected by location. Of note, an immunocompetent patient from the UK demonstrated a similar pattern to the Ukrainian donors with no obvious expansion of pathogenic taxa.

Further limitations include the inability to detect all analytes at measurable levels in all samples, and we were unable to measure neutrophil elastase in immunocompetent patients. We were also unable to measure other markers of NETosis and sputum cell counts due to sputum processing techniques and available volumes. It was not possible under current protocols to collect induced sputum samples from immunodeficient patients and this will be

valuable to further understand the respiratory tract biology of patients without overt respiratory symptoms. Furthermore, we could not collect samples from immunocompetent patients with bronchiectasis or other airways disease, e.g. non-cystic fibrosis bronchiectasis and COPD, which will be important in future work to discern the relative contribution of immune deficiency and airways disease to the findings in this study. Finally, proteases in sputum samples may have resulted in some minor degradation of other proteins (although we observed little objective evidence for this). However, as the proteases were more abundant in ID sputum samples, any degradation would have acted to weaken the majority of results (which tended to indicate increased abundance of other proteins in ID patients).

Overall, our results indicate that patients with antibody deficiency suffer from significant inflammation and dysbiosis in the airways. This is seen especially in patients with demonstrable airways disease but there are clear pathological differences from healthy controls even with apparently normal airway morphology. It may be possible in the future to measure inflammatory markers or indicators of dysbiosis in sputum (perhaps in relation to baseline values) in order to diagnose subclinical infection, even in the absence of a positive culture for pathogens.

Systemic replacement of IgG leads to good levels reaching the respiratory tract but this is insufficient to prevent pathology; in fact, higher concentrations of IgG correlated with more inflammation and lower microbial alpha diversity. In this context there is a need for further mechanistic studies to understand the exact localization of RT IgG with respect to epithelial lining fluid, mucus and relevant pathogens as well as the capacity of RT IgG to harness cellular and complement-based effector functions. Future strategies for host-directed therapy should focus on directly targeting inflammation (eg brensocatib which targets neutrophils [24]) or delivering localized therapy to the airways.

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571

572 **FIGURE LEGENDS**

573

574 **Figure 1**. **Immunoglobulins measured in paired serum and sputum of immunocompetent**
575 **and immunodeficient subjects.** Box plots showing median levels of IgA, IgM and IgG in the
576 IC (red), ID with normal airways (green) and ID with abnormal airways (blue) groups. Serum
577 levels are depicted in the upper panel, sputum in the lower panel. The lower limit of
578 quantification for IgA and IgM is indicated as a dotted black line. *p＜0.05, ***p＜0.001,
579 ****p＜0.0001, IC=immunocompetent, ID=immunodeficient.

580

581 **Figure 2**. **Cytokines measured in serum and sputum of immunocompetent and**
582 **immunodeficient subjects.** Box plots showing median concentrations of IL-1β, IL-6, IL-8,
583 TNFα, IL-5 and IL-13 measured in paired samples of IC (red) and ID subjects grouped
584 according to their airway morphology, normal airways (green) or abnormal airways (blue).
585 Serum levels are depicted in the upper panel and sputum levels in the lower panel, the
586 respective LLOQ is indicated as a dotted black line in each graph. IL-13 and IL-5 serum
587 levels were below the detection limit of the assay and were not considered for statistical
588 analysis. Extrapolated values below LLOQ are shown where applicable. *p＜0.05, **p＜
589 0.01, ***p＜0.001, ****p＜0.0001, IC=immunocompetent, ID=immunodeficient,
590 LLOQ=lower limit of quantification.

591

592 **Figure 3**. **Markers of tissue remodeling measured in serum and sputum of**
593 **immunocompetent and immunodeficient subjects**. A. MMP-9 was quantified in sputum
594 and serum of IC and ID subjects. B. Neutrophil elastase was quantified in sputum of ID

patients. Box plots with individual data of IC are shown in red, ID subjects with normal airways in green and ID with abnormal airways in blue. Extrapolated values below LLOQ (black dotted line) are shown where applicable. *p＜0.05, **p＜0.01, ****p＜0.0001, IC=immunocompetent, ID=immunodeficient, LLOQ=lower limit of quantification.

Figure 4. **Specific IgG against H. influenzae B (HiB) and S. pneumoniae (PCP)) quantified in serum and sputum.** Box plots with individual data of IC are shown in red, ID subjects with normal airways in green and ID with abnormal airways in blue. Serum levels are depicted in the upper panel and sputum levels in the lower panel, the respective LLOQ is indicated as a dotted black line in each graph. *p＜0.05, **p＜0.01, ***p＜0.001, IC=immunocompetent, ID=immunodeficient, LLOQ=lower limit of quantification

Figure 5. **Microbial abundance in the sputum of immunocompetent and immunodeficient patients, derived from 16S sequencing.** A. Taxonomy bar-plots detailing the top 20 most abundant genera across all samples, presented for IC, ID patients with abnormal airways or normal airways. Each bar represents one patient and samples are presented in each group according to the abundance of *Haemophilus*. Asterisk indicates sample from a non-ID asthmatic patient in the UK for comparability reasons. B. Summary bar-plots for each group presenting overall mean prevalence of top 20 genera. IC=immunocompetent, ID=immunodeficient

Figure 6. **Microbial alpha diversity in the sputum of immunocompetent and immunodeficient patients**. A. Alpha diversity as measured by number of species and Shannon index for IC, ID patients with abnormal airways or normal airways. B. Alpha

diversity metrics versus $\log_{10}$ MMP-9 concentration and $\log_{10}$ IgG concentration in sputum of immunodeficient patients. Green squares represent ID patients with abnormal airways, blue circles represent ID patients with normal airways. All correlations significant ($p<0.05$). ***p ＜0.001, IC=immunocompetent, ID=immunodeficient

Figure 7. Linear combination of serum IgG and MMP-9, sputum IgM, albumin, IgG and elastase for classification between normal and abnormal airways in immunodeficient patients. Variable selection for airways status response was carried out by elastic net, the final fit with selected biomarkers was performed with logistic regression. Coefficients can be found in supplementary table 3. ***p＜0.001, ID=immunodeficient

TABLES

Table 1. Patient demographics. Descriptive table with median [IQR] or counts (Percentages). CVID=common variable immunodeficiency, FEV=forced expiratory volume, IC=immunocompetent, ID=immunodeficient, i.v.=intravenous, IQR=inter quartile range, NA=not available, s.c.=subcutaneous, SID=secondary immune deficiency, unkn=unknown, XLA= X-linked agammaglobulinaemia

	IC (n = 31), median [IQR] or counts (%)	**ID normal (n = 18), median [IQR] or counts (%)**	**ID abnormal (n = 49), median [IQR] or counts (%)**
Age, years	33.00, [27.50, 40.00]	48.00, [36.25, 57.00]	57.00, [40.00, 71.00]
Diagnosis	Immunocompetent control - 31 (100.0%)	CVID - 12 (66.7%), XLA - 1 (5.6%), SID † - 4 (22.2%), Other - 1 (5.6%)	CVID - 26 (53.1%), XLA - 8 (16.3%), SID † - 10 (20.4%), Other - 5 (10.2%)
Immunoglobulin administration route	none - 31 (100.0%)	IV - 12 (66.7%), SC - 4 (22.2%), unkn - 2 (11.1%)	IV - 33 (67.3%), SC - 12 (24.5%), unkn - 4 (8.2%)
IgG trough, g/L	NA	9.07, [7.73, 10.35]	9.70, [8.45, 10.60]
Inhaled steroids intake	No steroids - 31 (100.0%)	No steroids - 14 (77.8%), Steroids - 4 (22.2%)	No steroids - 34 (69.4%), Steroids - 15 (30.6%)
Prophylactic antibiotics intake *	No Abx - 31 (100.0%)	Abx - 4 (22.2%), No Abx - 14 (77.8%)	Abx - 27 (55.1%), No Abx - 22 (44.9%)
Smoking status	No - 15 (48.4%), Yes - 16 (51.6%)	No - 13 (72.2%), Past - 5 (27.8%)	No - 29 (59.2%), Yes - 5 (10.2%), Past - 13 (26.5%), unreported- 2 (4.1%)
Self-reported respiratory infection frequency (categories specified below)	NA	1 - 4 (22.2%), 2 - 1 (5.6%), 3 - 3 (16.7%), 4 - 4 (22.2%), 5 - 5 (27.8%), 6 – 1 (5.6%)	1 - 11 (22.4%), 2 - 5 (10.2%), 3 - 8 (16.3%), 4 - 16 (32.7%) 5 - 6 (12.2%), 6 - 1 (2.0%), unkn - 2 (4.1%)
FEV1 % predicted (closest to sampling)	NA	101.0, [83.0, 107.0]	86.0, [72.0, 100.8]

Categories self-reported respiratory infection frequency: 1=at least once a month, **2**=less than once a month but at least 6 times per year, **3**=about 4 or 5 times per year, **4**=about 2 or 3 times per year, **5**=about once a year, **6**=less than once a year

* Prophylactic antibiotics comprised: amoxicillin (1), azithromycin (17), cephalexin (1), ciprofloxacin (1), clarithromycin (1), co-trimoxazole (3), doxycycline (4), nebulized colomycin plus azithromycin (1), co-trimoxazole plus azithromycin (1), unknown (1).

† Underlying diagnoses comprised: chronic lymphocytic leukaemia (2), eosinophilic granulomatosis with polyangiitis (1), chronic corticosteroid usage (1), non-Hodgkin's lymphoma (5), plasma cell dyscrasias (3),

	IC (n = 31), median [IQR] or counts (%)	ID normal (n = 18), median [IQR] or counts (%)	ID abnormal (n = 49), median [IQR] or counts (%)
rheumatoid arthritis (1), systemic lupus erythematosus (1).			

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Airway inflammation and dysbiosis in antibody deficiency despite the presence of IgG

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Conflict of interest: The following contributors are employees of CSL Behring AG Bern, Switzerland: A.S., M.S., S.G., I. I., C. v. A., I. S., A. Sch. or CSL Behring GmbH Marburg, Germany: M. H., C. W. and A. F. S.O.B and D.M.L have received travel and accommodation costs from CSL Behring. S.O.B has also received grant support from the European Union, National Institute of Health Research, UCLH and GOSH/ICH Biomedical Research Centers and personal fees or travel expenses from Immunodeficiency Canada/IAACI, Baxalta US Inc and Biotest. D.M.L. has also received grant support from LifeArc, Bristol-Myers-Squibb, Blood Cancer UK, Medical Research Council and the British Society for Antimicrobial Chemotherapy and personal fees from Merck. Other authors declare no conflicts of interest.

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Clinical implications statement

Antibody-deficient patients often suffer chronic respiratory symptoms. We demonstrate that, even with normal imaging and despite good levels of IgG in sputum, these patients have significant airway inflammation and dysbiosis.

Keywords: Immunodeficiency, immunoglobulin, sputum, respiratory tract, microbiome, inflammation

Abbreviations: CVID: Common variable immunodeficiency, FEV1: forced expiratory volume in 1 second, IC: immunocompetent, ID: Immunodeficient, IL: interleukin, MMP: matrix-metalloproteinase, PID: Primary antibody deficiency, RT: Respiratory tract, SID: Secondary immune deficiency, XLA: X-linked agammaglobulinaemia

ABSTRACT

Background: Patients with antibody deficiency suffer chronic respiratory symptoms, recurrent exacerbations and progressive airways disease despite systemic replacement of Immunoglobulin G. Little is known about the respiratory tract biology of these patients.

Objective: To measure immunoglobulin levels, inflammatory cytokines and mediators of tissue damage in serum and sputum from patients with antibody deficiency and healthy controls; to analyse the respiratory microbiome in the same cohorts.

Methods: We obtained paired sputum and serum samples from 31 immunocompetent subjects and 67 antibody deficient patients, the latter divided on computed tomography scan appearance into 'abnormal airways' (bronchiectasis or airway thickening) or 'normal airways'. We measured inflammatory cytokines, immunoglobulin levels, neutrophil elastase, matrix-metalloproteinase-9, urea, albumin and total protein levels using standard assays. We employed V3-V4 region 16S sequencing for microbiome analysis.

Results: Immunodeficient patients had markedly reduced Immunoglobulin A in sputum but higher concentrations of Immunoglobulin G compared to healthy controls. Inflammatory cytokines and tissue damage markers were higher in immunodeficient patients, who also exhibited dysbiosis with over-representation of pathogenic taxa and significantly reduced alpha diversity compared to immunocompetent individuals. These differences were seen regardless of airway morphology. Sputum matrix metalloproteinase-9 and elastase correlated inversely with alpha diversity in the antibody deficient group, as did sputum Immunoglobulin G, which correlated positively with several inflammatory markers, even after correction for albumin levels.

65 **Conclusion:** Patients with antibody deficiency, even with normal lung imaging, exhibit
66 inflammation and dysbiosis in their airways despite higher levels of Immunoglobulin G
67 compared to healthy controls.

68 **Capsule summary:** Persistent airway inflammation and dysbiosis despite adequate IgG
69 replacement highlights the need for better therapeutics to maintain lung health in antibody
70 deficient patients.

71

INTRODUCTION

Despite adequate substitution with systemic immunoglobulin G (IgG), recurrent respiratory tract infections and chronic respiratory symptoms constitute a significant disease burden in patients with primary and secondary antibody deficiency [1,2]. Although systemic IgG replacement therapy has significantly reduced mortality and severe bacterial pneumonia in these patient populations, repeated cycles of infection and inflammation drive chronic lung disease leading to bronchiectasis and lung function decline [1,3]. Quality of life in primary immunodeficiency conditions correlates closely with respiratory involvement, with both airflow obstruction and respiratory exacerbation frequency having an important impact [4].

