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Pro-inflammatory Markers Associated with COVID-19 Related Persistent Olfactory Dysfunction

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Abstract

Introduction: While localized inflammation has been implicated in the pathophysiology of acute COVID-19 olfactory dysfunction (OD), persistent COVID-19 OD remains poorly understood with limited therapeutics. Our prospective study evaluated olfactory cleft (OC) biomarkers as predictors of persistent OD in mucus sampling.

Methods: COVID-19 subjects with persistent OD >3months confirmed by psychophysical olfaction tests were compared to COVID-19 subjects with no OD and those with no prior infection. OC mucus samples were evaluated for 13 anti-viral and inflammatory biomarkers. Cohorts were compared using ANOVA and Mann-Whitney tests with multi-comparison adjustment. Viral RNA was assessed through RT-PCR using the COVID-19 N2 primer.

Results: 35 samples were collected (20 COVID persistent OD, 8 COVID no OD, 7 non-COVID no OD). Significant differences in IFN-λ1 (p=0.007) and IFN-γ (p=0.006) expression in OC mucus were found across all three groups, with the highest cytokine concentrations corresponding to COVID OD. IFN-α2 levels were elevated in COVID OD versus no OD (p=0.026). Mean IFN-γ levels were the highest in COVID OD, but there were higher levels found in COVID no OD compared to non-COVID no OD (p=0.008). No difference was seen in IL6. No N2 gene expression was detected in all cohorts.

Conclusion: IFN pathway cytokines were found elevated in the olfactory microenvironment of COVID-19 persistent OD compared to those with no OD and no prior history of COVID-19 infection.

Keywords

Persistent smell loss; COVID-19; olfactory dysfunction; pro-inflammatory; long haulers; mucus

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Introduction

Post-viral olfactory dysfunction (PVOD) has been associated with hundreds of viruses, most prominently SARS-CoV-2 with COVID-19.[1] PVOD can be temporary or permanent with few effective therapies[2–9] and unpredictable prognoses. Of those with acute COVID-19 OD,[10] up to 10% may experience persistent olfactory dysfunction (OD).[11,12] Given the pervasiveness of this disease, persistent PVOD remains an unmet medical need and a better understanding of the disease mechanisms may help guide research on targeted therapeutics.

Mechanisms of COVID-19 OD are continuing to be elucidated.[13] In animal models of acute SARS-CoV-2 infection, systemic proinflammatory responses induce transcriptional changes in the cells of the olfactory epithelium (OE) resulting in downregulation of the olfactory signaling pathways, specifically the odorant receptor genes.[14] Additionally, SARS-CoV-2–infected hamsters with clinical evidence of prolonged OD demonstrated upregulated IFN type I and II pathway gene signatures despite the absence of the virus detected in the OE.[15] In human studies, increased IFN-γ levels found in the nasal mucus of acute COVID-19 subjects were correlated to objective smell loss.[16] Given that these prior studies focused on the pathophysiology of acute COVID-19 OD, it was unknown if elevated nasal cytokine levels from nasal mucus during an acute infectious state would similarly reflect the olfactory microenvironment of those with persistent COVID-19 OD and if the inflammation was found in the OC itself. A recent study comparing subjects with persistent COVID-19 OD to non-COVID controls suggested persistent OD is influenced by an immune cell regulated pro-inflammatory response.[17] A diffuse infiltrate of T-cell expressing IFN-γ combined with an enrichment of $CD207^+$ dendritic cells and depletion of anti-inflammatory M2 macrophages resulted in gene expression changes of OE cells.[17] Thus our team sought to investigate the inflammatory profile of the OC microenvironment in persistent COVID-19 OD.

This study compared levels of OC inflammatory markers amongst subjects with COVID-19 related persistent OD, those with prior COVID-19 infection but no OD, and those with no prior COVID-19 infection.

Materials and Methods

Patient selection

The study was approved by the University of California San Diego Institutional Review Board (IRB# 210078). We recruited English-speaking subjects ≥18 years of age and queried their COVID-19 infection status and subjective sense of smell (intact, recovered, or absent/diminished). COVID-19 subjects demonstrated PCR-confirmed SARS-CoV2 infection at least 3 months prior to recruitment. Exclusion criteria included sinonasal inflammatory disease (e.g. rhinosinusitis, rhinitis), OD due to non-COVID etiologies, head trauma, neurological disease, and recent COVID-19 infection less than 3 months prior to enrollment. All subjects underwent nasal endoscopy for nasal sample collection and objective smell testing to characterize them as either persistent COVID-19 OD (smell loss), COVID-19 control (no smell loss), or non-COVID-19 control (no smell loss) based on objective olfactory function.

