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# Substance P and Prokineticin-2 are overexpressed in olfactory neurons and play differential roles in persons with persistent post-COVID-19 olfactory dysfunction

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ABSTRACT

Persistent olfactory dysfunction (OD) is one of the most complaining and worrying complications of long COVID-19 because of the potential long-term neurological consequences. While causes of OD in the acute phases of the SARS-CoV-2 infection have been figured out, reasons for persistent OD are still unclear.

Here we investigated the activity of two inflammatory pathways tightly linked with olfaction pathophysiology, namely Substance P (SP) and Prokineticin-2 (PK2), directly within the olfactory neurons (ONs) of patients to understand mechanisms of persistent post-COVID-19 OD.

ONs were collected by non-invasive brushing from ten patients with persistent post-COVID-19 OD and ten healthy controls. Gene expression levels of SP, Neurokinin receptor 1, Interleukin-1β (IL-1β), PK2, PK2 receptors type 1 and 2, and Prokineticin-2-long peptide were measured in ONs by Real Time-PCR in both the groups, and correlated with residual olfaction. Immunofluorescence staining was also performed to quantify SP and PK2 proteins.

OD patients, compared to controls, exhibited increased levels of both SP and PK2 in ONs, the latter proportional to residual olfaction.

This work provided unprecedented, preliminary evidence that both SP and PK2 pathways may have a role in persistent post-COVID-19 OD. Namely, if the sustained activation of SP, lasting months after infection's resolution, might foster chronic inflammation and contribute to hyposmia, the PK2 expression could instead support the smell recovery.

## 1. Introduction

The loss of smell is considered a salient, distinctive feature of SARS-CoV-2 associate disease (COVID-19). COVID-19 related olfactory dysfunction (hyposmia or anosmia) is very frequent (about 80 % of prevalence, depending on virus strains) and typically initial, brief, and transient. In most cases, olfaction recovers within weeks from the infection. However, in 20 % of cases, patients can develop a persistent form of olfactory dysfunction (OD), lasting more than six months (or years) after the resolution of COVID-19 (Butowt and von Bartheld, 2021; Doty, 2022; Esposito et al., 2022; Tan et al., 2022).

Persistent OD negatively impacts both the health and the quality of

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life of humans, increasing the risk for malnutrition, depression, cognitive decline, and mortality. Accordingly, OD results in one of the most annoying "long COVID" disturbances, potentially associated with neurological sequelae, deserving deeper understanding and appropriate management (Di Stadio et al., 2022b, 2022a; Doty, 2022; Schirinzi et al., 2020; Tan et al., 2022).

Causes of OD in COVID-19 include the nasal congestion impeding odorant access to the olfactory epithelium, the loss of the olfactory neurons, the viral invasion of the central olfactory pathway, the damage of sustentacular cells in the olfactory epithelium. The cytokine storm following SARS-CoV-2 infection, either at the systemic or nasal level, could be critical in all these events, representing a possible determinant of smell loss (Butowt and von Bartheld, 2021; Doty, 2022). In particular, the neuropeptide Substance P (SP) might be the main mediator of the inflammatory response to SARS-CoV-2 infection in the respiratory tract and the orofacial region, accounting for most of the COVID-19 symptoms, including OD (Ullah et al., 2021). Indeed, SP is expressed in both the olfactory system (Tirassa et al., 2021) and the trigeminal sensory fibers reaching the olfactory bulb, being involved in the olfaction processing of odorants and nasal irritant stimuli (Finger et al., 2002).

However, why some patients develop a long-lasting form of post-COVID-19 OD is still unclear. Female gender, higher initial severity of hyposmia, and nasal congestion increase the risk. As well other unexplored factors might play a role (Tan et al., 2022).

Prokineticin-2 (PK2) is a chemokine responsible for a broad array of functions in the CNS. In particular, PK2 is greatly expressed in the olfactory bulb, where it is critical for the maturation and migration of neurons in the olfactory system and the olfaction signalling overall (Cheng et al., 2006; Wen et al., 2019). Accordingly, the individual expression of PK2 might affect the capability of smell recovery in post-COVID-19 OD.

Therefore, because of their specific involvement in olfactory system pathophysiology, we could hypothesize that the SP and the PK2 pathways might be differently involved in post-COVID-19 OD, namely SP as a mediator of the noxious stimuli and PK2 as an olfactogenesis factor.