Recent observational studies have documented this residual respiratory disease burden and have investigated putative causes [5]. Besides more obvious risk factors like exposure to young children, low systemic levels of IgG, IgA and IgM were associated with increased infective burden in the airways. However, information on immunoglobulin levels in the respiratory tract itself and associations with both the characteristics of respiratory tract microbiota and clinical disease severity is scarce. In one study, the presence of IgM in serum and sputum in otherwise hypogammaglobulinaemic patients was shown to associate with reduced microbial burden, specifically of *Haemophilus influenzae*, as well as a lower incidence of acute airway infections[6].

In this study, we aimed to understand the respiratory tract biology in a large cohort of patients with primary and secondary antibody deficiency (immunodeficient ID), receiving long-term intravenous or subcutaneous immunoglobulin replacement. The cohort was compared to healthy donors (immunocompetent, IC) with regard to immunoglobulin levels, markers of inflammation and tissue remodeling as well as microbial diversity.

METHODS

Patients and Clinical data

Patients were recruited between September 2017 and March 2019 when they attended the department for routine clinic visits or immunoglobulin infusions. Patients were eligible for the study if they were aged ≥18 years and had any diagnosis of antibody deficiency made by a consultant immunologist. Data were collected on immunodeficiency diagnosis, the route and dose of immunoglobulin replacement therapy, the most recent serum IgG trough level and C-reactive protein concentration, and the use of prophylactic antibiotics and inhaled corticosteroids. Patients were asked to report respiratory infection frequency over the past year and their smoking habits. From the lung function tests performed closest to the time of sampling, we collected percentage predicted forced expiratory volume in 1 second (FEV1%). We also classified patients on the basis of their most recent computed tomography (CT) scan as having 'abnormal airways' or 'normal airways' (presence or absence of bronchiectasis or widespread airway thickening as diagnosed by a consultant radiologist).

At the RFH Immunology Department, patients on immunoglobulin replacement therapy are usually targeted to a minimum IgG trough level of 8 g/L, or 10 g/L in patients with X-linked agammaglobulinaemia (XLA).

Sputum Collection and Processing

While there are readily available methods for quantifying antibody isotypes in serum of individuals, the sampling of the respiratory space is not well standardized. Here we focused on spontaneous and induced sputum from immunodeficient patients (ID) and immunocompetent controls (IC) respectively, as opposed to bronchoalveolar lavage, saliva collection or airway brushing. Sputum sampling as defined in the European Respiratory Society guidelines[7] was practical to obtain suitable samples representative of the epithelial lining fluid in the conducting airways. Sputum samples reflect lower airway inflammation;

although viable cell counts may be lower in spontaneous compared to induced sputum, the concentrations of inflammatory markers such as Interleukin (IL)-8 have been reported as similar [8–10].

Spontaneous sputum from patients with antibody deficiencies was obtained during clinic visits and processed immediately at the Royal Free Hospital in London. Separation of soluble content from dense portions was achieved by centrifugation at 18'000g for 1h at 4°C. 500mcl RNALater (Sigma-Aldrich) was added to the pellet and it was frozen at -70°C for microbiome sequencing. The supernatant was stored at -70°C for protein quantification. Sputum for the healthy control cohort was collected in the Ukraine by a biosample management company (BioPartners Inc., Woodland Hills, CA, USA) according to a standardized induced sputum sampling protocol involving saline inhalation[8]. Frozen samples were shipped to CSL, where samples were thawed and mixed with complete protease inhibitors (Roche). Separation of soluble content from dense portions was achieved by centrifugation at 18'000g for 1h at 4°C. The pellet was snap frozen in liquid nitrogen and kept at -70°C for microbiome sequencing. The supernatant was stored at -70°C for protein quantification.

Serum Collection

Paired blood samples were collected at the same time point via venipuncture and serum was obtained by centrifugation according to standard protocols. Samples were not necessarily taken at the time of IgG trough. Serum samples were stored at -70°C until analysis.

Immuno-based assays

Immunoglobulin (Ig) and albumin concentrations (Abcam) in sputum as well as IL-8, MMP-9 (HS ELISA, R&D Systems), specific IgG against *Haemophilus Influenza*e type B (HiB) and *Streptococcus pneumoniae* (PCP: Pneumococcal capsular polysaccharide) (BL International) and IgG subclass levels (Invitrogen) in sputum supernatant as well as serum were quantified using commercial ELISA kits. A Cytokine Multiplex assay was used to simultaneously detect

the levels of cytokines (IL-1β, IL-5, IL-6, IL-13, TNFα) in sputum supernatant and serum (HS Cytokine premixed panel B, R&D Systems). Assay limits of quantification of the respective analytes are shown in Supplementary Table 1. The formula for albumin correction was calculated as the ratio of sputum analyte value/sputum albumin.

Nephelometry

Immunoglobulins G, A, M and albumin in serum were assessed by nephelometry using commercially available tests (Beckman Coulter).

Colorimetric quantification of total protein and urea

Total protein in sputum was measured based on the Bradford dye-binding method (Bio-Rad Laboratories). Urea was quantified in sputum and serum using a commercial urea assay kit (Abcam). Urea correction factor was calculated as the ratio of serum urea/sputum urea and was used to multiply the sputum analyte values.

Neutrophil elastase

Supernatant obtained from fresh sputum samples were used for quantification of neutrophil elastase via Protease Tag immunoassay (ProAxsis).

DNA isolation

Metagenomic DNA was extracted from approximately 250 uL sputum using DNeasy PowerLyzer PowerSoil kit following the instructions provided with an additional heat incubation at 70 °C for 10 minutes during lysis.

Microbiome Sequencing

The V3-V4 hypervariable region of 16S rRNA gene was amplified via a single-step PCR approach using universal 341F (5`- CCTACGGGNGGCWGCAG-3`) and 805R (5`-GACTACHVGGGTATCTAATCC-3`) primers fused with Nextera XT index and MiSeq adapter sequences (Sigma-Aldrich). To generate target region amplicons from approximately

5-10 ng metagenomic DNA, PCR was carried out as follows: initial denaturation at 95 °C for 5 minutes, then 30 cycles of denaturation at 95 °C for 30 sec, annealing at 58 °C for 40 seconds and extension at 72°C for 60 seconds plus a final extension at 72 °C for 10 minutes. The amplicons were cleaned using the high stringency Agencourt AMPure XP, and were quantified by Qubit™ dsDNA HS Assay Kit and pooled at equimolar concentrations to generate a 5 nM library. Subsequently, a 4 pM library containing 10% 12 pM PhiX (Illumina Inc. USA) was prepared and loaded according to Illumina`s instructions for MiSeq 2x250 bp paired-end sequencing run (Illumina Inc., v2 kit). Two sequencing runs were performed for a total of 95 samples.

Statistical Analysis

Individuals were grouped according to clinical diagnoses and the presence or absence of abnormal airways. The biomarker comparisons between groups immunocompetent (IC), immunodeficient with normal airways (ID normal) and immunodeficient with abnormal airways (ID abnormal) were performed using non-parametric Kruskal-Wallis, or Wilcoxon-Mann-Whitney (when only two groups were available) tests without overall adjustment for multiple testing. For pairwise post-hoc comparison after performing Kruskal-Wallis tests, we used pairwise Dunn test with Holm adjustment for multiple testing. For investigation of correlates of normal / abnormal airways in immunodeficient patients, variable selection was carried out by elastic net with logistic link [11]. The computations were run in R 3.5.3 under Windows 10.

Microbiome sequencing data was analysed using ‘Quantitative insights into microbial ecology 2’ (QIIME2 version 2021.2, https://qiime2.org/) [12]. For each sequencing run, raw sequences were de-multiplexed, and de-noised using the DADA2 algorithm with default parameters to create amplicon sequence variants (ASVs). After pre-processing, two data set were merged, and the resulting data set had a mean sequencing depth of 45,196 with a range of 15,769 –

77,284 sequences. Taxonomic classification of the ASVs was completed using the HOMD extended database. Taxonomy bar plots were generated using the 20 most abundant genera across all samples. For alpha diversity analysis, the merged data set was collapsed at species level to reduce variation arising from sequencing runs. Two alpha diversity metrics, observed species (number of unique species) and Shannon index, was calculated. Wilcoxon-Mann-Whitney test was used to compare the alpha diversity metrics between IC, ID normal ID abnormal groups.

To investigate associations between the sputum microbiome and host proteins, Spearman correlations were calculated.

Ethics

All patients provided written informed consent for the collection of samples and other data under a protocol approved by the NHS Research Ethics Committee (Reference 04/Q0501/119). Immunocompetent control subjects provided written informed consent for participation in bio-specimens collection study approved by Ethics Commission at Kyiv Municipal Blood Center (Protocol No. 20001501/17).

RESULTS

Study population

Table 1 details patient demographics and clinical details. To gain information about lung disease progression, ID patients were divided into subgroups according to their airway morphology as determined by their most recent CT scan. More patients in the abnormal airways group were receiving prophylactic antibiotics.

As sputum from immunocompetent donors was induced, we measured urea in serum and sputum, which should diffuse freely between compartments, to identify any dilution effect of exogenous fluid addition [9,13]. There was no significant difference in urea concentration between the induced (IC controls) and spontaneous (ID patients) sputum samples and correcting for the ratio of serum:sputum urea did not change key findings (data not shown). Total protein and albumin levels in IC sputum were lower than in ID sputum, which may represent a true biological difference related to airways disease (Supplementary Table 2). We therefore present unmodified sputum results but correct for albumin as a non-specific marker of protein leakage from the systemic circulation into the respiratory tract where applicable.

Patients with antibody deficiency have reduced airway IgA but increased airway IgG concentrations compared to healthy controls

Control subjects had normal IgA concentrations in serum (median=2.1 g/L) with median sputum levels of 0.25 g/L, whereas ID patients showed significantly reduced IgA in both serum and sputum (Figure 1, Supplementary Table 2). Similarly, IgM concentration in the serum of IC was within the normal range, median at 1.15 g/L whereas a distinct reduction of serum IgM was observed in ID patient groups. IgM concentrations in sputum did not differ and were very low across all groups.

IgG serum concentrations for healthy controls were in the normal range (median 10.8 g/L; normal range 7.0 – 16.0 g/L). Due to subcutaneous or intravenous replacement therapy, serum

IgG concentrations in ID patients were also normal at the time of sampling. Notably, ID subjects with abnormal airways had significantly increased serum IgG (median 12.6 g/L) in comparison to IC controls. There was no significant difference in trough IgG between ID subjects with normal airways versus ID with abnormal airway morphology, median IgG trough levels were 9.07g/L and 9.70g/L, respectively (Supplementary Figure 1). IgG concentration was considerably lower in sputum than in serum and for the IC controls the median was 0.029g/L. However, ID patients had significantly higher IgG concentration in sputum at 0.215g/L and 0.178g/L in the normal and abnormal airway groups, respectively (6 to 7-fold increase compared to IC).

To assess the contribution of local IgG synthesis versus serum leak, linear regression analysis was performed (Supplementary Figure 2). IgA and IgM levels in sputum associated with the respective serum levels in ID subjects (IgA: r^2=0.43, p<0.0001, IgM: r^2=0.49, p<0.0001), while no association was observed in IC controls (IgA: r^2=0.1, p=0.08, IgM r^2=0.11, p=0.064). No association was apparent between sputum and serum IgG in both groups.

We also investigated IgG subclass distribution in a subset of IC and ID samples. The distribution of the IgG subclasses in sputum and serum was very similar between compartments and was as expected in terms of relative abundance. No statistical differences were found between samples from IC and ID subjects (Supplementary Figure 3), but we had less power to detect differences here due to the small number of patients and division of IgG into subclasses.

The sputum of patients with antibody deficiency demonstrates marked increases in markers of inflammation and airway damage compared to healthy controls

To characterize the extent of inflammation in the respiratory tract we quantified inflammatory cytokines and proteolytic markers in sputum and serum. In all groups, absolute cytokine

concentrations were considerably higher in sputum compared to serum. Inflammatory cytokines were increased in serum of ID patients compared to IC controls irrespective of the airway morphology (Figure 2, Supplementary Table 2). The release of inflammatory cytokines (IL-1β, IL-6, IL-8, TNFα) by resident cells is crucial in the response to respiratory infections but also implicated in the pathogenesis of airways disease. ID patients showed significantly elevated pro-inflammatory markers in sputum in comparison to IC. The only significant difference between the ID groups was in IL-8 (higher in those with abnormal airways). However, the median concentrations of all markers tended to be higher in those with abnormal airways and this group demonstrated more marked differences compared to healthy controls. Interestingly, IL-6 levels in IC sputum differed significantly according to smoking status in sputum, but not in serum (Supplementary Figure 4). Measured Th2 cytokines included IL13 and IL5; sputum and serum levels of both cytokines were close to the detection limits of the assay and may not be as statistically reliable as the other markers. Nevertheless, similar trends towards higher levels in ID patients were observed.

Median C-reactive protein (CRP) levels in patient serum were 4 mg/L (1.0-38.5 mg/L) for ID with normal airways and 5 mg/L (0.9-26 mg/L) for ID with abnormal airways (Supplementary Figure 5). No statistical difference was seen between the two groups. CRP levels in IC controls were not available.

Recurrent infections and activation of inflammatory cells in the lung lead to airway remodeling and progressive damage of the lung tissue, in part mediated by matrix metalloproteinases[14]. MMP-9 (pro- and active enzyme) concentration was highly increased in sputum of ID patients (Figure 3A). Serum MMP-9 levels were much lower, but also markedly increased in both ID groups and significantly higher in ID patients with abnormal airway morphology in comparison to normal airways. Neutrophil elastase is another marker for tissue damage driven by infiltrating neutrophils and highly implicated in the development of

bronchiectasis. This was quantified in ID sputum only. The median elastase concentration in the sputum was 136 ng/mL (ID with normal airways) and 172 ng/mL (ID with abnormal airways) (Figure 3B). Despite the large variability, elastase concentration was significantly higher in ID patients with abnormal airways.