Subjective and objective smell assessment

Participant olfactory function was evaluated subjectively via a self-assessment of his or her current smell function as intact/never experienced OD, recovered from COVID-19 related OD, and COVID-19 related persistent OD. Objective smell assessment was obtained via the self-administered, well-validated, 40-item University of Pennsylvania Smell Identification Test (UPSIT).[23] Out of 40 possible points, scores greater than 34 in males and greater than 35 in females were categorized as normosmia. Participants were placed into cohorts based on objective smell testing at the time of recruitment.

Olfactory cleft mucosal sampling

Olfactory cleft mucosal sampling under nasal endoscopy was performed in the clinic and a sterile filter membrane (Leukosorb, Pall Corporation) was placed into the OC bilaterally under direct visualization, as previously published.[18–20] After 2 minutes, the membrane was removed, placed directly on ice and centrifuged for 10mins at 13,000rpm in 4°C. The mucus from both sides were combined, 1x protease inhibitor (Halt, Thermo Fisher, Waltham, MA) added, and stored at −20°C prior to assay conduction.

Multiplex ELISA for cytokine panel assessment

A 13-plex ELISA for human anti-virus response panel kit was used that measured IL-β, IL-6, TNF-α, IP-10, IFN-λ1 IL-8, IL-12p70, IFN-α2, IFN-λ2/3, GM-CSF, IFN-β, IL-10, IFN-γ (Biolegend, LEGENDplex, #740390, San Diego, CA). OC mucus samples were diluted 1:2 and the assay was performed per protocol of the manufacturer. In brief, the sample/standard, assay buffer, and mixed beads were added to each well in a 96-well V-bottom plate. Plates were sealed with a clear plate sealer, covered in aluminum foil, and placed on a shaker plate for 2 hrs at 800rpm in room temperature. Plate was centrifuged for 5 min at 1050rpm, supernatant discarded, and 200ul of wash buffer was added. Centrifugation step was repeated. Supernatant was discarded and detection antibodies were added. Plate was covered similarly to previously described and placed on shaker plate for 1 hr at 800rpm in room temperature. SA-PE was added directly to each well, sealed, and placed on shaker plate for 30min at 800rpm in room temperature. Centrifugation and wash step was repeated. After supernatant was discarded, final volume of wash buffer was added to each well and pellet resuspended.

Flow cytometry (Agilent NovoCyte, Santa Clara, CA) was performed in technical triplicates for each sample with device configuration per manufacturer protocol. FCS files were analyzed on the Biolegend LEGENDplex Data Analysis Software Suite.

COVID-19 qPCR

In a subset of participants selected at random, a second brushing of OC epithelial cells was collected for RNA analysis. Briefly, OC epithelial swabs were immediately placed in TRIzol (Invitrogen, Carlsbad, CA) with the ratio of sample volume to TRIzol volume per manufacturer protocol. Samples were snap frozen and stored at −80°C. RNA was extracted using TRIzol/chloroform protocol and RNeasy Mini Kit (Qiagen, Germantown, Maryland) and transformed to cDNA using iScript (Bio-Rad, Hercules, CA). Quantitative RT-PCR for SARS-CoV-2 detection was used per the US Centers for Disease Control panel assay.[24]

COVID-19 primer (N2, GENEWIZ, La Jolla, CA) plus human GAPDH gene (Qiagen, Germantown, Maryland) were used while a previously validated internal COVID-19 positive control was used. Relative gene expression was normalized to that of GAPDH using $\Delta\Delta C_t$. Melting curve was obtained to ensure the correct amplicon size. Ct values >38 or undetectable were considered negative.