By the non-invasive brushing of the olfactory mucosa, olfactory neurons (ONs) can be easily collected and analysed to examine molecular events related to pathological conditions (Brozzetti et al., 2020; Orrú et al., 2014; Schirinzi et al., 2022). In this study, we went to characterize the activation profile of SP and PK2 pathways in ONs of patients with persistent post-COVID-19 OD, aimed at dissecting the underlying mechanisms of such a disabling phenomenon.

## 2. Materials and methods

### 2.1. Study population

The study involved ten patients with persistent post-COVID-19 OD and ten healthy sex/age matched controls (CTRLs) enrolled at Tor Vergata University Hospital (Rome, Italy) in 2021–22. All patients had SARS-CoV-2 from April to November 2021 in Rome region. Although no virus typing has been performed in the acute phase, in those periods the dominant variants were Alpha B.1.1.7 (April-June 2021) and Delta B.1.167.2 (July-November 2021), as reported by the National Health Institute of Italy. To avoid any potential biases due to acute inflammation, all patients were negative for SARS-CoV-2 from six months at least. Controls were healthy volunteers without olfaction complaints, history and clinical signs of neurological and *ENT* diseases, never affected by COVID-19. Individuals with main acute/chronic infectious/inflammatory/internal diseases or under medications potentially interfering with data interpretation were excluded.

For all subjects, demographics, anthropometrics, and medical history were collected. Patients underwent olfaction quantitative assessment by the "identification score" (IS) of the Sniffing Sticks test (Burghardt®, Wedel, Germany). Briefly, 16 odorant pens have been presented and patients had to make a forced choice from a list of four proposals. The "identification score" corresponded to the number of correct responses (Rumeau et al., 2016).

The study was approved by the local EC (protocol n° 16.21), following the principles of Helsinki declaration. All participants signed an informed consent.

### 2.2. ONs sampling

All participants underwent non-invasive olfactory mucosa brushing for ONs collection by expert otolaryngologists as described in (Brozzetti et al., 2020; Schirinzi et al., 2022; Stefani et al., 2021). After inspection of the nose under nasal endoscopic view, ONs were collected through a specifically designed flocked nasal brush (FLOQBrushTM, Copan Italia Spa, Brescia, Italy).

Two samples were collected from each patient (from one or both nostrils). One swab was immediately placed in tubes containing Cytofix fixation buffer (4 % paraformaldehyde, from Diacyte, Diapath, Italy) for immunofluorescence analysis; the second swab was placed in Eppendorf vials containing TRIzol reagent (Invitrogen, Carlsbad, CA) for RNA extraction. Cells were recovered from the swab by vortexing. The swab was then removed, and samples collected in Cytofix fixation buffer were stored at 4 °C, while samples collected in TRIzol were frozen and stored (-80 °C) until assayed.

### 2.3. Biochemical assays

This work examined both the SP and the PK2 pathways in ONs of different clinical groups. Real Time-PCR was used to quantify the gene expression of SP, Neurokinin receptor 1 (NK1R), Interleukin-1β (IL-1β), PK2, PK2-long (PK2L), PK receptor 1 and 2 (PKR1 and PKR2). Immunofluorescence staining was, instead, performed to investigate the ONs expression pattern and cellular localization of SP and PK2. Assays were run in blind as previously described (Schirinzi et al., 2022).

