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Association of endopeptidases, involved in SARS-CoV-2 infection, with microbial aggravation in sputum of severe asthma

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To the Editor,

COVID-19 can be a serious multi-system disease caused by the SARS-CoV-2 coronavirus, and the current pandemic has affected more than 80 million people and caused nearly two million deaths worldwide. The SARS-CoV-2 virus attaches to angiotensin-converting enzyme 2 (ACE2) receptors on the host cell membrane, with the help of dipeptidyl peptidase 4 (DPP4), both exopeptidases [1]. Cleavage of the virus spike protein (S-protein) by endopeptidases, such as transmembrane protease, serine 2 (TMPRSS2) and Furin, occurs following which the virus enters the host cell leading to virus replication [1]. Other enzymes, such as the

sialyltransferases; ST6GAL1 and ST3GAL4, play a role for the synthesis of influenza A virus entry receptors [2], however their role in SARS-CoV-2 infection has not been elucidated.

Asthma is a chronic inflammatory airway disease affecting 350 million people worldwide. It has not been linked to serious outcomes when presenting with COVID-19 infection, although a higher risk of death has been reported in severe asthma populations [3]. The heterogeneous inflammatory nature of asthma raises the possibility that the type of asthmatic inflammation might determine the outcome of SARS-CoV-2 infection in asthma. Type-2 (T2) inflammatory markers have been associated with decreased ACE2 expression in asthma [4, 5], that could underlie the reduced risk of SARS-CoV-2 infection in asthmatics. In contrast, non-T2 asthma, particularly neutrophilic asthma, has been associated with higher ACE2 and endopeptidases (TMPRSS2 and Furin) expression as compared with the T2-high phenotype [4, 5], that might imply a worse outcome with COVID-19 infection.

Airway microbial imbalances has been reported in asthma, particularly in severe non-T2 asthma, and is characterized by decreased microbial α-diversity with increased pathogenic bacterial abundances in association with neutrophilia [6]. Endopeptidases involved in S-protein cleavage such as Furin may also play a role in the cleavage of pathogenic bacteria such *Streptococcus pneumoniae* and *Pseudomonas aeruginosa* or bacterial toxins [7, 8]. High expression of such endopeptidases may be associated not only with a higher risk of SARS-CoV-2 infection but also with microbial imbalances in severe asthma. Therefore, the aim of this study is to investigate associations of sputum endopeptidases gene expression with metagenomics composition and whether they could be used to stratify asthma patients according to risk of SARS-CoV-2 infection.

We examined the relation of SARS-CoV-2-associated endopeptidases with the airway bacterial composition, SARS-CoV-2-associated exopeptidases and sialyltransferases, and inflammatory profile (cells and proteins) in 120 sputum samples collected from severe non-smoking asthmatics, severe smoking asthmatics, mild-moderate asthmatics and healthy controls of the Unbiased BIOmarkers in PREDiction of respiratory disease outcomes (U-BIOPRED) adult cohort[9]. Definition of asthma severity within the U-BIOPRED cohort has been presented in details elsewhere[9]. Sputum transcriptomics, SomaScan® proteomics and metagenomics were assayed as described previously [5, 6]. Gene set variation analysis (GSVA) was performed to obtain the enrichment score (ES) of the endopeptidase genes (TMPRSS2, and Furin). Spearman correlation coefficients were computed between endopeptidases ES and sputum inflammatory markers and metagenomics α-diversity measures. The median ES is equal to zero. Subsequently, subjects were subdivided into two groups

according to their ESs, i.e., endopeptidase-high (ES > 0, n=60) and endopeptidase-low expression group (ES < 0, n=60). These were compared according to sputum inflammatory markers, metagenomics α-diversity measures, and gene expression of the exopeptidases, ACE2, DPP4 and sialyltransferases (ST3GAL4 and ST6GAL1). The two groups were also compared with respect to current intake of antibiotics, oral corticosteroid (OCS), OCS normalized dosage (in mg), and history of hypertension and diabetes diagnoses. The differential bacterial abundance between endopeptidase groups was computed using edgeR after relative log expression normalization, while proteomics differential abundance was computed using limma. Pathway enrichment analysis of differentially abundant proteins in the endopeptidase-high group was performed using the Reactome database in g:Profiler.