Statistical analysis

Mann Whitney test was used to compare the cytokine levels between COVID persistent OD, COVID no OD, and non-COVID control cohorts given its ability to withstand outliers. ANOVA was performed to compare across all three cohorts. Linear regression analysis was performed on COVID persistent OD and COVID no OD cohort with UPSIT as the dependent variable. Statistical significance was determined by p-value<0.05. Benjamin Hochberg (BH) adjustment was performed to adjust for a 5% false discovery ratio denoted by q-values. Statistical analysis was performed using SPSS (IBM, Chicago, IL) and RStudio (Boston, MA).

Results

This prospective study recruited a total of 35 participants. There were three cohorts in the study—COVID persistent OD (n=20), COVID no OD control (n=8), and non-COVID no OD control (n=7) as determined by objective smell function at the time of recruitment. Participant demographics and post-acute sequelae SARS-CoV-2 infection (PACS) symptoms are described in Table 1. There was a significant difference in UPSIT scores (p=0.004) between the persistent OD (average ±SD: 27.6±7.0) and COVID control (36.0±1.5), but no difference between COVID control and non-COVID controls (36.2±2.2, p=0.870). Concordance between objective and subjective smell assessment was highest in the non-COVID control (100%, 7 of 7) and lowest in persistent OD (30%, 6 of 20). The persistent OD cohort reported other PACS symptoms including fatigue (20%, 4 of 20), sleep disorder (15%, 3 of 20), anxiety/depression (10%, 2 of 20), and tachycardia/POTS (5%, 1 of 20).

All cytokines obtained from OC mucus were detectable and within range of the analyte's standard curve provided by the manufacturer. Comparisons of OC mucus across COVID persistent OD, COVID non-OD control, and non-COVID no OD control samples revealed significant differences in TNF-α (p=0.049), IFN-λ1 (p=0.007), IFN-λ2/3 (p=0.012), and IFN-γ (p=0.006) expression before multiple comparisons correction, with the highest cytokine concentrations noted in the COVID OD cohort (Table 2, Figure 1). The COVID OD cohort demonstrated higher levels of IFN-α2 compared to the COVID no-OD cohort (24.56±39.99 pg/ml vs. 3.64±2.08pg/ml, p=0.026). IFN-γ expression was the highest in the COVID OD cohort (72.61±50.23 pg/ml) with levels of IFN-γ higher in the COVID no OD (41.55±43.16 pg/ml) compared to the no COVID control group (5.19±5.27pg/ml, p=0.008). After adjustments for multiplicity using the BH procedure, significant differences across groups were identified in IFN-λ1 and IFN-γ expression levels. There was no difference in IL-6 OC mucus expression across the three cohorts and between group comparisons. Linear regression was performed with UPSIT as a continuous variable for the COVID persistent

OD and COVID no OD cohorts, and no significant difference was observed (data not shown).

SARS-CoV-2 RNA expression was assayed from a random subset of subjects (COVID persistent OD n=3, COVID no OD n=2, and non-COVID control n=1). No virus was detected from any of the samples.

Discussion

Our study highlights the presence of inflammatory markers in the OC microenvironment in persistent COVID OD subjects. Although acute OD associated with COVID-19 has been studied,[14,25] our understanding of the inflammatory OC profile in persistent OD is limited. We found that persistent COVID OD was associated with increased concentrations of multiple inflammatory cytokines, in particular those associated with the Type I and Type II interferon pathways. Both types of IFNs play critical roles in viral infections with IFN type I (α and β) commonly associated with mounting an acute antiviral response and activation of the innate immune cells and IFN type II (γ) implicated in modulating the inflammatory state through innate and adaptive immune responses.[26]

Higher concentrations of IFN-γ secreted in the OC mucus were found in COVID persistent OD subjects compared to COVID no OD control and non-COVID no OD control subjects. These findings corroborate a previous study that evaluated nasal (non-OC) mucus in acutely infected COVID subjects and found that elevated IFN-γ levels correlated with acute COVID OD.[16] The specific cell types in the OC secreting the inflammatory cytokines requires further elucidation. In a recent study, Finlay and colleagues compared single cell transcriptomes using OC tissue obtained from humans with persistent COVID OD and non-COVID OD subjects[17] to find a diffuse increase in IFN-γ expressing T cells in subjects with COVID OD. The immune cell infiltration resulted in a change in sustentacular cell gene expression and an ultimate reduction in olfactory sensory neurons. Together these findings demonstrate that pro-inflammatory signals in the olfactory cleft microenvironment are implicated in persistent COVID OD. Further, these elevated cytokines were found in the absence of persistent SARS-CoV2 viral infection, consistent with findings by Finlay et al.[17] While the highest IFN-γ levels were noted in the COVID OD cohort, the COVID non OD group had elevated concentrations compared to the non-COVID control, perhaps suggesting that a history of COVID infection results in persistent type II IFN response independent of OD. Significant difference was not identified after multiplicity adjustment which may reflect the underpowered statistical significance with a larger cohort.