#### 2.3.1. RNA extraction and Real Time-PCR

Total RNA was isolated from ONs using TRIzol reagent (Invitrogen Carlsbad, CA) following the manufacturer's instructions and quantified using the D30 BioPhotometer spectrophotometer (Eppendorf AG, Hamburg, Germany). One microgram of RNA was reverse transcribed into cDNA using the SensiFAST cDNA Synthesis Kit (Bioline Meridian Bioscience, USA). The cDNAs were amplified by RT -qPCR (Real-Time Quantitative Polymerase Chain Reaction) (iCycler; Bio-Rad) using SensiMix SYBR & Fluorescein Kit (Bioline Meridian Bioscience, USA) and gene-specific human primers (SP, NK1R, IL-1β, PK2, PK2L, PKR1, PKR2) synthesised by the supplier Biomers.net (Ulm, Germany) or Eurofins Genomics (Ebersberg, Germany). All reactions were performed in triplicate under the same thermal cycling conditions as follows: 95 °C for 10 min (polymerase activation), followed by 40 cycles at 95 °C for 30 s, 52–60 °C for 30 s, and 72 °C for 30 s. A reaction mixture without cDNA was used as a control. After amplification, dissociation curves were generated to verify the presence of a single amplification product and the absence of genomic DNA contamination. Gene expression was analysed by the comparative ($2^{-\Delta\Delta Ct}$) method and results were presented as fold increase of the target gene compared to the control group, normalised to the house-keeping gene GAPDH. The primer sequences used in the present study were: PK2 Fw: 5′-ATGTGCTGTGCTGTCAGTAT-3′, Rev: 5′-AAAATGGAACTTTACGAGTCA-3′; SP Fw: 5′-CGACCAGATCAAGGAGGAACTG-3′, Rev: 5′-CAGCATCCCGTTTGCCCATT-3′; PK2L Fw: 5′-ATGTGCTGTGCTGTCAGTAT-3′, Rev: 5′-TGCCTTCCATTTCCAAAA-3′; PKR1 Fw: 5′-CCTGGTCCGCTACAAG-3′, Rev: 5′-GGCACTTCATCCGTGG-3′; PKR2 Fw: 5′-GCCATCTCCGACTTCC-3′, Rev: 5′-GGAGGCCGTTTGATAATTCA-3′; NK1R Fw: 5′-TCTTCTTCCTCCTGCCCTACATC-3′, Rev: 5′-AGCACCGGAAGGCATGCTTGAAGCCCA-3′; IL-1β Fw: 5′-CCACAGACCTTCCAGGAGAATG-3′, Rev: 5′-GTGCAGTTCAGTGATCGTACA GG-3′. GAPDH Fw: 5′-TGCACCACCAACTGCTTAGC-3′, Rev: 5′-GGCATGGACTGTGG

TCATGAG-3′,

#### 2.3.2. Immunofluorescence

The ONs immersed in Cytofix solution were washed by serial passages in phosphate saline solution (PBS) to remove excess of fixative, and then, the cell suspension was cytocentrifuged onto microscope slides (Menzel Gläser, SuperFrost® Plus), a procedure that allows to the cells to adhere to the microscope slides. Slides were then washed with PBS, permeabilized with 0.3 % Triton X-100 in PBS, and blocked in 5 % normal donkey serum for 1 h. Primary antibodies were diluted in blocking solution and incubated overnight at 4 °C. The following primary antibodies were used for the immunostaining: rabbit anti-PK2 (1:300; Abcam, Cambridge, UK, ab76747), mouse anti-SP (1:300, sc-517213; Santa Cruz Biotechnology;), mouse anti-β3-tubulin (TU-20) (1:300; Cell Signalling Technology; #4466) and rabbit anti-β3-tubulin (1:300; Invitrogen ThermoFisher Scientific; #PA5-85639) binding to β3-tubulin protein expressed by neuronal cells. After three 10-min washes in PBS, slides were incubated, for 1 h at room temperature, with anti-species IgG secondary antibodies coupled to Alexa Fluor-488 or 555 (Immunological Sciences). Nuclei were stained with DAPI (Sigma Aldrich), for 10 min at room temperature. After PBS rinse slides were mounted with Fluoromount aqueous mounting medium (Sigma Aldrich). The stained cells were visualized with a fluorescence microscope (Eclipse E600; Nikon Instruments, Rome, Italy) connected to a QImaging camera with NIS-Elements BR 3.2 64-bit software. To quantify the immunofluorescence intensity of SP and PK2, images were acquired at high magnification with a 100X objective, and exposure parameters, such as gain and time, were kept constant to avoid observing differences between experimental groups due to artifacts. A number of 5–6 images were taken from each sample and an average was taken. The analysis of IF staining for each antibody (in terms of emitted fluorescence) was performed using the ImageJ software (version 1.53, National Institutes of Health, USA https://imagej.nih.gov/ij/download.html). The fluorescence was quantified with RGB (red, green, blue) method, which uses brightness values for calculation.

### 2.4. Statistical analysis

Distribution of variables was examined preliminarily by Shapiro-Wilk test, and the non-normally distributed were $Log_{10}$ + 1 transformed for analysis when necessary. Categorical variables were compared by chi-square test, the continuous ones instead by parametric (Student's T-test) or non-parametric tests, as appropriate; correlations were evaluated by linear regression analysis or Spearman's test. Statistical significance was set at $p < 0.05$. Analysis was run in blind, by using IBM-SPSS-23 and Graphpad Prism 7.