Severe non-smoking (n=61) and smoking (n=23) asthmatics showed the highest median expression ES of endopeptidase as compared to mild-moderate asthmatics (n=20) and healthy controls (n=16) (Figure 1A), consistent with previous findings [5]. The endopeptidases ESs were significantly correlated with sputum neutrophil absolute counts (r_s=0.55, p=7.7x10^{-11}) and percentages (r_s=0.58, p=2.4x10^{-12}), which suggests that the endopeptidases were neutrophil-derived. The endopeptidases ESs were inversely associated with bacterial α-diversity measures (r_s for observed species=-0.44, Shannon=-0.38, Chao1=-0.46, Simpson=-0.35, all ps<1x10^{-4}). The endopeptidase-high group (mean age=50.9±13.2, 53.3% females) had higher sputum neutrophils (Figure 1B), with no differences in sputum eosinophils (Figure 1C), and exhibited reduced bacterial α-diversity measures as compared with the endopeptidase-low group (mean age=48.2±14.6 yrs, 53.3% females) (Figure 1D). In addition, the endopeptidase-high group had a higher abundance of pathogenic bacteria, such as *Moraxella catarrhalis*, *Haemophilus influenzae* displaying a pattern of pathogenic bacterial aggravation compared with endopeptidase-low group (Figure 2A), while the latter had a higher abundance of commensal bacteria, such as *Rothia and Prevotella* species. The endopeptidase-high group showed higher sputum expression of the exopeptidases, ACE2 and DPP4 (Figure 1E), and sialyltransferase ST3GAL4 (but not ST6GAL1) (Figure 1F) compared with the endopeptidase-low group, which might indicate higher risk of SARS-CoV-2 infection and possible associated COVID-19 morbidity. No significant differences were found between both groups considering current antibiotic and normalized OCS dose (data not shown). 250 proteins were differentially-abundant between the high and low endopeptidase groups particularly with a higher abundance of inflammatory markers, such as interleukin-6 (IL-6), tumor necrosis factor (TNF) superfamily member 4 (LIGHT), tissue inhibitor of metalloproteinases 2 (TIMP2), macrophage migration inhibitor factor (MIF), TNF-stimulated gene 6 protein (TSG-6) and IL-8 proteins in endopeptidase-high group. Enrichment analysis in the

endopeptidase-high group showed up-regulation of several pathways including innate immunity, neutrophil degranulation, cytokines signaling, Toll-like receptor and platelet activation (Figure 2C). In serum, there was a higher levels of IL-6, IL-18 and C-reactive protein in the endopeptidase-high group ($p < 0.05$, data not shown).

These findings suggest that appropriate stratification of asthma patients is necessary to adequately estimate risk and/or morbidity of SARS-CoV-2 infection. The neutrophilia observed in the endopeptidase-high group might be directly associated with pathogenic bacteria aggravation in this group. This may suggest that these pathogenic bacteria presence or "blooming" is aggravating the immune system and changing the overall microbial population. In addition, we speculate that the presence of airway bacterial imbalances might be a consequence of the disturbed immune system in severe asthma such as inadequate phagocytic capacity of macrophages [10], which might lead to higher risk of infections. In this cohort, clusters of severe asthma patients which exhibited bacterial aggravation were relatively stable after 12-18 months [6], which suggest impairment of immune system over relatively long periods of time. Second, this bacterial aggravation might be associated with comorbid-conditions, such as hypertension and diabetes, which are known risk factors for more severe COVID-19. In our study, the endopeptidase-high group showed higher gene expression of exopeptidases ACE2 (associated with hypertension) and DPP4, and the sialyltransferase ST3GAL4 (associated with diabetes) compared with the endopeptidase-low group, which might indicate the pathophysiologic involvement of both diseases in the endopeptidase-high group. However, there were no significant associations between endopeptidases high/low groups and reported history of diabetes and hypertension diagnosis in the included subjects (data not shown). Therefore, future studies are needed to explore whether both diseases may influence the airway microbiome composition in asthmatics.

The present findings suggest that personalized therapies, such as those targeting neutrophils (e.g. anti-IL-17), endopeptidase inhibitors (e.g. neprilysin inhibitors) and/or antimicrobial compounds might be tailored to asthma patients with high risk of SARS-CoV-2 infection.

In conclusion, these findings in sputum highlight that it is important to assess overall microbial profile in relation to SARS-CoV-2 associated proteases in order to adequately assess risk of infection in patients with severe neutrophilic asthma.

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List of author contributions:

MIA and NZK have performed the analysis and drafted the manuscript. MIA, NZK, FC and IMA have contributed to the design of the study and the analysis plan. All co-authors have contributed to the acquisition of data, interpretation of the analysis, revision, critical appraisal and ensuring accuracy and integrity of the analysis. All co-authors have provided final approval of the version to be published. IMA is the

corresponding author. In addition, the U-BIOPRED is a consortium effort and we wish to acknowledge the help and expertise of the individuals and groups who participated in the study.

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Conflicts of interest:

SED reports personal fees from AZ, Cayman Chemicals, GSK, Merck, Novartis, Regeneron, Sanofi, Teva, outside the submitted work. RD reports receiving fees for lectures at symposia organised by Novartis, AstraZeneca and TEVA, consultation for TEVA and Novartis as member of advisory boards, and participation in a scientific discussion about asthma organized by GlaxoSmithKline. RD is a co-founder and current consultant, and has shares in Synairgen, a University of Southampton spin out company. PJS reports grants from Innovative Medicines Initiative (IMI) covered by the European Union and the European Federation of Pharmaceutical industries and Associations (EFPIA), during the conduct of the study. AHM has received research grants outside the submitted work from GSK, Boehringer Íngelheim and Vertex, she is the PI of a P4O2 (Precision Medicine for more Oxygen) public private partnership sponsored by Health Holland involving many private partners that contribute in cash and/or in kind (Boehringer Ingelheim, Breathomix, Fluidda, Ortec Logiqcare, Philips, Quantib-U, Smartfish, SODAQ, Thirona, TopMD and Novartis), and she has served in advisory boards for AstraZeneca, GSK and Boehringer Ingelheim with money paid to her institution. KFC has received honoraria for participating in Advisory Board meetings of GSK, AZ, Roche, Novartis, Merck, BI and Shionogi regarding treatments for asthma, chronic obstructive pulmonary disease and chronic cough and has also been remunerated for speaking engagements. All other co-authors have nothing to disclose.