High abundance of proteases such as MMP-9 and elastase in sputum potentially leads to non-specific protein digestion. IgG fragmentation patterns in sputum samples were visually assessed by immunoblotting: the results indicate that in some sputum samples minor fragmentation might occur, but the major proportion of the IgG is structurally intact (Supplementary Figure 6).

Sputum IgG concentration correlates with markers of inflammation and tissue damage

We next investigated for correlations between the measured concentrations of immunoglobulins and inflammatory markers. The analysis was performed within and between compartments for the IC and the ID subjects separately (Supplementary Figure 7). Only weak relationships were observed between sputum and serum, indicating compartmentalization of inflammation. However, there were several positive correlations between sputum and serum immunoglobulins that were assessed by linear regression in more detail to study the contribution of passive ‘leakage’ from serum (see above and Supplementary figure 2).

A cluster of positive correlations was found between proinflammatory cytokines in ID serum but not in IC controls. In sputum, inflammatory cytokines generally correlated positively, especially in the IC group (although we note that many of these levels were very low). Of note, IgG in sputum also correlated positively with inflammatory markers in both groups. However, when sputum IgG was normalized with sputum albumin to correct for non-specific protein leak, the correlations were lost in IC and weaker in ID subjects. Nevertheless, IgG

corrected for albumin still showed significant positive correlation with IL-1β, IL-8, TNFα, MMP-9 and neutrophil elastase in ID patients. In the IC group, IgA and IgM also correlated positively with inflammatory markers but no similar relationship was seen in ID patients. Sputum elastase in ID patients also correlated positively with multiple other sputum inflammatory markers, which is reflective of respiratory tract neutrophilia as a driver of chronic lung disease.

Lung function of ID patients as assessed by % predicted FEV1 negatively and significantly correlated with total protein, IgG, IgG/albumin and elastase in sputum.

Concentrations of specific antibodies against respiratory pathogens correlate with the total IgG in the ID sputum

Patients suffering from antibody deficiency have very limited capability to produce specific antibodies against important respiratory pathogens. We measured specific IgG against *H. influenzae B* (HiB) and *S. pneumoniae* (pneumococcal capsular polysaccharide (PCP)) in serum and sputum (Figure 4). In serum, anti-PCP IgG was increased in ID subjects with abnormal airways in comparison to IC controls. No difference between the groups was observed for anti-HiB IgG. In sputum, total IgG levels were low and specific IgG was detectable only when a threshold of total IgG around 25-50 µg/ml was reached. Specific sputum IgG levels were higher in ID abnormal compared to IC controls, consistent with the higher total sputum IgG in the ID patients with abnormal airways. There was no significant difference in specific antibody concentration between the IC group and the patients with normal airways, and the overall magnitude of difference appeared lower than for total IgG. However, many values were at the limit of detection.

Immunodeficient patients demonstrate greater prevalence of pathogenic bacterial species and have reduced microbial alpha diversity, which correlates negatively with markers of tissue destruction and sputum IgG concentration

We proceeded to analyse the sputum microbiome via 16S sequencing. As demonstrated in Figure 5A (individual taxa bar plots) and Figure 5B (summary bar plots), many immunodeficient patients exhibited a marked expansion of *Haemophilus* spp. compared to immunocompetent controls. In other patients, *Streptococcus* spp. or other pathogens (eg *Pseudomonas*) were noticeably enriched. As most of the healthy samples were obtained in the Ukraine, we included one sample from a non-PID asthmatic patient in the UK for comparability reasons. The microbiome in that sample was similar to the other immunocompetent donors (Figure 5A).

As anticipated from the bar plots, alpha diversity was significantly lower in ID patients than the IC cohort ($p<0.001$ for comparison of number of species or Shannon index; Figure 6A). Interestingly, there were no clear differences between the ID patients with normal or abnormal airways. As an interplay between dysbiosis and inflammatory or destructive airways disease is suspected, we correlated the alpha diversity measures with MMP-9 concentration. As seen in Figure 6B, there was a strong negative correlation of MMP-9 levels with number of observed species ($r= -0.32$, $p=0.013$) and Shannon index ($r= -0.47$, $p=0.0002$). There was also a negative correlation of elastase with number of observed species ($r= -0.32$, $p=0.02$; data not shown). Given the apparent correlation we had demonstrated between sputum IgG concentration and markers of inflammation, we also investigated the relationship between IgG and alpha diversity. Again, we observed a significant negative correlation ($r= -0.26$, $p=0.04$ for number of species and $r= -0.44$, $p=0.0004$ for Shannon index; Figure 6B).

There was no significant difference in alpha diversity measures according to the use of inhaled corticosteroids or antibiotics.

Immunoglobulins, elastase, albumin and MMP-9 may be suitable markers for prediction of abnormal airways status in immunodeficient subjects.

While multiple analytes were significantly different between the ID normal and ID abnormal patients, it remains unclear whether any biomarkers are able to accurately predict airway inflammation. We therefore searched for a combination of biomarkers separating as far as possible the ID patients with normal airways from those with abnormal airway morphology. First, we performed variable selection by fitting a sparse logistic regression by elastic net with airways status as the dependent variable and all available biomarkers as covariates; second, we fitted a multivariable logistic regression with airways status as the dependent variable and, as explanatory variables, the 6 most important analytes selected by elastic net (serum IgG and MMP-9, sputum IgM, albumin, IgG and elastase). Even though we did not have a separate cohort for validation, the model revealed a promising linear combination separating ID normal from ID abnormal (Figure 7, estimated coefficients of the combination in Supplementary Table 3).

DISCUSSION

To our knowledge, we have here conducted the most detailed study to date on the biology of the respiratory tract of immunodeficient patients.

Lung mucosal immunity is primarily mediated by secretory IgA and to a lesser extent IgM [15]. Therefore, it was not surprising to find IgA as the most abundant immunoglobulin in the sputum of healthy controls. IgG is the most abundant Ig in serum, and our study demonstrated that IgG reaches the respiratory tract and can be measured in sputum samples not only of IC subjects, but also of ID patients on IgG replacement. These patients with antibody deficiency conditions have significantly lower IgA but actually have conspicuously higher concentrations of IgG in the airways compared to healthy individuals.

It is not well understood how systemic IgG reaches the airways [16,17]. No obvious correlation between serum and sputum IgG was seen in our study. However, we demonstrated that specific IgG against Hib and PCP, which cannot be generated by antibody-deficient patients, could be found in the respiratory tract of people receiving systemic IgG when total sputum IgG reaches a certain threshold; in fact, the concentrations of these specific antibodies tended to be higher than in immunocompetent individuals (consistent with the higher total IgG). There was also a strong positive correlation between sputum IgG and albumin supporting a predominant mechanism of passive transfer, although we hypothesise that the degree – and potentially pattern – of IgG leakage may be associated with local inflammation. In contrast, IgA and IgM levels were correlated between sputum and serum in ID patients, suggesting a simple mechanism of passive transfer. The same relationship was not seen in healthy controls who are presumably capable of local IgA and IgM synthesis.

Patients with antibody deficiency had significantly higher concentrations of inflammatory cytokines compared to healthy individuals in both serum and sputum, especially in those with abnormal airways. However, we did not observe clear correlations between the serum and

sputum levels which suggests compartmentalisation of inflammation. There was evidence of localized inflammation due to smoking in the IC cohort, indicated by elevated levels of the pro-inflammatory cytokine IL-6 in sputum of IC smokers.

We also measured higher levels of MMP-9 in the sputum of ID patients, consistent with a tissue damage phenotype. MMP-9 in serum of ID patients with abnormal airways was higher than in ID with normal airways, which may reflect a greater disease severity affecting not only the lung, but also the systemic circulation. Although we could not measure neutrophil elastase in the IC cohort, we did also observe higher elastase levels in patients with abnormal airways, in line with the putative role of this enzyme in the development of bronchiectasis [18].

Collectively, these results indicate a state of chronic inflammation in the airways of patients with immune deficiency, which helps to explain the persistent respiratory symptoms which compromise quality of life [4]. It is interesting to note that this is seen even in the absence of demonstrable abnormalities on cross-sectional imaging, albeit to a somewhat lesser degree. However, all immunodeficient patients in this study were able to spontaneously produce sputum which might imply either acute symptoms or a cohort with risk of progression to structural lung disease: for example, we note that MMP-9 levels were similar in patients with or without radiological lung disease. We would therefore recommend close monitoring of patients with chronic sputum production who do not currently have evidence of airways disease. Studying induced sputum from ID patients who cannot spontaneously expectorate would be interesting in future work.

Intriguingly, the concentration of IgG in the sputum correlated positively with several inflammatory markers. In large part these relationships disappeared after correction for albumin, suggesting that a passive leak of protein is the major explanation and is presumably increased in the context of inflammation. However, there were still significant correlations of IgG (after correction for albumin) with IL-1β, IL-8, TNFα and MMP-9 in ID patients. This

may indicate some mechanism of either enhanced recruitment or retention of IgG in the context of inflammation. We considered whether patients with worse symptoms may be treated with more immunoglobulin by the clinical teams, but there were no clear relationships between inflammatory markers and serum IgG concentration or IgG trough.

Unlike in the immunodeficient cohort, we observed positive correlations of inflammatory markers with IgA and IgM in immunocompetent individuals: this presumably indicates an appropriate local response to inflammation without leakage of systemic proteins.

We also assessed the airway microbiome in the study participants. Many patients with immunodeficiency exhibited marked dominance of pathogenic organisms, especially *Haemophilus* spp., in comparison to immunocompetent individuals. Correspondingly, the immunodeficient group also demonstrated far lower alpha diversity measures (indicating lower richness and evenness of the bacterial species). The alpha diversity metrics correlated negatively with MMP-9 and neutrophil elastase suggesting that dysbiosis is directly associated with host damage in inflammatory airway disease [19]. As with the inflammatory markers, alpha diversity also correlated negatively with sputum IgG concentration. Again, this suggests that sputum IgG paradoxically associates with airway pathology, presumably due to greater protein leak in the context of inflammation and dysbiosis.

These findings are underlined by the negative correlation of lung function (FEV1% predicted) with IgG, total protein and elastase.

A previous study[20] identified IgA concentration as a key driver of oropharyngeal microbiome (although intriguingly alpha diversity was actually increased in the absence of IgA, unlike observations in the gut)[21]. In our study, using sputum rather than oropharyngeal samples, we did not replicate this result and could not differentiate samples on the basis of IgA concentration.

Instead, IgG (and IgM) concentration in sputum was among the variables which helped to differentiate immunodeficient patients with normal airways from those with abnormal airways, with higher levels correlating with an increased risk of radiological airway pathology. As expected, sputum elastase and MMP-9 also helped to differentiate the groups, while lower serum IgG and sputum albumin were the final analytes in our model. If this is replicated in other cohorts, these could be useful biomarkers to predict the progression of lung disease.

Our study does have limitations. Most significantly, the healthy controls and immunodeficient patients were recruited from different sites and sputum was collected via different techniques. However, we extensively investigated the potential confounding impact of sputum induction and did not confirm any significant dilutional effect. Furthermore, the key findings remained robust even after correcting for the serum:sputum urea ratio. We do not believe there is any reason to expect that levels of immunoglobulins and inflammatory markers would be different according to country of origin of donors. Taxa identified via 16S sequencing were consistent with organisms expected in the airways, and although gastrointestinal microbiome may vary according to geographical area, the respiratory microbiome appears to be largely similar worldwide in health [22] and with airways disease [23]. Even if there were minor geographical differences, alpha diversity and the presence of pathogens should not be affected by location. Of note, an immunocompetent patient from the UK demonstrated a similar pattern to the Ukrainian donors with no obvious expansion of pathogenic taxa.

Further limitations include the inability to detect all analytes at measurable levels in all samples, and we were unable to measure neutrophil elastase in immunocompetent patients. We were also unable to measure other markers of NETosis and sputum cell counts due to sputum processing techniques and available volumes. It was not possible under current protocols to collect induced sputum samples from immunodeficient patients and this will be

valuable to further understand the respiratory tract biology of patients without overt respiratory symptoms. Furthermore, we could not collect samples from immunocompetent patients with bronchiectasis or other airways disease, e.g. non-cystic fibrosis bronchiectasis and COPD, which will be important in future work to discern the relative contribution of immune deficiency and airways disease to the findings in this study. Finally, proteases in sputum samples may have resulted in some minor degradation of other proteins (although we observed little objective evidence for this). However, as the proteases were more abundant in ID sputum samples, any degradation would have acted to weaken the majority of results (which tended to indicate increased abundance of other proteins in ID patients).

Overall, our results indicate that patients with antibody deficiency suffer from significant inflammation and dysbiosis in the airways. This is seen especially in patients with demonstrable airways disease but there are clear pathological differences from healthy controls even with apparently normal airway morphology. It may be possible in the future to measure inflammatory markers or indicators of dysbiosis in sputum (perhaps in relation to baseline values) in order to diagnose subclinical infection, even in the absence of a positive culture for pathogens.

Systemic replacement of IgG leads to good levels reaching the respiratory tract but this is insufficient to prevent pathology; in fact, higher concentrations of IgG correlated with more inflammation and lower microbial alpha diversity. In this context there is a need for further mechanistic studies to understand the exact localization of RT IgG with respect to epithelial lining fluid, mucus and relevant pathogens as well as the capacity of RT IgG to harness cellular and complement-based effector functions. Future strategies for host-directed therapy should focus on directly targeting inflammation (eg brensocatib which targets neutrophils [24]) or delivering localized therapy to the airways.