## 3. Results

### 3.1. Population features

Sex (Post-COVID-19 OD: 7 females, 3 males; Controls: 6 females, 4 males) and age distribution (Post-COVID-19 OD: mean ± St.Dev = 43 ± 13 years; Controls: 50 ± 14) were not different between the groups. Patients had significant smell impairment measured with IS of the Sniffing Sticks test (Post-COVID-19 OD: 10.5 ± 3.2 vs Controls: 15.6 ± 0.7, U = 2.5, $p < 0.001$). Table 1 summarizes other clinical data of Post-COVID-19 OD patients.

### 3.2. Gene expression and clinical correlations

Analysis of mRNA expression showed that SP mRNA levels in ONs were significantly higher in OD patients (mean ± St.Dev. = 50.9 ± 44.3 fold increase) than in controls (CTRLs) (2.4 ± 2.9; U = 1.5, $p < 0.0001$) (Fig. 1A). In contrast, neither NK1R levels (OD = 3.5 ± 5.6 vs CTRLs = 1.9 ± 2.7) (Fig. 1B) nor IL-1β levels (OD = 5.1 ± 6.8 vs CTRLs = 1.2 ± 0.8) (Supplementary Fig. 1) reached statistical significance. In OD patients but not in controls, NK1R levels and IL-1β directly correlated with SP levels (R = 0.9, $p < 0.001$ and R = 0.8, p = 0.002, respectively).

**Table 1**
Summary of main clinical and neuroimaging findings of post-COVID-19 OD patients. F = female, M = male, NP = not performed, age is expressed in years.

| Patient | Age | Sex | Other neurological complaints | Brain MRI |
|---|---|---|---|---|
| 1 | 29 | F | No | NP |
| 2 | 57 | F | No | NP |
| 3 | 47 | F | No | NP |
| 4 | 29 | F | Transient insomnia | NP |
| 5 | 61 | F | Persisting depression | Minimal periventricular gliosis |
| 6 | 50 | F | Transient headache | Normal |
| 7 | 30 | F | Transient headache | Normal |
| 8 | 33 | M | No | NP |
| 9 | 36 | M | No | Normal |
| 10 | 58 | M | No | NP |

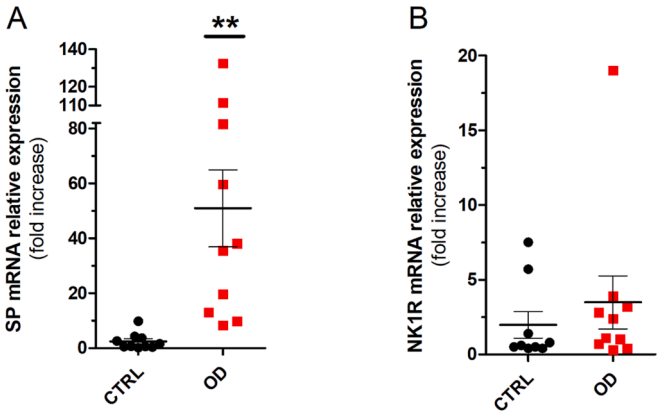


**Fig. 1.** mRNA expression levels of SP and NK1R genes in ONs from OD and control groups. Graphs show differences of SP (A) and NK1R (B) mRNA expression levels (measured by RT-PCR) between CTRLs (n = 10) and OD patients (n = 10). Data-points represent the mRNA fold increase value and the bars the mean value ± SEM.**$p < 0.01$ OD vs CTRL.

PK2 mRNA levels were also significantly higher in OD (7.8 ± 8.9 fold increase, U = 22.5, p = 0.04) than in CTRLs (2.0 ± 2.1) (Fig. 2A), while PK2L (OD = 2.9 ± 4.8 vs CTRLs = 1.7 ± 1.4), PKR1 (1.1 ± 1.4 vs 1.4 ± 1.5), and PKR2 (9.2 ± 11.9 vs 5.2 ± 7.7) levels did not reach statistical significance (Fig. 2B, C, D). In OD patients but not in controls, PKR2 levels directly correlated with PK2 levels (R = 0.8, p = 0.001).

In both the groups, neither gender differences nor correlations with age resulted.

In OD patients, IS correlated directly with PK2 levels ($Log_{10}$ + 1 transformed value) [$F(1,8) = 5.2$, p = 0.05, $R^2 = 0.32$; t = 2.3; p = 0.05] but not with SP levels ($Log_{10}$ + 1 transformed value).