TNF-α was found elevated in the COVID persistent OD cohort compared to the control cohorts in the unadjusted analysis though significance was not reached post-adjustment. This trend however may suggest that pro-inflammatory signals remain following COVID-19 in those with objective olfactory loss. TNF-α was similarly elevated in the acute immune response in the olfactory environment of those with immediate post-COVID.[27] Consistent with OE gene expression data presented by Finlay and colleagues,[17] our study did not show any differences in the expression of IL-1β, a mediator of severe cytotoxic inflammatory response. The association of IL-6 with persistent OD is controversial in the current

literature.[28–30] In our evaluation of OC mucus IL-6 levels, we found no such significance between OD and the cytokine expression.

Other etiologies of persistent OD such as chronic rhinosinusitis (CRS) have also been linked to increased IFN-γ and TNF-α expression in preclinical models.[31,32] Chronic IFN-γ expression by sustentacular (supporting) cells in the OE via a transgenic mouse model resulted in decreased odorant responsiveness without visible inflammatory tissue injury.[31] However, IFN-γ is a T helper 1 (Th1) cytokine typically linked to CRS without nasal polyps, a phenotype less commonly associated with OD. Comparably, persistent TNF-α expression has also been implicated in CRS-related OD in preclinical models,[32,33] although no correlation was found between its olfactory mucus expression and objective olfaction in human CRS subjects.[34] In a CRS mouse model, persistent elevation of TNF-α reduced olfactory basal stem cell regeneration which was then restored when TNF-α expression normalized.[33] Similar to CRS models, chronic localized inflammation may be contributing to sustained damage or impeded recovery of the OE and the olfactory circuitry in COVID-19 persistent OD. Pro-inflammatory signaling may play a role in CRS and chronic post-viral OD, but the specific pathways require further research for both etiologies.

There was a high concordance between subjective and objective olfactory function in the non-COVID-19 no OD control group and a moderate concordance in the COVID-19 no OD control group. The persistent OD cohort, however, had low concordance between their subjective and objective olfactory assessment. Several subjects who self-reported normosmia were tested to have objective mild-hyposmia and thus characterized as COVID persistent OD. The low concordance is not surprising as many studies have shown poor self-identification of OD.[35,36] However, we suspect that some discordance may be due to the limitations of the psychophysical testing utilized in the study and subject odorant familiarity.

One of the limitations of our study is the high variability in cytokine measurements. Increasing the sample size of the cohorts, especially the control groups, would reduce the variability. Additionally, increasing the sample size may recruit participants with a history of COVID with and without OD, and concurrent long-hauler symptoms to further study its correlation to smell function and biomarkers. Multi-comparisons across the cytokines were adjusted for the false discovery ratio using the BH correction. The loss of significance may also point to the underpowered nature of the study. In this study, the original UPSIT was used, however, the commercially available revised UPSIT may be more appropriate for determining OD, particularly those on the border between hyposmia and normosmia. There may be selection bias due to participants volunteered to participate in the study. It was also considered that participants had different strains of COVID-19 that have differing viral infectivity of the OE and prevalence of OD.

Conclusion

Our study investigated OC inflammatory markers associated with persistent COVID OD and found a correspondence between Type I and II interferon pathway cytokines and OD. This data supports the current literature that persistent inflammation in the peripheral olfactory epithelial microenvironment may be contributing to the persistent loss of olfactory

function. Future studies elucidating the specific signaling mechanisms and the type of cells responsible for cytokine secretion may shed insight into designing an efficacious treatment for PVOD associated with COVID-19.