Ethical approval/consent:

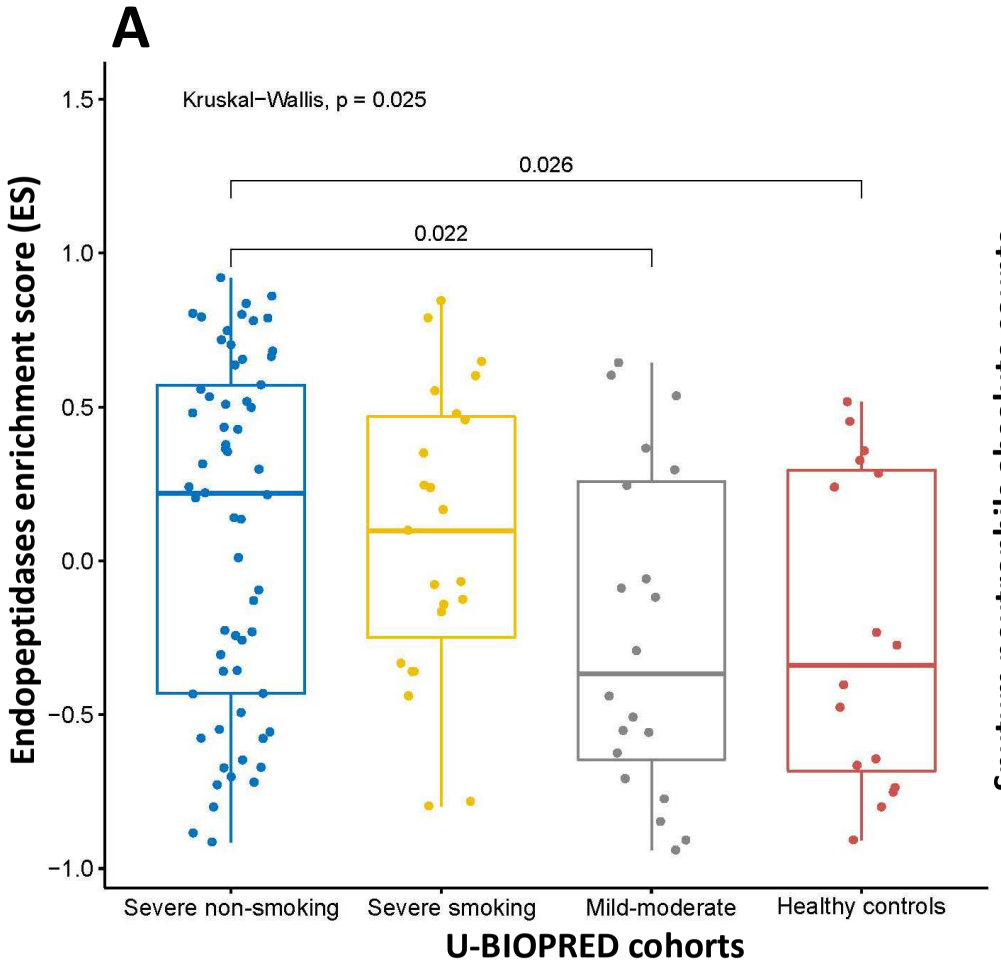
The study was approved by the local ethics committee for each participating clinical institution, and adhered to the International Conference on Harmonisation and Good Clinical Practice standars. The study is registered