### 3.3. Protein quantification

Immunofluorescence analysis showed that SP and PK2 proteins were also increased in ONs from OD patients. Immunofluorescence staining of SP showed a higher immunofluorescence signal in the ONs of all samples examined from the OD group compared with the CTRLs samples, in which the signal was weak (Fig. 3A). The SP positivity was expressed mainly in neuronal cells, as shown by colocalization with the neuronal marker β3-tubulin, but also in non-neuronal cells. SP was found in the cell cytosol and showed a predominantly granular pattern. Quantitative analysis of SP immunofluorescence staining (RGB pixel analysis) confirmed this significant difference: (OD = 29.3 ± 16.8 vs CTRLs = 7.4 ± 6.4) (Fig. 3B).

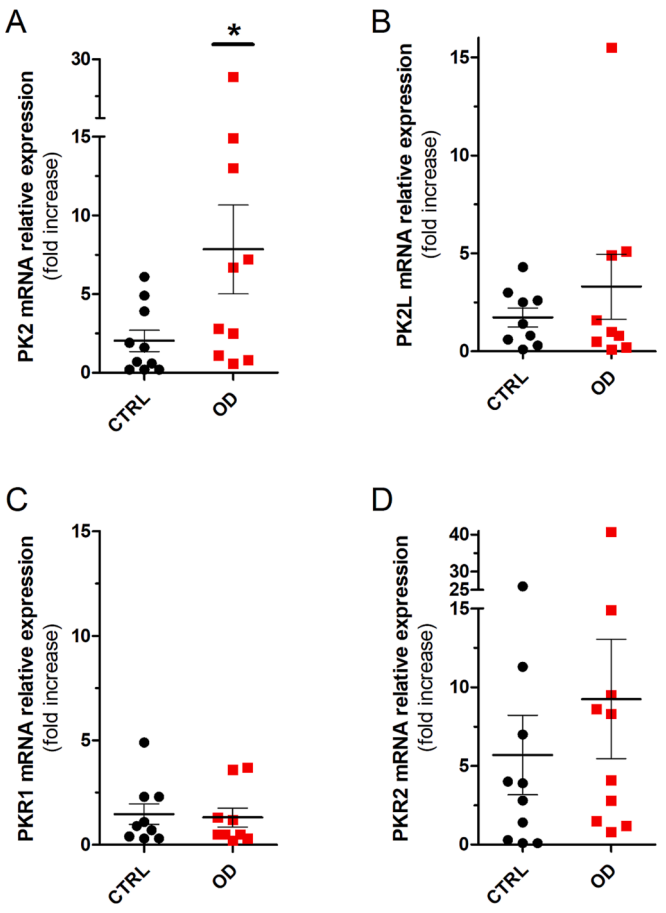


**Fig. 2.** mRNA expression levels of PK2 pathway genes in ONs from OD and control groups. Graphs show differences of PK2 (A), PK2L(B), PKR1 (C) and PKR2 (D) mRNA expression levels (measured by RT-PCR) between CTRLs (n = 10) and OD patients (n = 10). Data-points represent the mRNA fold increase value and the bars the mean value ± SEM.*p < 0.05 OD vs CTRL.

PK2 immunofluorescence signal was increased in ONs of the OD group compared with CTRLs (Fig. 4A), both in neuronal cells that were β3-tubulin positive and in non-neuronal cells, with a cytosolic localization (Fig. 4C). This increase was also confirmed by RGB immunofluorescence analysis: (OD = 19.5 ± 6.1 vs CTRLs = 4.3 ± 1.7) (Fig. 4B).

## 4. Discussion

This study deepened into molecular events underlying persistent post-COVID-19 OD by assessing, in ONs of patients, the activity of two inflammatory pathways tightly linked with olfaction pathophysiology. We found that either SP or PK2 were overexpressed in patients, the latter proportionally with residual olfaction.

SP is a neuroactive peptide (belonging to the tachykinin family), widely expressed through the human body, which exerts plenty of functions by binding the G-protein coupled Neurokinin receptor 1 (NK1R) (Severini et al., 2002). At the peripheral level, SP operates as a mediator of neurogenic inflammation (Schirinzi et al., 2021b; Tirassa et al., 2021). In particular, in the airways, SP is released by the trigeminal nerve in response to any kind of noxious stimuli, including viral infections. Via the NK1R, SP promotes a series of changes involving the vessels, the circulating immune cells, and the native local cells, leading to a massive, possibly harmful, inflammatory response (Mehboob, 2021; Ullah et al., 2021).