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571

572 **FIGURE LEGENDS**

573

574 **Figure 1**. **Immunoglobulins measured in paired serum and sputum of immunocompetent**
575 **and immunodeficient subjects.** Box plots showing median levels of IgA, IgM and IgG in the
576 IC (red), ID with normal airways (green) and ID with abnormal airways (blue) groups. Serum
577 levels are depicted in the upper panel, sputum in the lower panel. The lower limit of
578 quantification for IgA and IgM is indicated as a dotted black line. *p＜0.05, ***p＜0.001,
579 ****p＜0.0001, IC=immunocompetent, ID=immunodeficient.

580

581 **Figure 2**. **Cytokines measured in serum and sputum of immunocompetent and**
582 **immunodeficient subjects.** Box plots showing median concentrations of IL-1β, IL-6, IL-8,
583 TNFα, IL-5 and IL-13 measured in paired samples of IC (red) and ID subjects grouped
584 according to their airway morphology, normal airways (green) or abnormal airways (blue).
585 Serum levels are depicted in the upper panel and sputum levels in the lower panel, the
586 respective LLOQ is indicated as a dotted black line in each graph. IL-13 and IL-5 serum
587 levels were below the detection limit of the assay and were not considered for statistical
588 analysis. Extrapolated values below LLOQ are shown where applicable. *p＜0.05, **p＜
589 0.01, ***p＜0.001, ****p＜0.0001, IC=immunocompetent, ID=immunodeficient,
590 LLOQ=lower limit of quantification.

591

592 **Figure 3**. **Markers of tissue remodeling measured in serum and sputum of**
593 **immunocompetent and immunodeficient subjects**. A. MMP-9 was quantified in sputum
594 and serum of IC and ID subjects. B. Neutrophil elastase was quantified in sputum of ID

patients. Box plots with individual data of IC are shown in red, ID subjects with normal airways in green and ID with abnormal airways in blue. Extrapolated values below LLOQ (black dotted line) are shown where applicable. *p＜0.05, **p＜0.01, ****p＜0.0001, IC=immunocompetent, ID=immunodeficient, LLOQ=lower limit of quantification.

Figure 4. **Specific IgG against H. influenzae B (HiB) and S. pneumoniae (PCP)) quantified in serum and sputum.** Box plots with individual data of IC are shown in red, ID subjects with normal airways in green and ID with abnormal airways in blue. Serum levels are depicted in the upper panel and sputum levels in the lower panel, the respective LLOQ is indicated as a dotted black line in each graph. *p＜0.05, **p＜0.01, ***p＜0.001, IC=immunocompetent, ID=immunodeficient, LLOQ=lower limit of quantification

Figure 5. **Microbial abundance in the sputum of immunocompetent and immunodeficient patients, derived from 16S sequencing.** A. Taxonomy bar-plots detailing the top 20 most abundant genera across all samples, presented for IC, ID patients with abnormal airways or normal airways. Each bar represents one patient and samples are presented in each group according to the abundance of *Haemophilus*. Asterisk indicates sample from a non-ID asthmatic patient in the UK for comparability reasons. B. Summary bar-plots for each group presenting overall mean prevalence of top 20 genera. IC=immunocompetent, ID=immunodeficient

Figure 6. **Microbial alpha diversity in the sputum of immunocompetent and immunodeficient patients**. A. Alpha diversity as measured by number of species and Shannon index for IC, ID patients with abnormal airways or normal airways. B. Alpha

diversity metrics versus $\log_{10}$ MMP-9 concentration and $\log_{10}$ IgG concentration in sputum of immunodeficient patients. Green squares represent ID patients with abnormal airways, blue circles represent ID patients with normal airways. All correlations significant ($p<0.05$). ***p ＜0.001, IC=immunocompetent, ID=immunodeficient

Figure 7. Linear combination of serum IgG and MMP-9, sputum IgM, albumin, IgG and elastase for classification between normal and abnormal airways in immunodeficient patients. Variable selection for airways status response was carried out by elastic net, the final fit with selected biomarkers was performed with logistic regression. Coefficients can be found in supplementary table 3. ***p＜0.001, ID=immunodeficient

TABLES

Table 1. Patient demographics. Descriptive table with median [IQR] or counts (Percentages). CVID=common variable immunodeficiency, FEV=forced expiratory volume, IC=immunocompetent, ID=immunodeficient, i.v.=intravenous, IQR=inter quartile range, NA=not available, s.c.=subcutaneous, SID=secondary immune deficiency, unkn=unknown, XLA= X-linked agammaglobulinaemia

	IC (n = 31), median [IQR] or counts (%)	**ID normal (n = 18), median [IQR] or counts (%)**	**ID abnormal (n = 49), median [IQR] or counts (%)**
Age, years	33.00, [27.50, 40.00]	48.00, [36.25, 57.00]	57.00, [40.00, 71.00]
Diagnosis	Immunocompetent control - 31 (100.0%)	CVID - 12 (66.7%), XLA - 1 (5.6%), SID † - 4 (22.2%), Other - 1 (5.6%)	CVID - 26 (53.1%), XLA - 8 (16.3%), SID † - 10 (20.4%), Other - 5 (10.2%)
Immunoglobulin administration route	none - 31 (100.0%)	IV - 12 (66.7%), SC - 4 (22.2%), unkn - 2 (11.1%)	IV - 33 (67.3%), SC - 12 (24.5%), unkn - 4 (8.2%)
IgG trough, g/L	NA	9.07, [7.73, 10.35]	9.70, [8.45, 10.60]
Inhaled steroids intake	No steroids - 31 (100.0%)	No steroids - 14 (77.8%), Steroids - 4 (22.2%)	No steroids - 34 (69.4%), Steroids - 15 (30.6%)
Prophylactic antibiotics intake *	No Abx - 31 (100.0%)	Abx - 4 (22.2%), No Abx - 14 (77.8%)	Abx - 27 (55.1%), No Abx - 22 (44.9%)
Smoking status	No - 15 (48.4%), Yes - 16 (51.6%)	No - 13 (72.2%), Past - 5 (27.8%)	No - 29 (59.2%), Yes - 5 (10.2%), Past - 13 (26.5%), unreported- 2 (4.1%)
Self-reported respiratory infection frequency (categories specified below)	NA	1 - 4 (22.2%), 2 - 1 (5.6%), 3 - 3 (16.7%), 4 - 4 (22.2%), 5 - 5 (27.8%), 6 – 1 (5.6%)	1 - 11 (22.4%), 2 - 5 (10.2%), 3 - 8 (16.3%), 4 - 16 (32.7%) 5 - 6 (12.2%), 6 - 1 (2.0%), unkn - 2 (4.1%)
FEV1 % predicted (closest to sampling)	NA	101.0, [83.0, 107.0]	86.0, [72.0, 100.8]

Categories self-reported respiratory infection frequency: 1=at least once a month, **2**=less than once a month but at least 6 times per year, **3**=about 4 or 5 times per year, **4**=about 2 or 3 times per year, **5**=about once a year, **6**=less than once a year

* Prophylactic antibiotics comprised: amoxicillin (1), azithromycin (17), cephalexin (1), ciprofloxacin (1), clarithromycin (1), co-trimoxazole (3), doxycycline (4), nebulized colomycin plus azithromycin (1), co-trimoxazole plus azithromycin (1), unknown (1).

† Underlying diagnoses comprised: chronic lymphocytic leukaemia (2), eosinophilic granulomatosis with polyangiitis (1), chronic corticosteroid usage (1), non-Hodgkin's lymphoma (5), plasma cell dyscrasias (3),

	IC (n = 31), median [IQR] or counts (%)	ID normal (n = 18), median [IQR] or counts (%)	ID abnormal (n = 49), median [IQR] or counts (%)
rheumatoid arthritis (1), systemic lupus erythematosus (1).			