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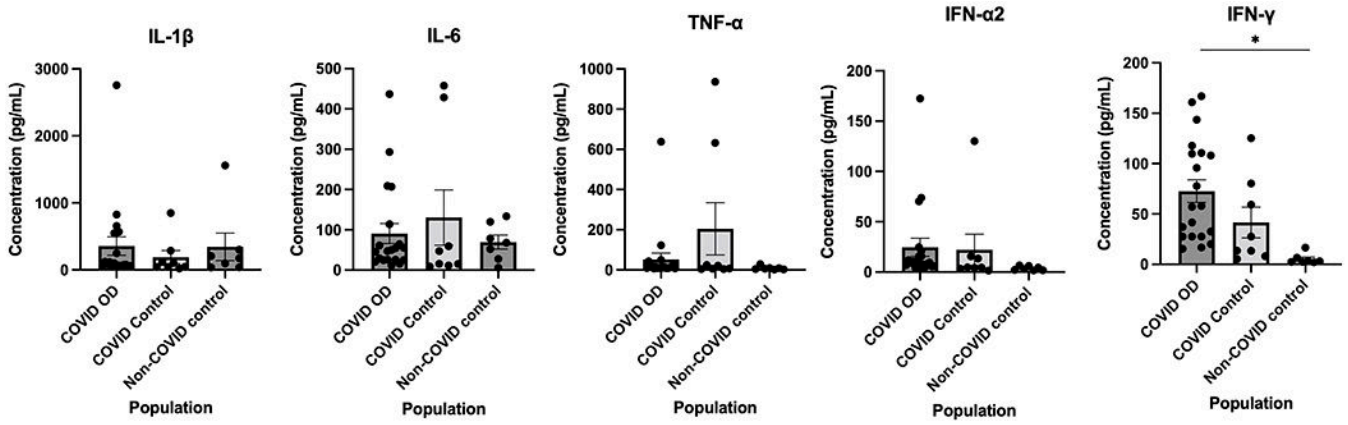

Figure 1.

Comparison of olfactory mucus inflammatory cytokine levels demonstrates significant differences in IFN-γ expression across three cohorts (COVID persistent OD, COVID no OD control, non-COVID no OD control) with the highest expression in the COVID persistent OD group. * adjusted $p<0.05$ after multiple comparison group correction

Table 1.

Study population demographics and long hauler/post-acute sequelae SARS-CoV-2 infection symptoms

	COVID Persistent OD (n=20)		COVID No OD Control (n=8)		Non-COVID No OD Control (n=7)		All groups*	COVID only†	No OD only¶
	n	%	n	%	n	%	*p*-value		
Age (yrs, stdev)	46.7	15.9	38.9	11.1	36.6	9.8	0.078	0.241	0.642
Gender (male)	8	40.0%	4	50.0%	2	28.6%	0.619	0.907	0.738
Ethnicity							0.074	0.453	0.125
Hispanic	2	10.0%	1	12.5%	2	28.6%			
White, non-Hispanic	16	80.0%	7	87.5%	2	28.6%			
Black, non-Hispanic	1	5.0%	0	0.0%	0	0.0%			
Two or more races	1	5.0%	0	0.0%	0	0.0%			
Asian or Pacific Islander	0	0.0%	0	0.0%	3	42.9%			
American Indian or Alaskan Native	0	0.0%	0	0.0%	0	0.0%			
Smoking							0.980	0.846	0.887
Never smoker	16	80.0%	7	87.5%	5	71.4%			
Current smoker	2	10.0%	0	0.0%	0	0.0%			
Former smoker	2	10.0%	1	12.5%	2	28.6%			
UPSIT (average, stdev)	27.6	7.0	36.0	1.5	36.2	2.2	<0.001**	0.004*	0.870
Parosmia‡	3	15.0%	1	12.5%	0	0.0%	0.326	0.870	0.369
Duration post-COVID (mo, stdev)	10.7	7.4	5.1	2.4	-	-	-	0.064	-
Subjective smell assessment							0.006**	0.352	<0.001**
No smell loss	11	55.0%	6	75.0%	7	100.0%			
Recovered smell loss	3	15.0%	1	12.5%	0	0.0%			
Persistent smell loss	6	30.0%	1	12.5%	0	0.0%			
Past medical history‡									
Diabetes	3	15.0%	0	0.0%	1	14.3%	0.741	0.263	0.302
Hypertension	4	20.0%	0	0.0%	1	14.3%	0.502	0.185	0.302
Chronic lung disease	0	0.0%	0	0.0%	1	14.3%	0.085	-	0.302
Allergies	4	20.0%	2	25.0%	2	28.6%	0.635	0.781	0.887
Depression/Anxiety	1	5.0%	0	0.0%	0	0.0%	0.438	0.537	-