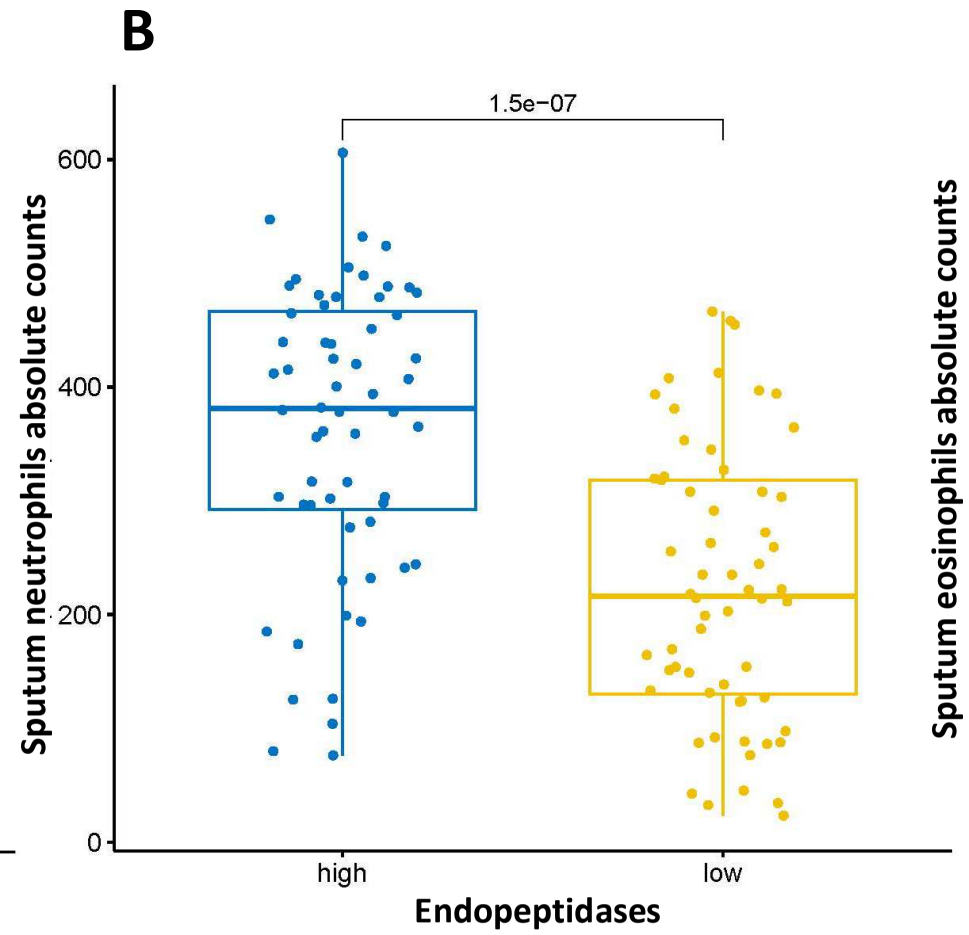
on ClinicalTrials.gov (identifier: NCT01976767). All study participants gave written and signed informed consent.