635

Figure No. 1

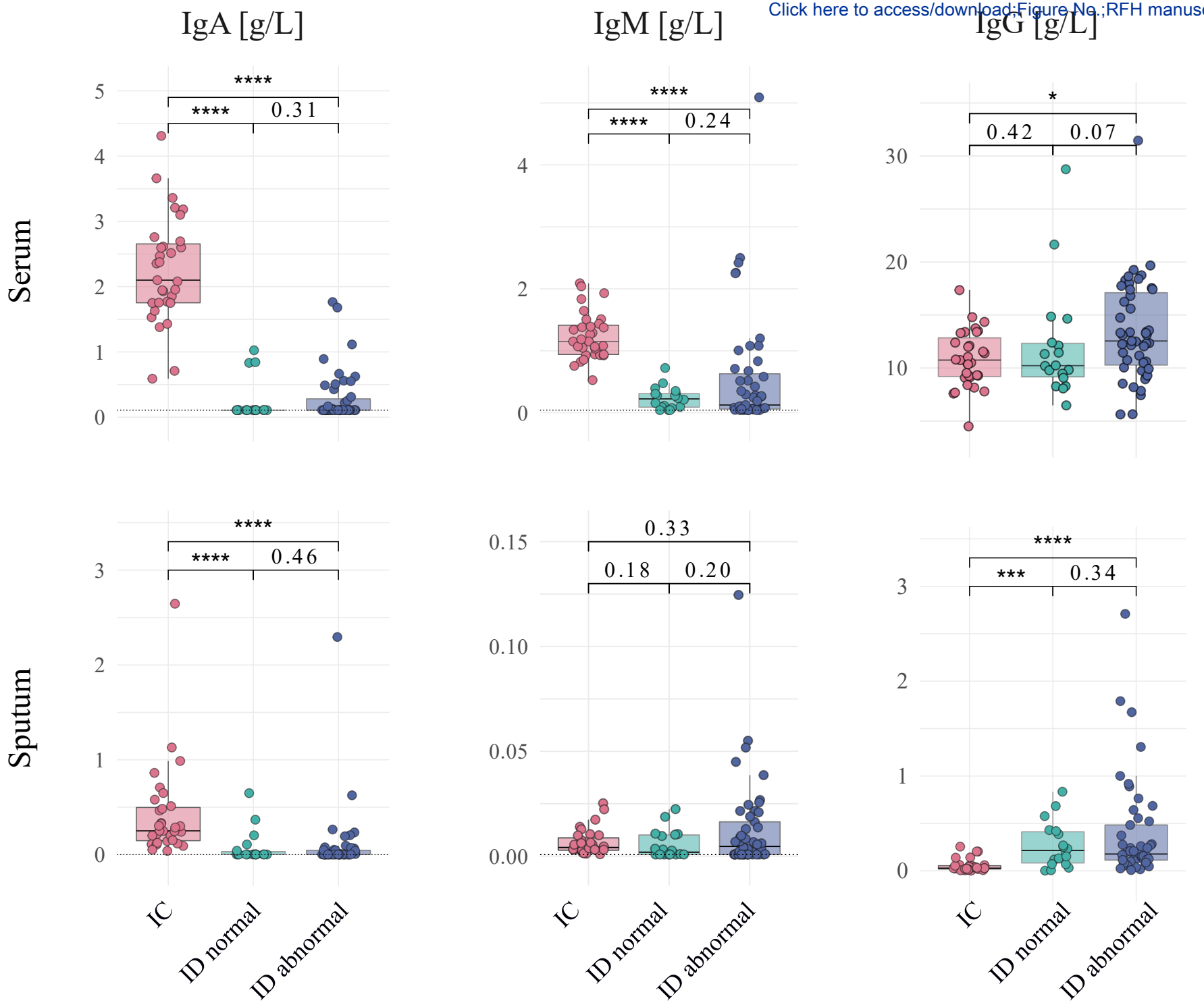


Figure 1

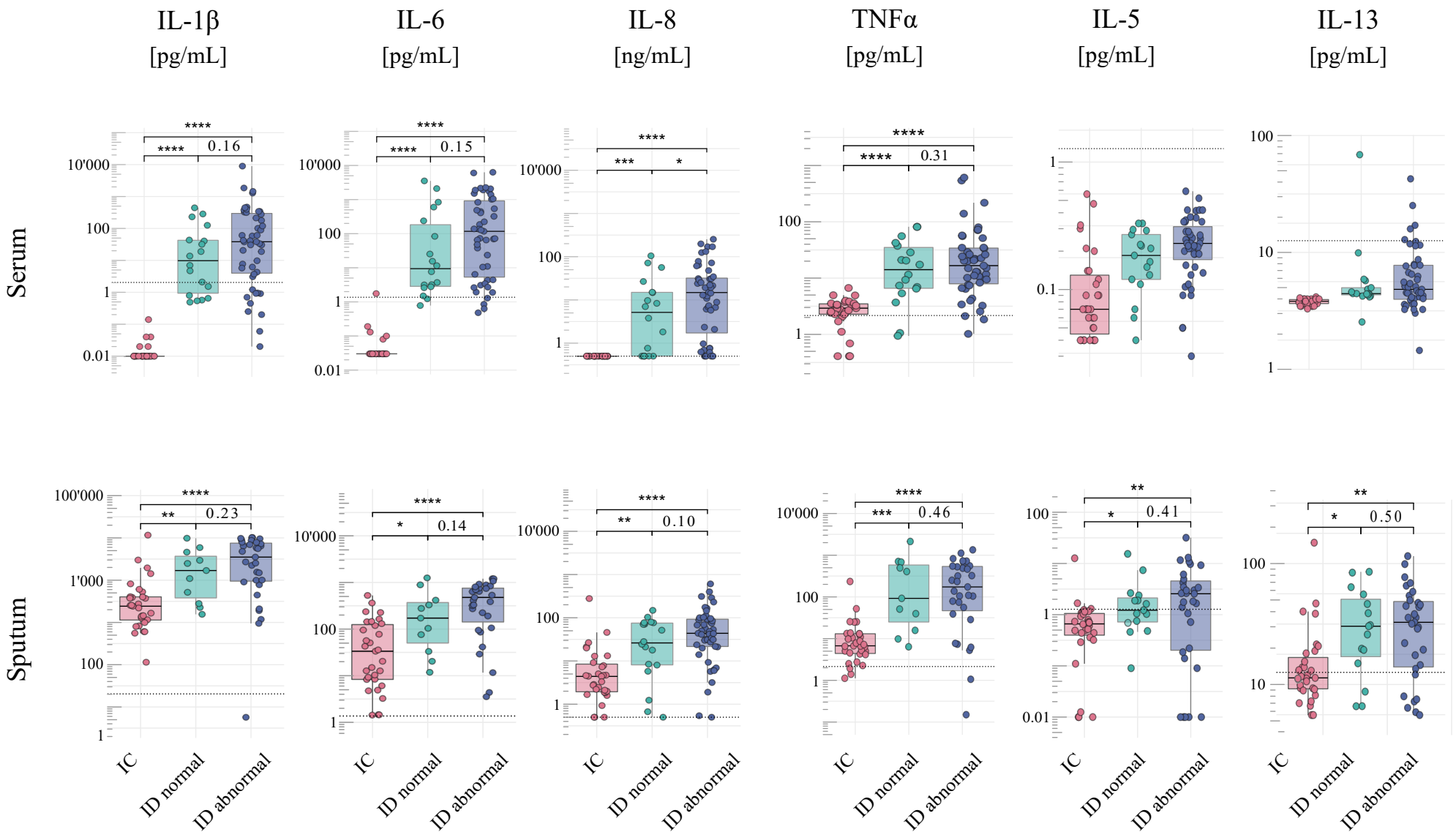
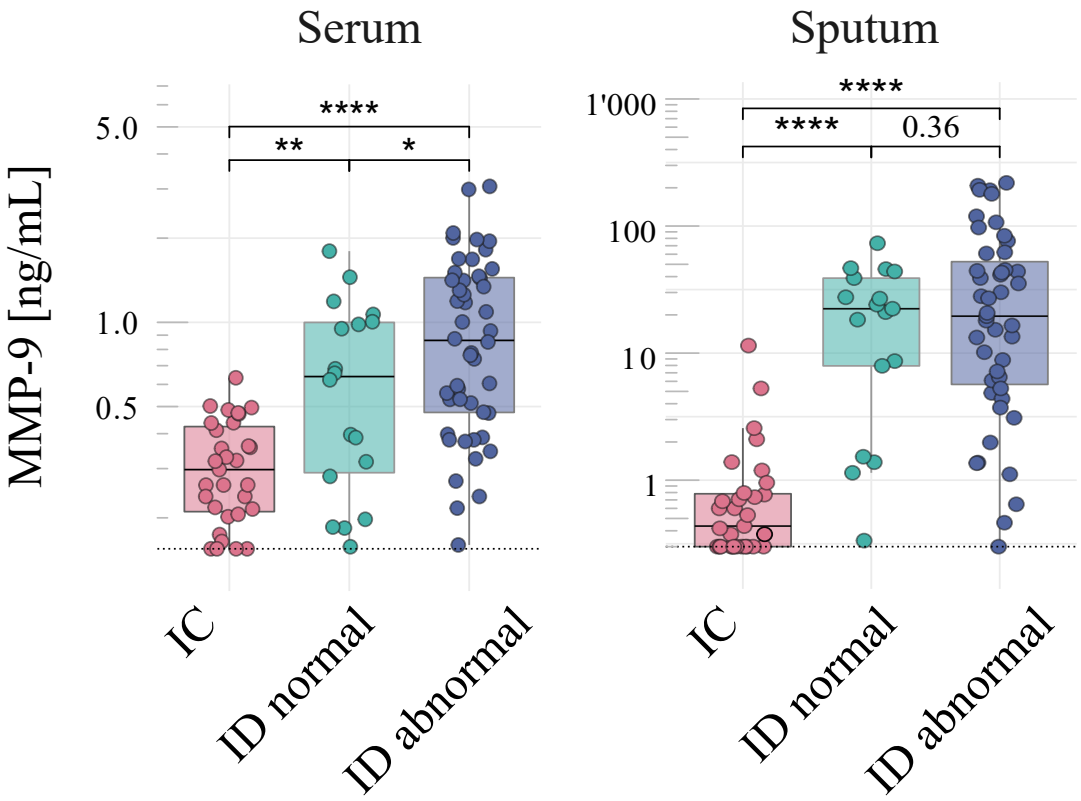


Figure 2

A



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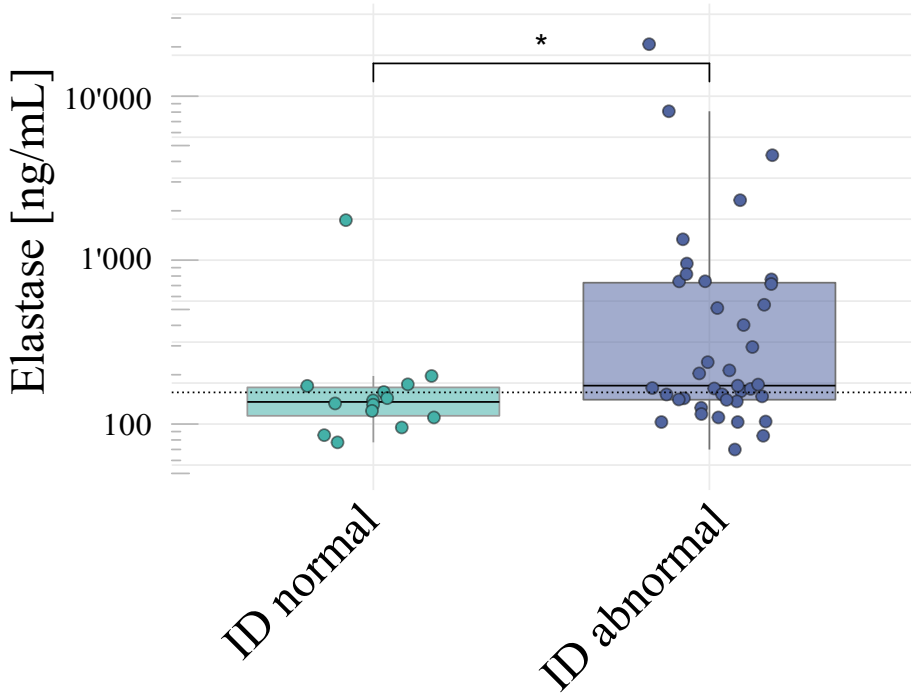

Figure 3

Figure No. 4

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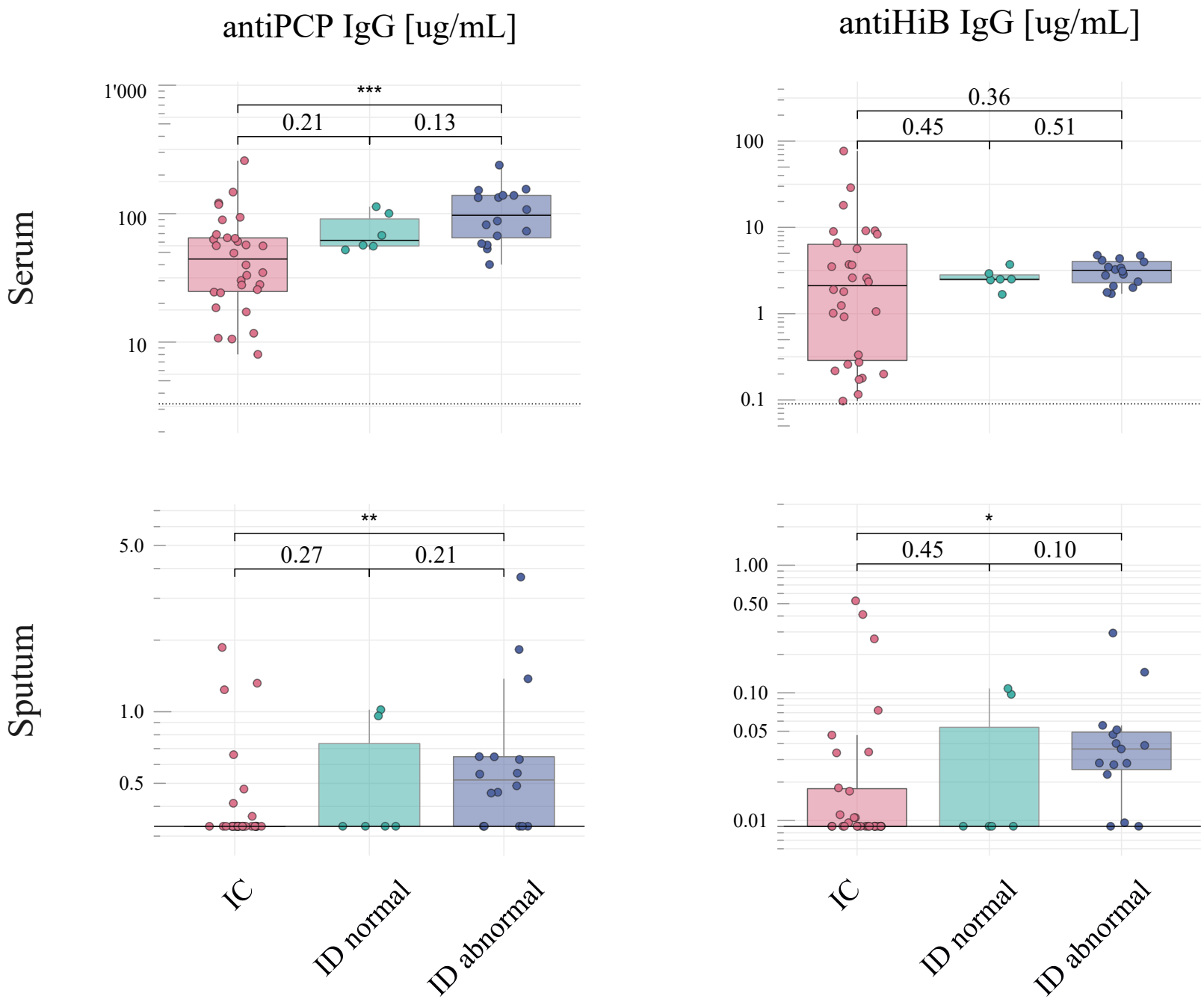