	COVID Persistent OD (n=20)		COVID No OD Control (n=8)		Non-COVID No OD Control (n=7)		All groups*	COVID only†	No OD only¶
	n	%	n	%	n	%	*p*-value		
Long-hauler symptoms‡									
Shortness of breath	0	0.0%	0	0.0%	0	0.0%	-	-	-
Fatigue	4	20.0%	0	0.0%	0	0.0%	0.099	0.185	-
Brain fog	0	0.0%	0	0.0%	0	0.0%	-	-	-
Sleep disorder	3	15.0%	0	0.0%	0	0.0%	0.162	0.263	-
Anxiety/depression	2	10.0%	0	0.0%	0	0.0%	0.263	0.372	-
Tachycardia/POTS	1	5.0%	0	0.0%	0	0.0%	0.438	0.537	-

Note: OD- olfactory dysfunction, COVID- coronavirus disease of 2019, UPSIT- University of Pennsylvania Smell Identification Test, POTS- postural orthostatic tachycardia syndrome

*All groups = COVID persistent OD, COVID no OD control, Non-COVID no OD control

†COVID only = COVID persistent OD, COVID no OD control

¶No smell loss only = COVID no OD control, Non-COVID no OD control

‡Present or absent

**p-value <0.05

Table 2.

Average olfactory mucus cytokine levels across COVID cohorts

Cytokine	COVID Persistent OD (n=20)		COVID No OD Control (n=8)		Non-COVID No OD Control (n=7)		All groups*		COVID only†		Controls only¶	
	Average	Stdev	Average	Stdev	Average	Stdev	*p*-value	*q*-value	*p*-value	*q*-value	*p*-value	*q*-value
IL-1β	356.21	617.59	189.13	279.84	344.29	543.75	0.317	0.375	0.094	0.306	0.563	0.563
IL-6	90.81	111.51	130.49	193.96	69.50	46.08	0.438	0.475	0.541	0.781	0.563	0.563
TNF-α	52.34	140.33	204.49	366.95	9.12	9.10	0.049**	0.159	0.710	0.839	0.298	0.430
IP-10	906.26	2068.00	442.54	578.90	198.82	134.98	0.188	0.272	0.214	0.397	0.563	0.563
IFN-λ1	144.80	134.99	651.07	1458.64	37.77	18.35	0.007**	0.046**	0.623	0.810	0.028**	0.182
IL-8	10097.28	5619.95	7114.14	5413.48	13142.83	16337.47	0.279	0.363	0.201	0.397	0.355	0.462
IL-12p70	14.04	28.25	15.58	30.40	1.38	0.87	0.505	0.505	0.817	0.885	0.064	0.277
IFN-α2	24.56	39.99	22.10	43.92	3.64	2.08	0.127	0.236	0.026**	0.306	0.247	0.430
IFN-λ2/3	486.70	594.93	256.23	185.82	150.75	146.80	0.012**	0.052	0.071	0.306	0.203	0.430
GM-CSF	5.80	11.26	4.02	4.41	1.93	1.76	0.169	0.272	0.214	0.397	0.203	0.430
IFN-β	29.96	27.07	28.46	23.27	9.99	2.88	0.072	0.187	1.000	1.000	0.296	0.430
IL-10	6.56	5.18	5.13	3.16	3.03	2.72	0.094	0.204	0.414	0.673	0.132	0.429
IFN-γ	72.61	50.23	41.55	43.16	5.19	5.27	0.006**	0.046**	0.071	0.306	0.008**	0.104

Notes: unit of measurement is pg/ml.

ANOVA was performed across all groups. Mann Whitney test was performed between COVID OD and COVID control, and between COVID control and non-COVID control

p-value- unadjusted statistical significance set at α=0.05

q-value- based on Benjamini-Hochberg (BH) procedure adjusted for false positive ratio with significance set at α=0.05

*All groups = COVID OD, COVID control, Non-COVID control

†COVID only = COVID OD, COVID control

¶No smell loss only = COVID control, Non-COVID control

**p-value<0.05 or q-value<0.05 (adjusted BH)