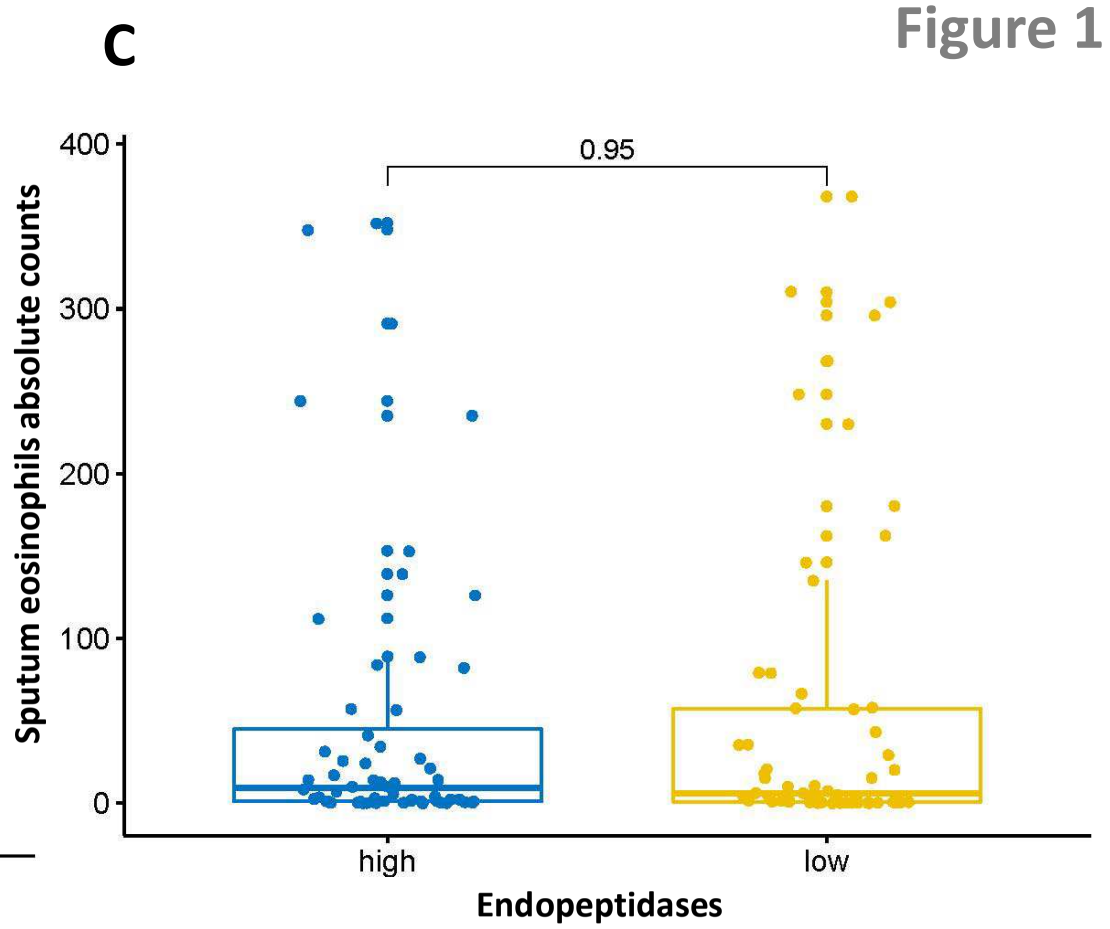
Figure legends

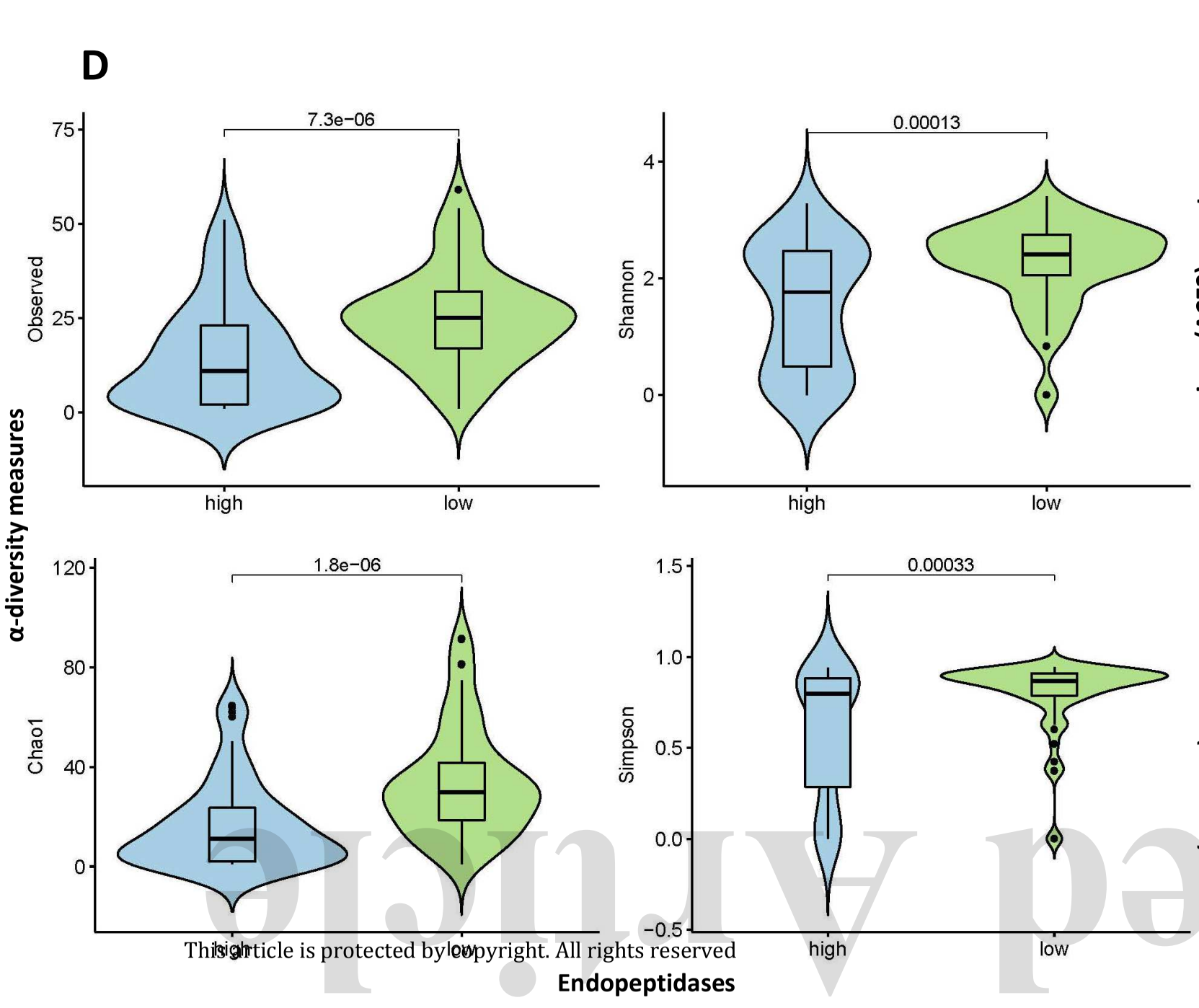
Figure 1: A; Protease (endopeptidases) genes enrichment scores (ES) in induced sputum were compared between the 4 U-BIOPRED adult sub-cohorts. **B;** Sputum neutrophils (in absolute counts) were compared between endopeptidase-high and endopeptidase-low groups. **C;** Sputum eosinophils (in absolute counts) were compared between endopeptidase-high and endopeptidase-low groups. **D;** Different metagenomics α-diversity measures (observed, Shannon, Chao1 and Simpson) were compared between endopeptidase-high and protease-low groups. **E;** ACE2 and DPP4 expression in induced sputum was compared between endopeptidase-high and endopeptidase-low groups. **F;** ST3GAL4 and ST6GAL1 gene expression in induced sputum were compared between endopeptidase-high and endopeptidase-low groups. Analysis was performed using two-tailed Mann-Whitney U and Kruskal-Wallis H tests as appropriate.