Figure 4

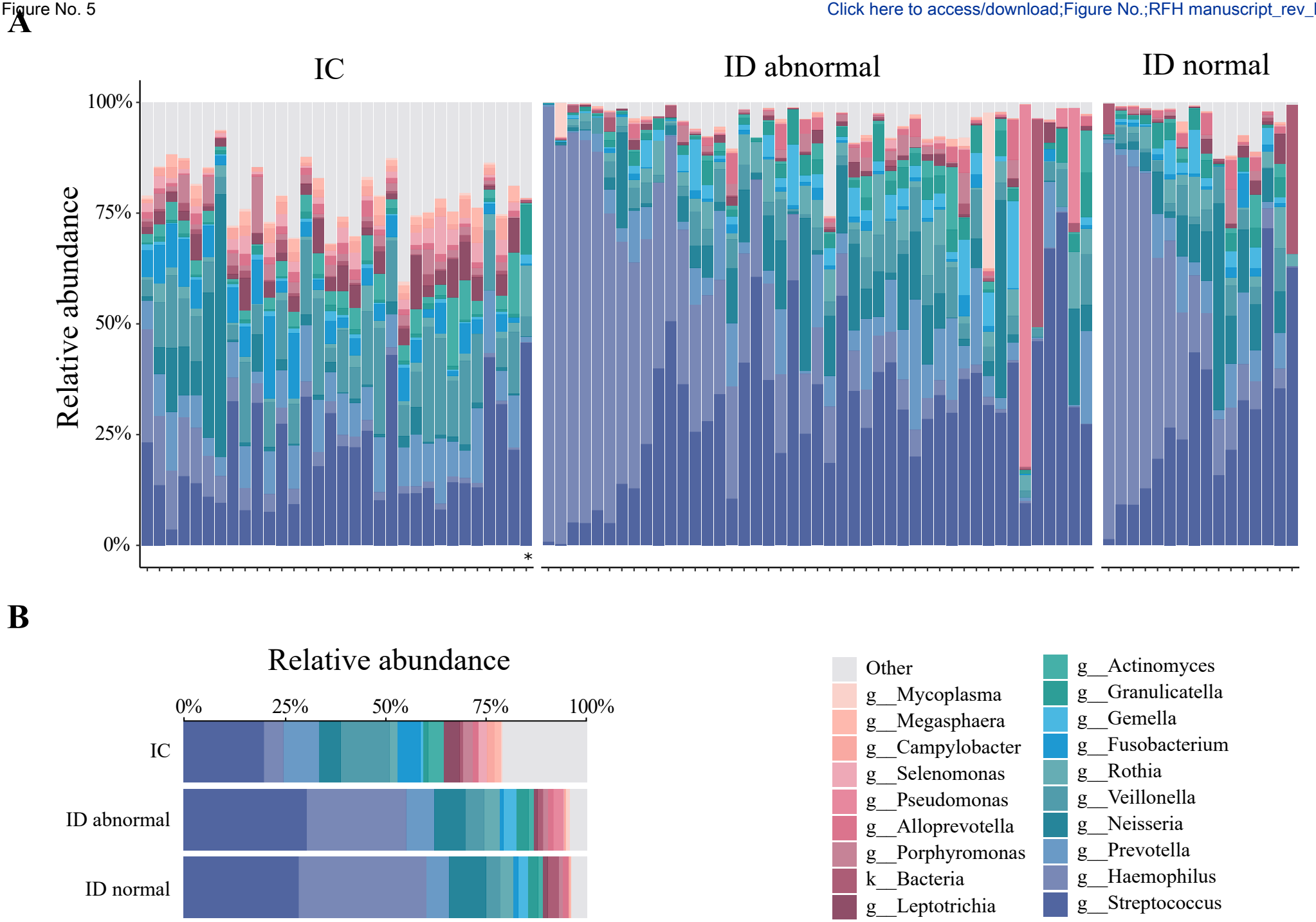


Figure 5

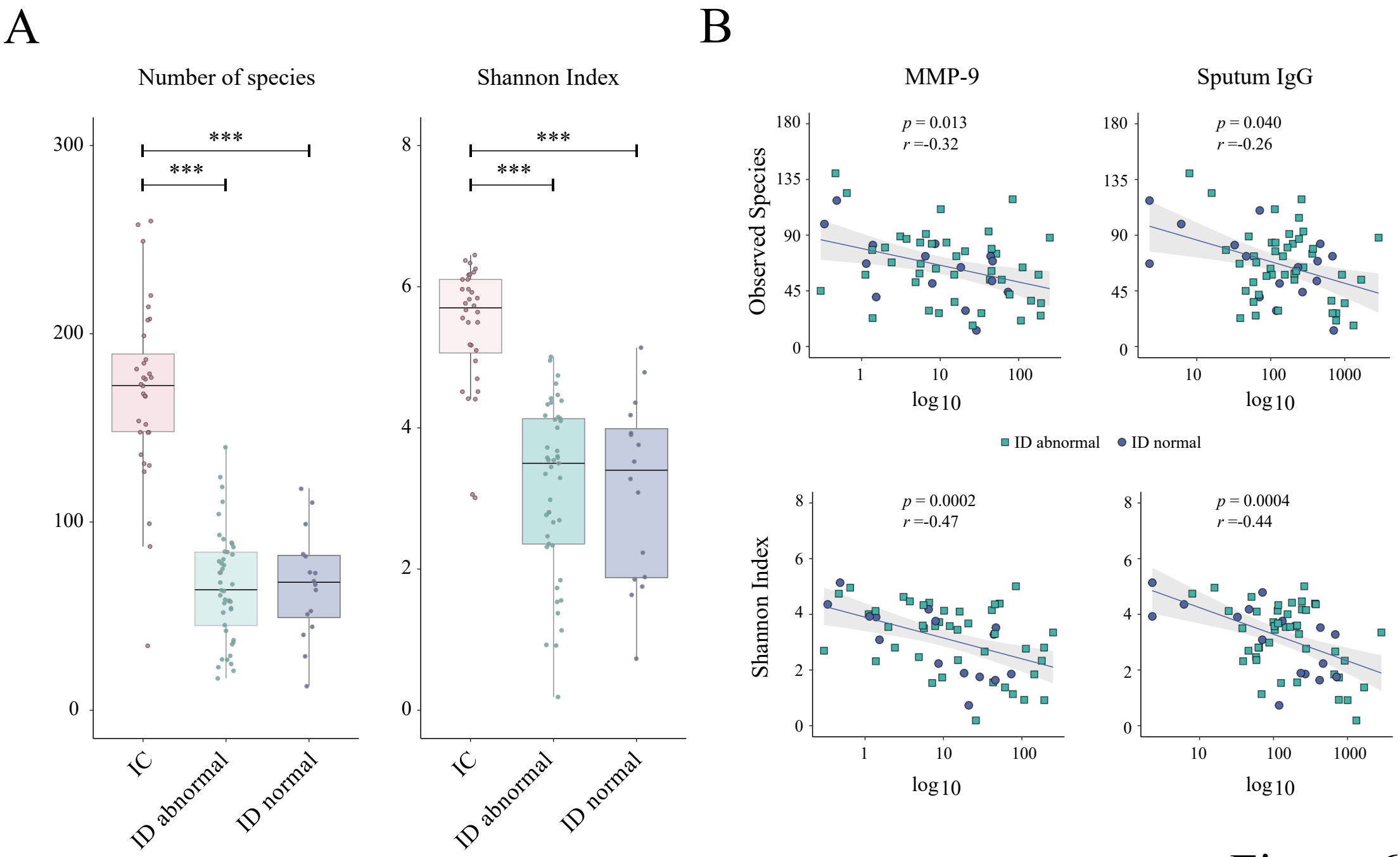


Figure 6

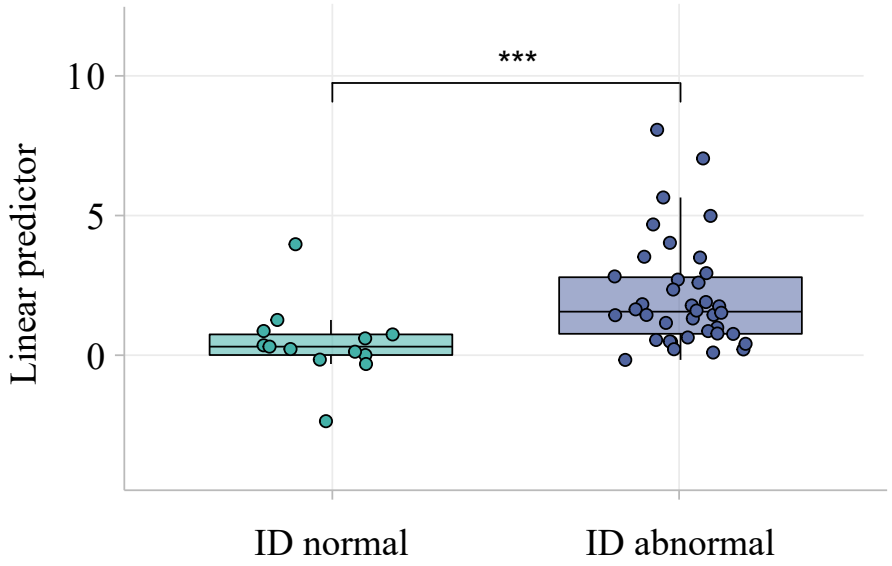


Figure 7

Supplementary material

Supplementary Figure 1. IgG trough levels in immunodeficient (ID) patients with normal (green) and abnormal airways (blue). Comparison not significant, $p > 0.05$. Note one outlier with very high IgG trough due to IgG paraprotein (grey dot).

Supplementary Figure 2. Linear regression analysis of Ig levels in sputum and serum of immunocompetent subjects (IC, red) and immunodeficient patients (ID, blue). Goodness of fit shown by r^2 and p-value in the corresponding plots.

Supplementary Figure 3. Subclass IgG distribution in serum and sputum of a subset of immunocompetent (IC) and immunodeficient (ID) subjects. No significant differences between IC and ID groups, $p > 0.05$.

Supplementary Figure 4. Immunocompetent (IC) subjects were sub-grouped into non-smokers (pink dots) and smokers (red dots) to assess the effect of smoking on the immunological status of the lung. Among the pro-inflammatory cytokines investigated, a significant increase was observed for IL-6 ($p = 0.022$) in sputum of smokers, while no difference was seen for IL-8, MMP-9, TNFα, IL-1β, IL-5 and IL-13.

Supplementary Figure 5. C-reactive protein (CRP) levels in serum of immunodeficient (ID) patients grouped according to airway morphology. Comparison not significant, $p > 0.05$.

Supplementary Figure 6. Immunoblot comparing IgG fragmentation pattern in sputum of IC and ID subjects. Normal human IgG was digested by increasing amounts of human neutrophil elastase (HNE) as a control for IgG cleavage (lanes 2-4) and compared to sputum samples from IC controls (lanes 5-7) and sputum from ID patients (lanes 8-14). All samples were loaded at an equal IgG concentration of 0.25µg IgG per lane and run under non-reduced conditions. Polyclonal rabbit anti-human IgG/HRP (Dako) was used. Full length IgG band (~180 kDa), combinations of Fab, Fc-fragments (between 150 and 75kDa) and Fab, Fc fragment (~50kDa) were observed. Additional bands were visible when IgG was digested with HNE and also appear weakly in some sputum samples (arrows). Markers (lanes 1 and 15) were run on both sides of the gel for size comparison.

Supplementary Figure 7. Spearman correlation matrices of variables measured in sputum and serum of immunodeficient (A) and immunocompetent (B) subjects. Age and lung function (FEV1 % predicted) were included. Significant correlation coefficients after spearman are shown ($p < 0.05$), positive correlations are shown in red, negative correlation in blue. Asterisks marks IgG sputum corrected with sputum albumin. FEV= forced expiratory volume

Supplementary Table 1. Lower limits of quantification of immuno-based methods in sputum and serum samples. LLOQ=lower limit of quantification.

Parameters	LLOQ	units
IgA serum	0.108	g/L
IgA sputum	0.00156	g/L
IgM serum	0.0417	g/L
IgM sputum	0.00078	g/L
MMP9 sputum	0.3	ug/ml
MMP9 serum	0.155	ug/ml
IL8 serum/ sputum	0.5	ng/ml
IL6 serum/ sputum	0.001366	ng/ml
TNFa serum/ sputum	0.002144	ng/ml
IL1b serum/ sputum	0.002038	ng/ml
IL5 serum/ sputum	0.001274	ng/ml
IL13 serum/ sputum	0.012598	ng/ml
Elastase sputum	156	ng/ml
aHiB IgG serum	0.09	ug/ml
aHiB IgG sputum	0.009	ug/ml
aPCP IgG serum	3.3	ug/ml
aPCP IgG sputum	0.33	ug/ml

Supplementary Table 2. Median and IQR of variables quantified in sputum and serum of immunocompetent (IC) subjects and immunodeficient (ID) subjects with normal and abnormal airways. Kruskal-Wallis p-value and pairwise Dunn adjusted p-values are shown in a separate column; (a) IC vs. ID normal, (b) IC vs. ID abnormal, (c) ID normal vs. ID abnormal. Normal ranges for serum immunoglobulins (d) IgG 6-16 g/L, (e) IgA 0.8-3 g/L, (f) IgM 0.4-2.5 g/L (source: https://www.ouh.nhs.uk/immunology/diagnostic-tests/tests-catalogue/immunoglobulins.aspx)

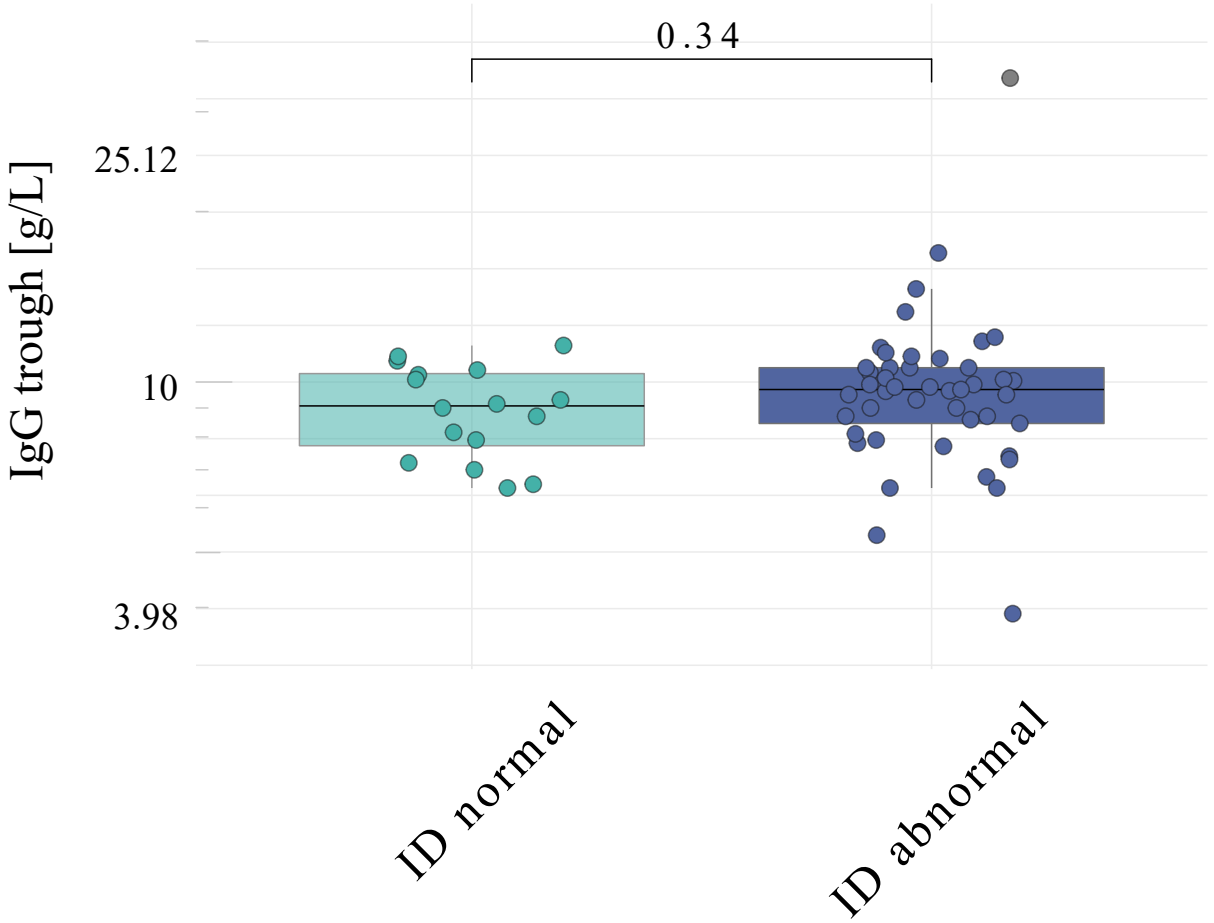
	IC (n = 31), median [IQR]	**ID normal (n = 18), median [IQR]**	**ID abnormal (n = 49), median [IQR]**	**Kruskall-Wallis p-value and pairwise Dunn adjusted p-values**
Total protein sputum, mg/ml	1.16, [0.85, 1.74]	2.94, [1.27, 3.87]	2.95, [2.28, 6.13]	< 0.005 ; (a) < 0.005; (b) 0.007; (c) 0.073
Albumin sputum, ug/mL	135.58, [65.56, 184.11]	473.92, [295.57, 1538.40]	406.50, [274.46, 980.74]	< 0.005 ; (a) < 0.005; (b) < 0.005; (c) 0.297
IgG sputum, ug/mL	28.89, [19.25, 54.44]	214.48, [83.02, 411.72]	177.50, [113.64, 484.15]	< 0.005 ; (a) < 0.005; (b) < 0.005; (c) 0.340
IgA sputum, ug/mL	250.66, [146.59, 497.22]	1.56, [1.56, 31.35]	1.56, [1.56, 46.13]	< 0.005 ; (a) < 0.005; (b) < 0.005; (c) 0.461
IgM sputum, ug/mL	4.10, [2.80, 8.70]	1.85, [0.78, 10.06]	4.60, [0.78, 16.34]	0.284 ; (a) 0.326; (b) 0.181; (c) 0.195
IL8 sputum, ng/mL	4.40, [1.94, 8.50]	26.23, [8.14, 75.35]	43.73, [21.66, 92.45]	< 0.005 ; (a) < 0.005; (b) < 0.005; (c) 0.099
MMP9 sputum, ug/mL	0.44, [0.30, 0.78]	22.35, [7.94, 38.93]	19.54, [5.69, 53.03]	< 0.005 ; (a) < 0.005; (b) < 0.005; (c) 0.357
IL6 sputum, pg/mL	33.52, [8.33, 124.98]	171.93, [54.64, 373.36]	477.79, [148.34, 755.05]	< 0.005 ; (a) < 0.005; (b) 0.019; (c) 0.138
TNF-alpha sputum, pg/mL	6.72, [4.41, 13.07]	92.35, [27.70, 589.15]	173.36, [47.37, 537.97]	< 0.005 ; (a) < 0.005; (b) < 0.005; (c) 0.463
IL1beta sputum, pg/mL	242.23, [114.14, 403.35]	1691.74, [398.41, 3779.39]	3517.56, [949.74, 7530.91]	< 0.005 ; (a) < 0.005; (b) 0.009; (c) 0.233
IL5 sputum, pg/mL	0.66, [0.40, 1.06]	1.22, [0.72, 2.13]	2.56, [0.20, 4.56]	0.008 ; (a) 0.005; (b) 0.033; (c) 0.412
IL13 sputum, pg/mL	11.34, [9.20, 16.77]	30.33, [17.14, 50.95]	32.67, [13.97, 48.56]	< 0.005 ; (a) < 0.005; (b) 0.012; (c) 0.498