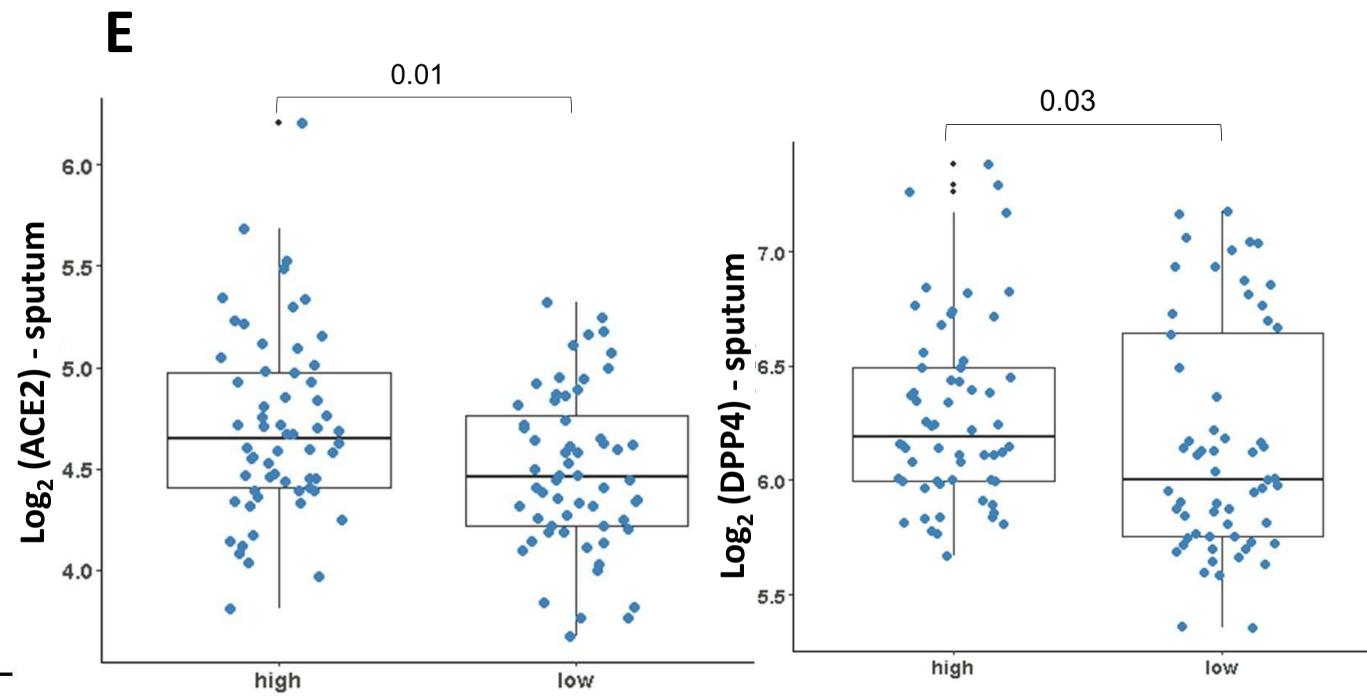
Figure 2: A; Bacterial species differential abundance in induced sputum between endopeptidase-high and endopeptidase-low groups. Values in positive $\log_2$ fold change demonstrate higher abundance of bacterial species in endopeptidase-high group relative to endopeptidase-low group. Only statistically significant differentially abundant bacterial species with false discovery rate (FDR) $\alpha < 0.05$ are depicted. **B;** SomaScan® proteomics differential abundance in induced sputum between endopeptidase-high and endopeptidase-low groups. Values in positive $\log_2$ fold change demonstrate higher abundances of proteins in endopeptidase-high group relative to endopeptidase-low group. Only labels of sputum proteins with at least twofold change are depicted on the figure. **C;** Pathway enrichment analysis of differentially-abundant proteins (DAPs) in the endopeptidase-high group using the Reactome pathways database. Only the top 15 significant pathways are depicted.