	IC (n = 31), median [IQR]	ID normal (n = 18), median [IQR]	ID abnormal (n = 49), median [IQR]	Kruskall-Wallis p-value and pairwise Dunn adjusted p-values
Elastase sputum, ng/ml	NA	136.53, [112.38, 167.50]	171.56, [140.84, 728.36]	0.029 ;
Urea sputum, mmol/l	1.67, [1.24, 2.30]	2.44, [2.04, 4.38]	1.96, [1.17, 2.70]	0.028 ; (a) 0.413; (b) 0.019; (c) 0.015
Albumin serum, mg/mL	47.60, [45.48, 52.67]	45.55, [42.84, 49.44]	46.15, [43.87, 48.95]	0.120 ; (a) 0.087; (b) 0.109; (c) 0.449
IgG serum, g/L, (d)	10.75, [9.19, 12.85]	10.22, [9.17, 12.33]	12.55, [10.28, 17.10]	0.027 ; (a) 0.021; (b) 0.422; (c) 0.066
IgA serum, g/L, (e)	2.10, [1.75, 2.66]	0.11, [0.11, 0.11]	0.11, [0.11, 0.28]	< 0.005 ; (a) < 0.005; (b) < 0.005; (c) 0.313
IgM serum, g/L, (f)	1.15, [0.94, 1.41]	0.22, [0.09, 0.31]	0.12, [0.06, 0.63]	< 0.005 ; (a) < 0.005; (b) < 0.005; (c) 0.238
IL8 serum, ng/ml	0.50, [0.50, 0.50]	5.39, [0.50, 14.99]	14.81, [1.80, 32.00]	< 0.005 ; (a) < 0.005; (b) < 0.005; (c) 0.047
MMP9 serum, ug/ml	0.30, [0.21, 0.42]	0.64, [0.29, 1.00]	0.86, [0.48, 1.44]	< 0.005 ; (a) < 0.005; (b) 0.007; (c) 0.028
IL6 serum, pg/ml	0.03, [0.03, 0.03]	9.60, [2.87, 199.55]	117.19, [5.42, 925.42]	< 0.005 ; (a) < 0.005; (b) < 0.005; (c) 0.145
TNF-alpha serum, pg/ml	2.92, [2.28, 3.48]	14.40, [6.60, 35.21]	16.64, [7.93, 34.09]	< 0.005 ; (a) < 0.005; (b) < 0.005; (c) 0.312
IL1-beta serum, pg/ml	0.01, [0.01, 0.01]	10.34, [0.98, 42.66]	38.36, [3.98, 296.52]	< 0.005 ; (a) < 0.005; (b) < 0.005; (c) 0.158
IL5 serum, pg/ml	0.07, [0.04, 0.13]	0.18, [0.12, 0.27]	0.23, [0.17, 0.31]	NA
IL13 serum, pg/ml	3.82, [3.65, 3.95]	4.44, [4.33, 4.99]	4.83, [3.97, 7.76]	NA
IgG trough, g/L	NA	9.07, [7.73, 10.35]	9.70, [8.45, 10.60]	0.341 ;
Urea serum, mmol/L	5.32, [4.79, 6.34]	4.87, [3.32, 8.54]	6.09, [4.66, 7.65]	0.518 ; (a) 0.391; (b) 0.389; (c) 0.473
CRP serum, mg/L	NA	4.00, [2.25, 7.88]	5.00, [2.50, 8.00]	0.771 ;

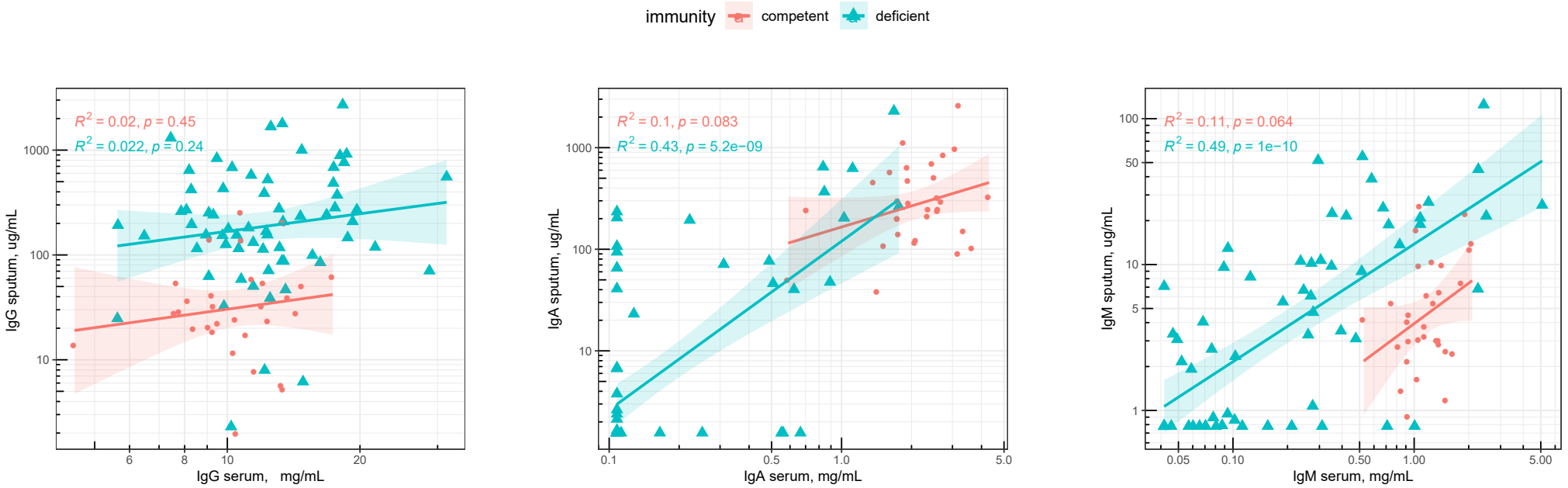
	IC (n = 31), median [IQR]	ID normal (n = 18), median [IQR]	ID abnormal (n = 49), median [IQR]	Kruskall-Wallis p-value and pairwise Dunn adjusted p-values
aPCP serum, ug/mL	44.57, [24.77, 64.74]	62.23, [56.06, 92.19]	97.64, [64.94, 138.73]	< 0.005 ; (a) < 0.005; (b) 0.212; (c) 0.127
aPCP sputum, ug/mL	0.33, [0.33, 0.33]	0.33, [0.33, 0.80]	0.52, [0.33, 0.65]	0.017 ; (a) 0.006; (b) 0.266; (c) 0.205
aHiB serum, ug/mL	2.12, [0.29, 6.38]	2.51, [2.47, 2.82]	3.18, [2.28, 4.03]	0.489 ; (a) 0.355; (b) 0.453; (c) 0.513
aHiB sputum, ug/mL	0.01, [0.01, 0.02]	0.01, [0.01, 0.08]	0.04, [0.03, 0.05]	0.026 ; (a) 0.012; (b) 0.453; (c) 0.104

Supplementary Table 3. Estimated coefficients of the model predicting abnormal airways status in immunodeficient subjects. Variable selection for airways status as the dependent variable was carried out by elastic net, the final fit with selected biomarkers was performed with logistic regression

	Airways status response		
Predictors	*Odds Ratios*	*CI*	*p*
(Intercept)	0.37	0.00 – 6341.62	0.842
IgM sputum	1.39	0.81 – 2.61	0.254
IgG serum	0.73	0.06 – 9.11	0.798
Albumin sputum	0.42	0.12 – 1.23	0.132
MMP9 serum	2.04	0.71 – 6.87	0.206
IgG sputum	1.60	0.72 – 3.83	0.255
Elastase sputum	3.05	0.98 – 17.48	0.121
Observations	51		
R^2 Adj	0.225		



Suppl. Figure 1



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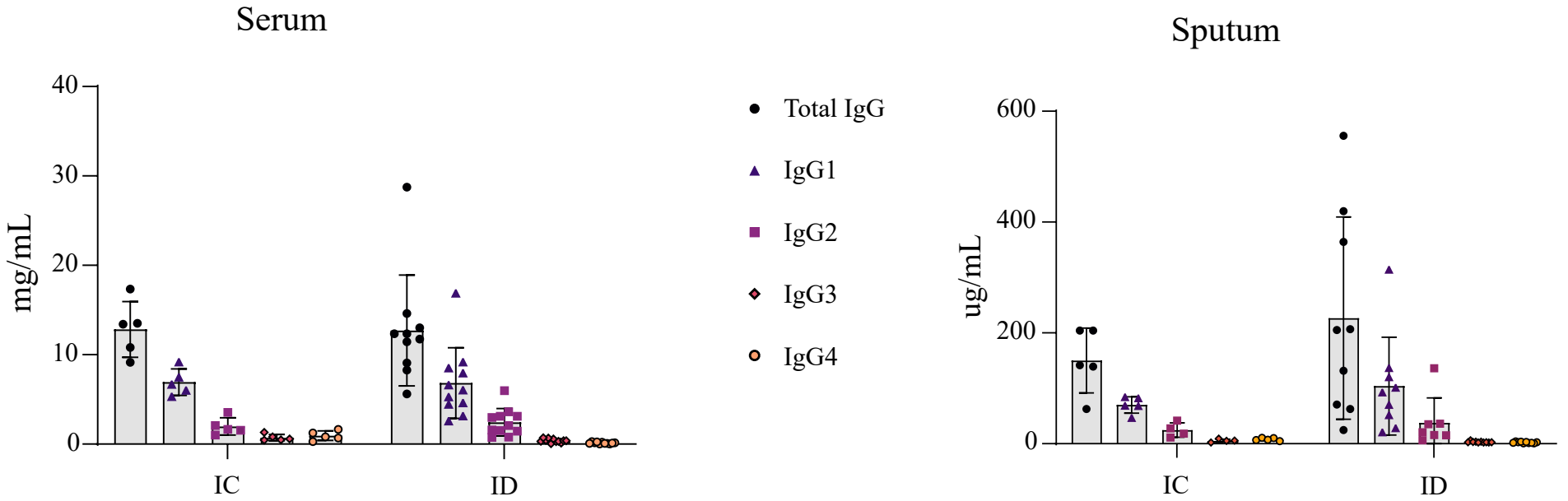