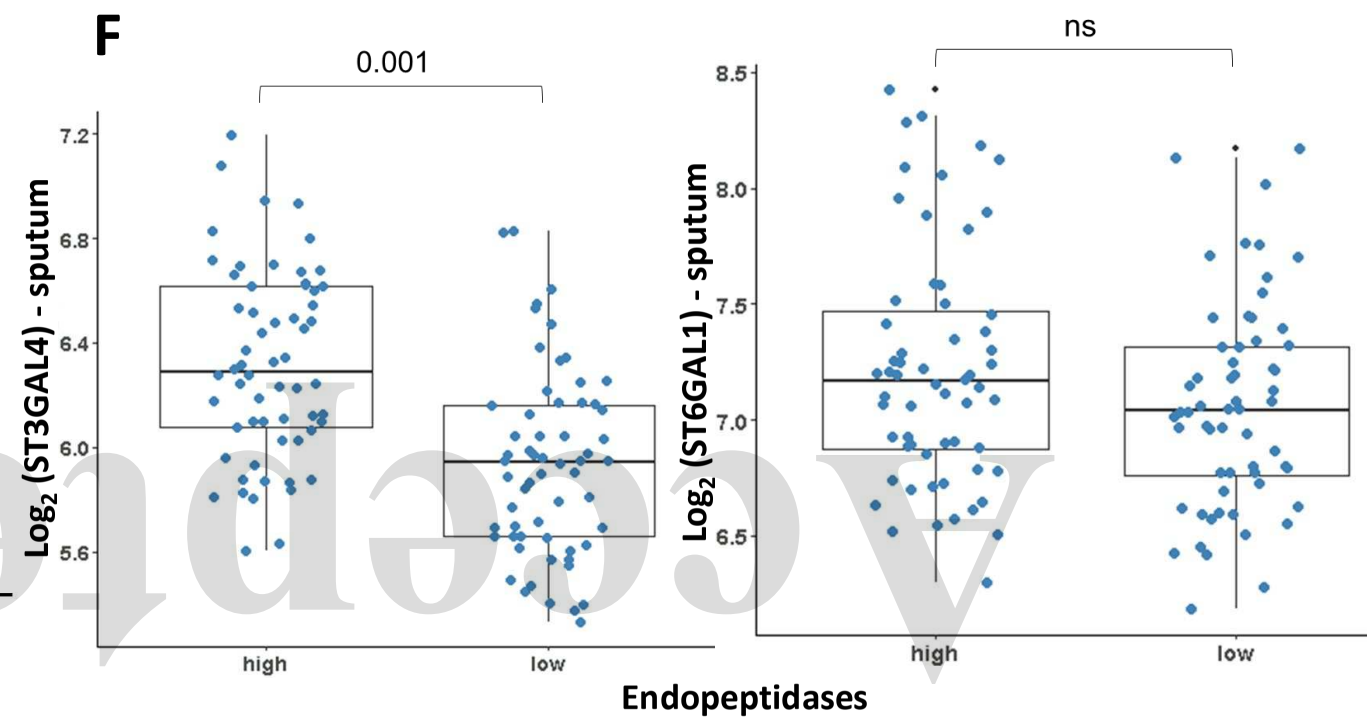
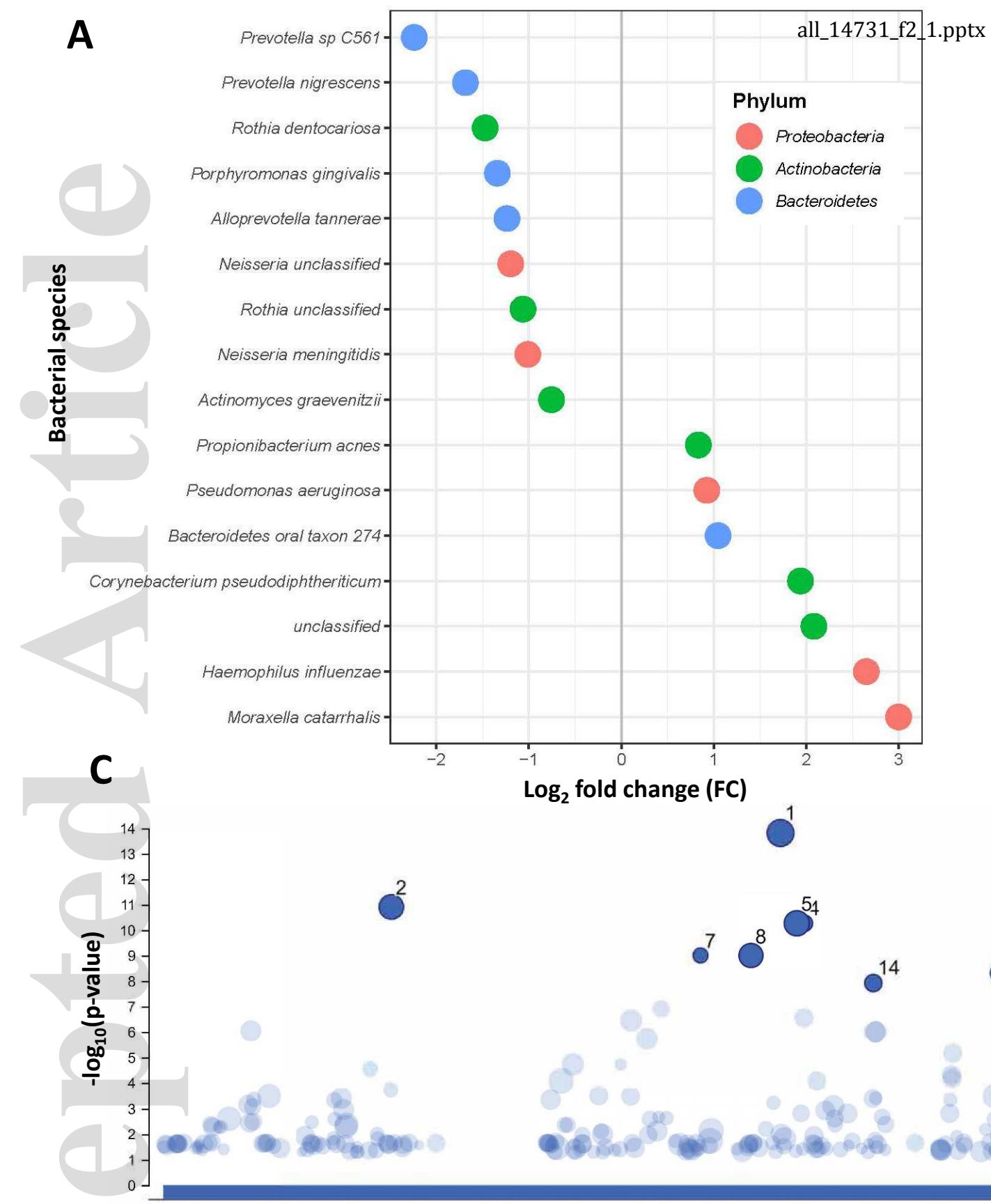
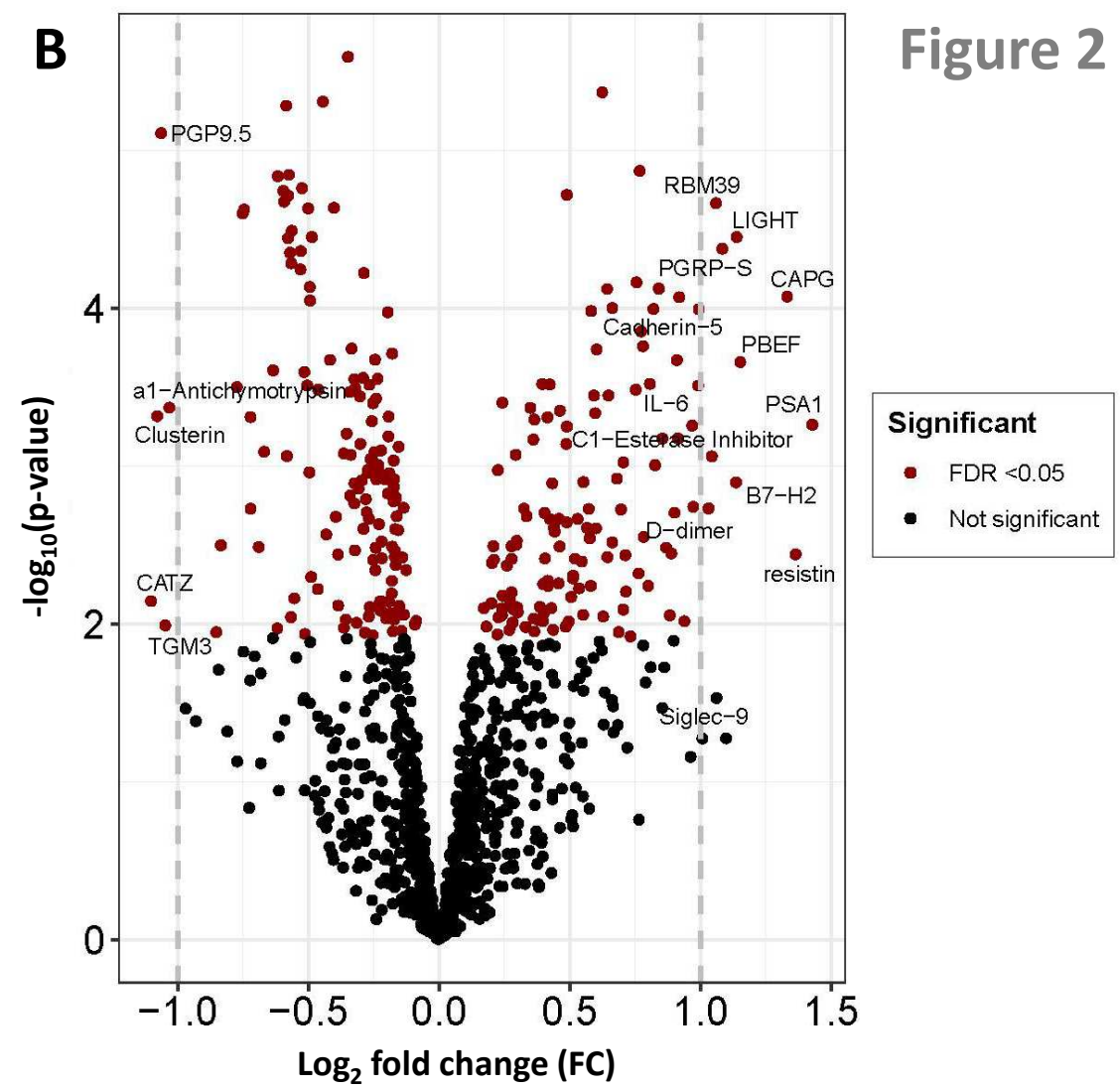


Figure 2





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3	REAC	REAC:R-HSA-5686938	Regulation of TLR by endogenous ligand	5.415×10^{-11}
4	REAC	REAC:R-HSA-354192	Integrin signaling	5.415×10^{-11}
5	REAC	REAC:R-HSA-168249	Innate Immune System	5.415×10^{-11}
6	REAC	REAC:R-HSA-76009	Platelet Aggregation (Plug Formation)	9.497×10^{-10}
7	REAC	REAC:R-HSA-354194	GRB2:SOS provides linkage to MAPK signaling for Integrins	9.918×10^{-10}
8	REAC	REAC:R-HSA-109582	Hemostasis	9.918×10^{-10}
9	REAC	REAC:R-HSA-372708	p130Cas linkage to MAPK signaling for integrins	1.346×10^{-9}
10	REAC	REAC:R-HSA-6798695	Neutrophil degranulation	4.834×10^{-9}
11	REAC	REAC:R-HSA-449147	Signaling by Interleukins	4.953×10^{-9}
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13	REAC	REAC:R-HSA-76002	Platelet activation, signaling and aggregation	9.635×10^{-9}
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