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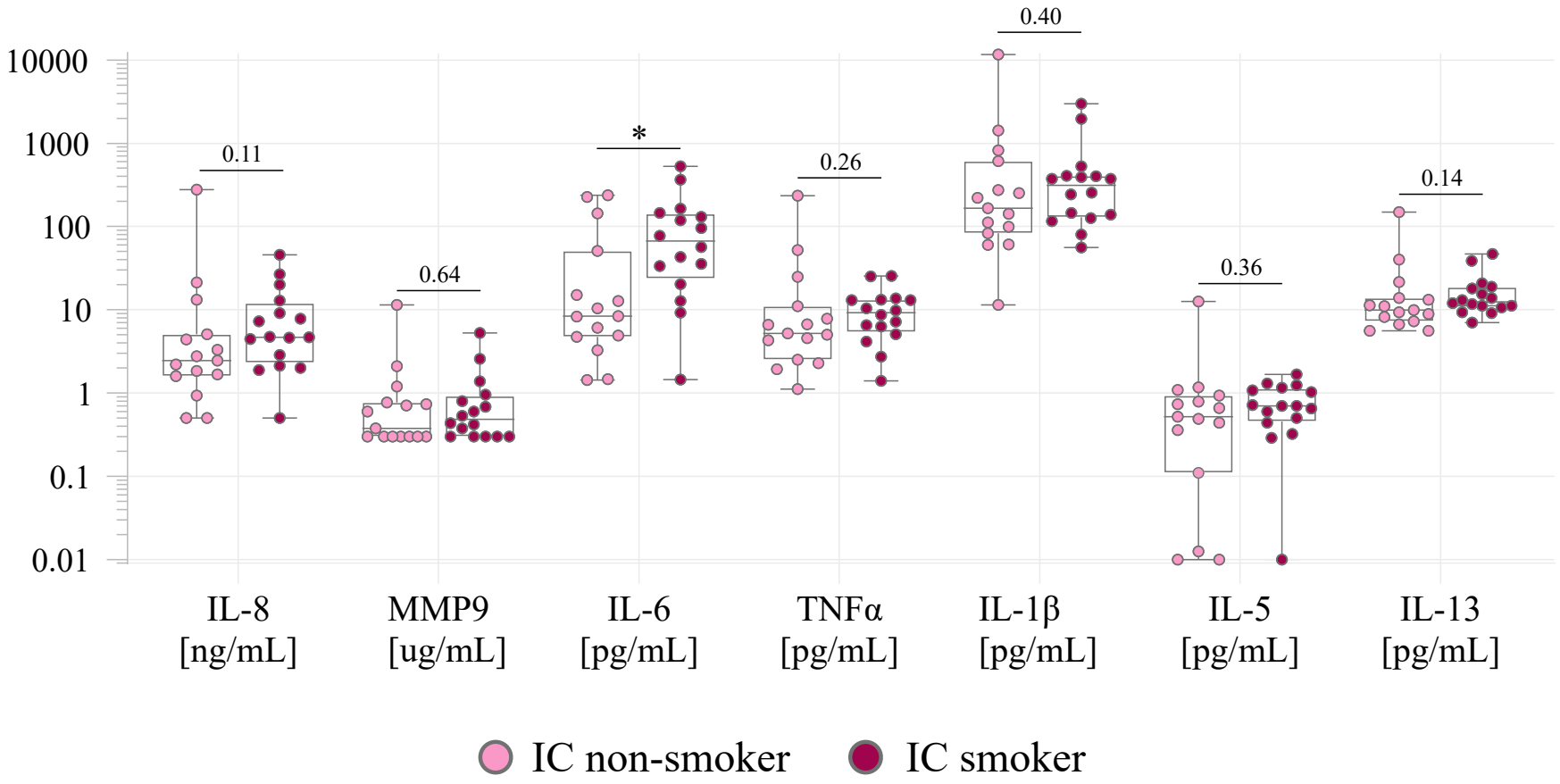

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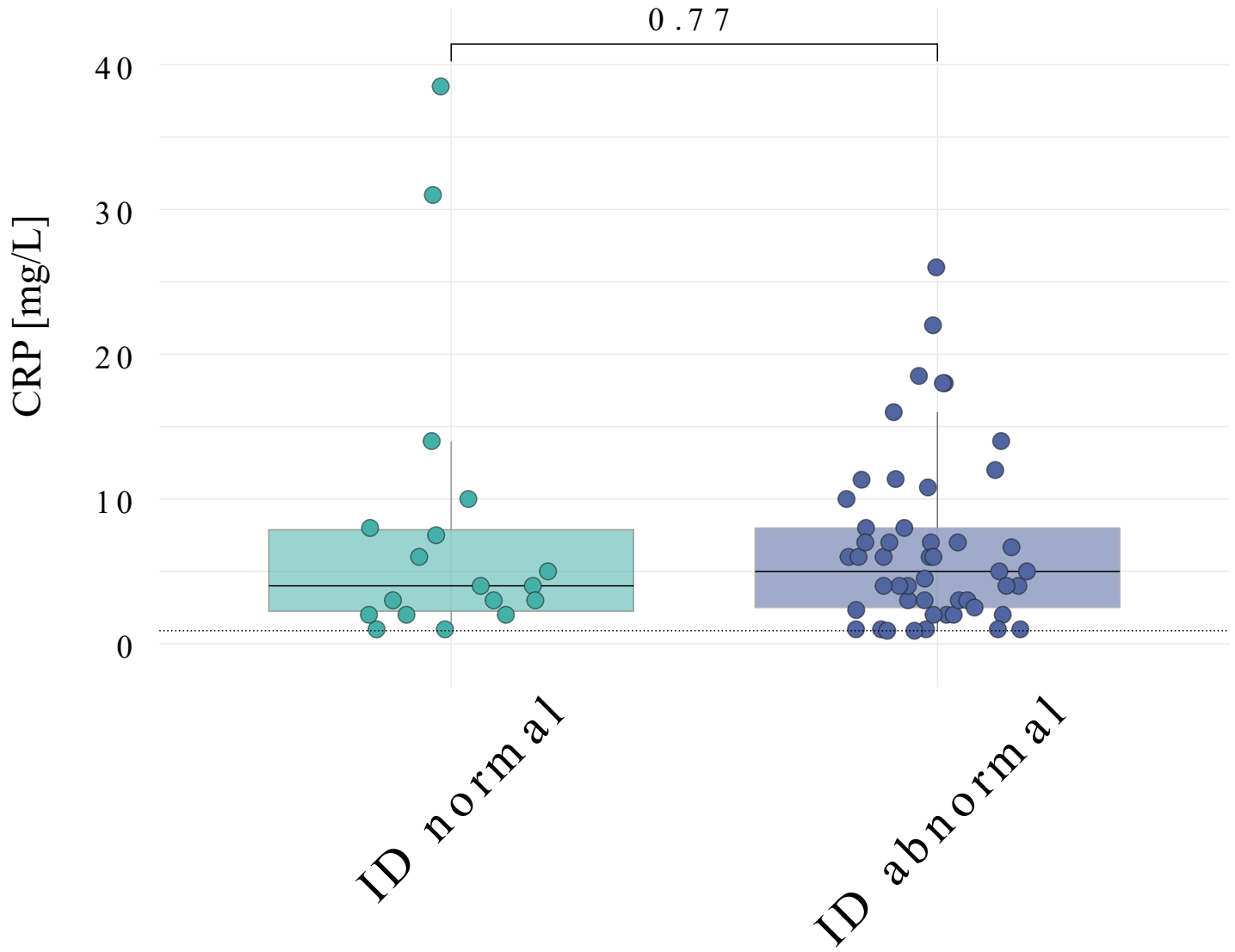

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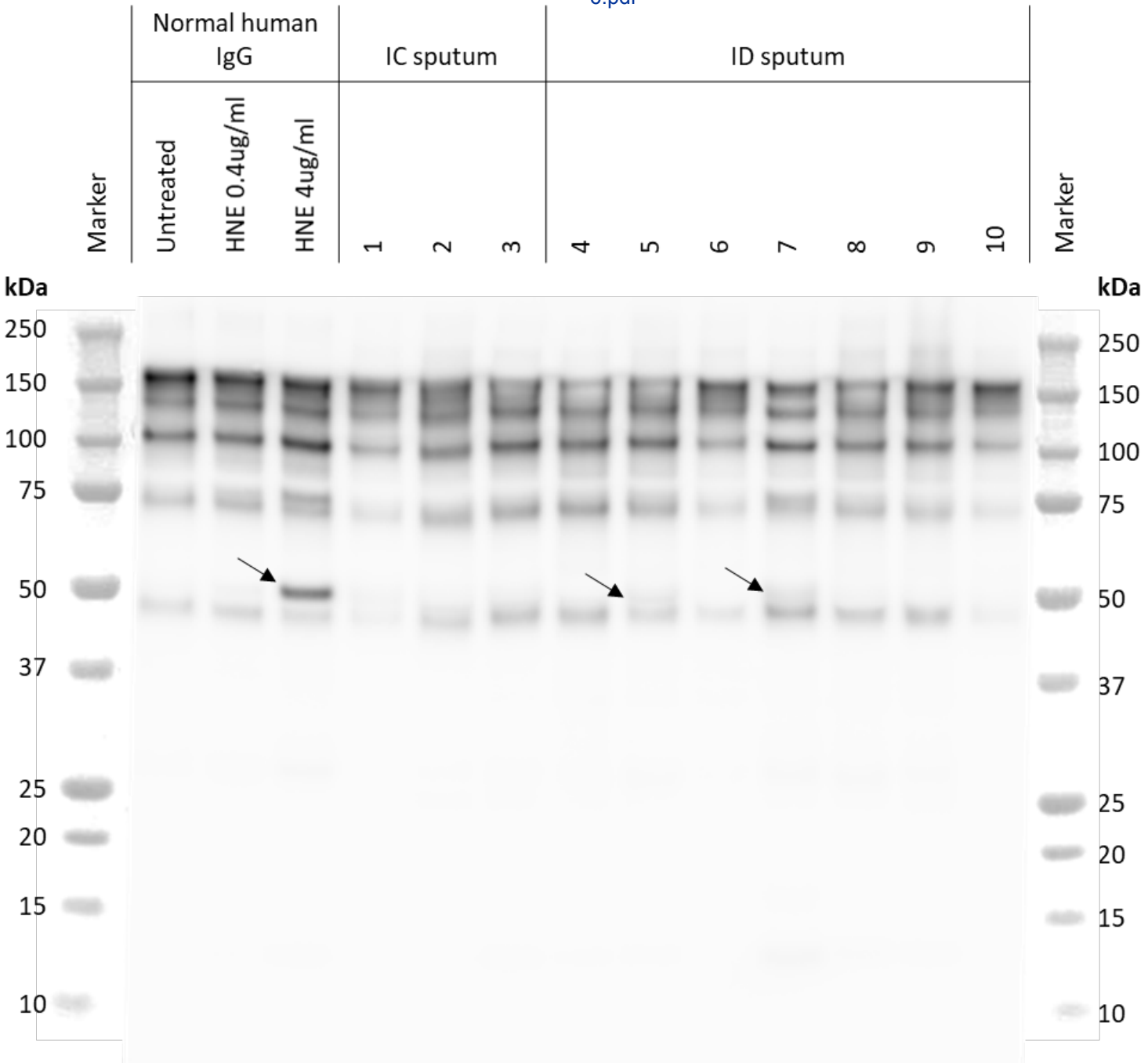

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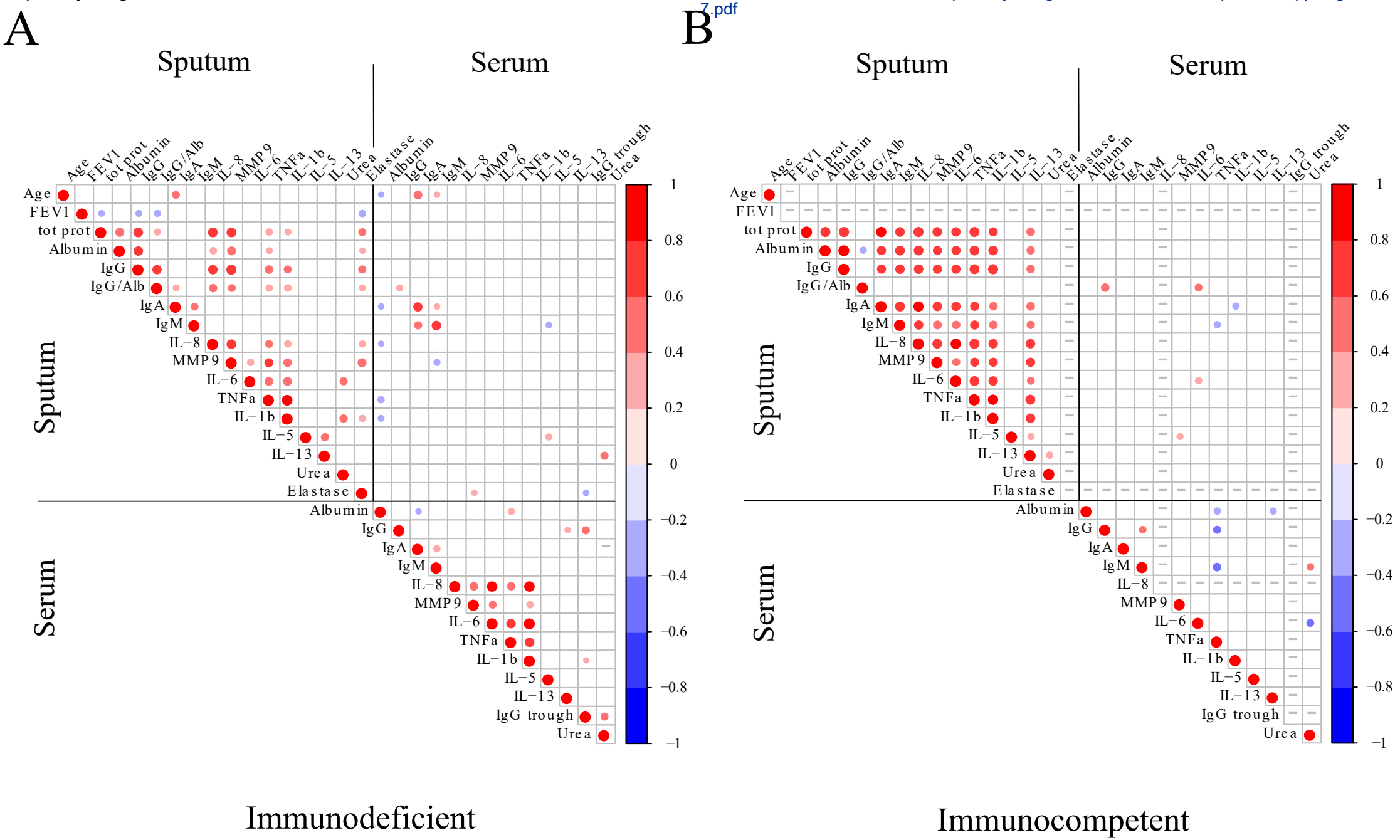

Suppl. Figure 7