Here, we observed that patients with persistent post-COVID-19 OD maintained very high levels of SP expression in ONs, even long after the SARS-CoV-2 infection recovery. Albeit not statistically significant, also the expression of NK1R and IL-1β, one of the main downstream factors of SP (Cuesta et al., 2002; Mashaghi et al., 2016), remained higher, suggesting a sustained activation of the SP pathway. Such a persisting SP signalling within the olfactory structures might thus contribute to long-lasting OD. Mechanisms by which SP impairs smell or prevents the full recovery in post-COVID-19 patients can not be established here, but inflammation might be crucial. In fact, SP can trigger cytokine production and immune cell activation (Johnson et al., 2017), which are detrimental to olfaction (Doty, 2022). SP is involved in chronic rhinitis (Hanf et al., 2000), which in turn is often associated with hyposmia (Passali et al., 2022). Moreover, high SP levels have been measured in the nasal fluid of individuals with impaired smell (Steelant et al., 2018). Besides inflammation, SP might also directly affect neuronal transmission into the olfactory pathway. Indeed, the olfactory bulb expresses NK1R (Johnson et al., 2017), whereas SP serves as a neurotransmitter in the olfactory epithelium (Lucero, 2013) and the bulb, where depresses the neuronal firing (Olpe et al., 1987). However, factors leading to sustained SP pathway activation in post-COVID-19 OD patients remain unknown, although either genetic or environmental determinants should be considered (Tavano et al., 2013).

PK2 belongs to Prokineticins, a family of highly-conserved, secreted chemokines. PK2 primary transcript undergoes two alternative splice variants, PK2 and PK2-long (PK2L). PK2L is expressed only in peripheral tissues, while PK2 also in the brain (Lattanzi and Miele, 2022; Negri and Ferrara, 2018), in the olfactory system in particular. PK2 participates in several physiological and neurodevelopmental processes via two different G-protein coupled receptors (PKR1 and PKR2), ubiquitously present in the entire CNS (Negri and Ferrara, 2018). PK2 has a prominent role in olfactogenesis, contributing to the proper development and functioning of the olfactory system (Wen et al., 2019). In addition, PK2 serves as an inducible mediator, overexpressed in the presence of injurious insults to promote defensive reactions (Lattanzi et al., 2021, 2019,2018; Maftei et al., 2022; Schirinzi et al., 2021a).

In ONs of patients with post-COVID-19 OD, PK2 expression was significantly increased. As well, PKR2 was also augmented, although not significantly. PK2 pathway was thus activated in patients with persistent OD. Moreover, the PK2 levels were directly correlated with their residual olfaction (quantified through Sniff's test IS), suggesting that a greater PK2 expression in ONs might contribute recovering OD better. Indeed, the PK2 pathway could be activated in ONs by pathological triggers, including inflammation, and participate in a wide neuroprotective response, as it occurs in other neurological conditions (Bao et al., 2021; Gordon et al., 2016; Lattanzi et al., 2021; Lattanzi and Miele, 2022; Maftei et al., 2022; Negri and Ferrara, 2018). There is experimental evidence that PK2 prevents neuronal death, enhances mitochondrial bioenergetics, promotes protective Nrf-2 mediated effects, and finally induces a down-toning of inflammation, favouring a phenotypic shift in immune cells even at the CNS level (Neal et al., 2018). All these events associated with PK2-pathway activation might potentially play a role in smell recovery, although no direct proofs have been provided here. In particular, because efficient ONs mitochondria are critical for proper olfactory signalling (Fluegge et al., 2012), we could hypothesize that PK2, by the stimulation of mitochondrial activity, may improve olfaction, counteracting detrimental effects of chronic inflammation and SP, which instead aggravate oxidative stress and mitochondrial impairment (Wang et al., 2014). Indeed, the potential of PK2 as a neuroprotective mediator has been recently presented in Parkinson's disease, where patients at the early stages of the disease exhibit increased levels within the ONs (Schirinzi et al., 2022).

Otherwise, the higher PK2 expression could be simply related to the regeneration of the olfactory structures, consistently with the physiological role of the chemokine (Lattanzi and Miele, 2022; Ng et al., 2005).

Despite limitations due to the sample size or lack of genetic typing of virus, this study brought light on some molecular mechanisms underlying persistent OD in patients with long-COVID. We observed that SP was stably elevated in these patients' ONs, as for a continuous

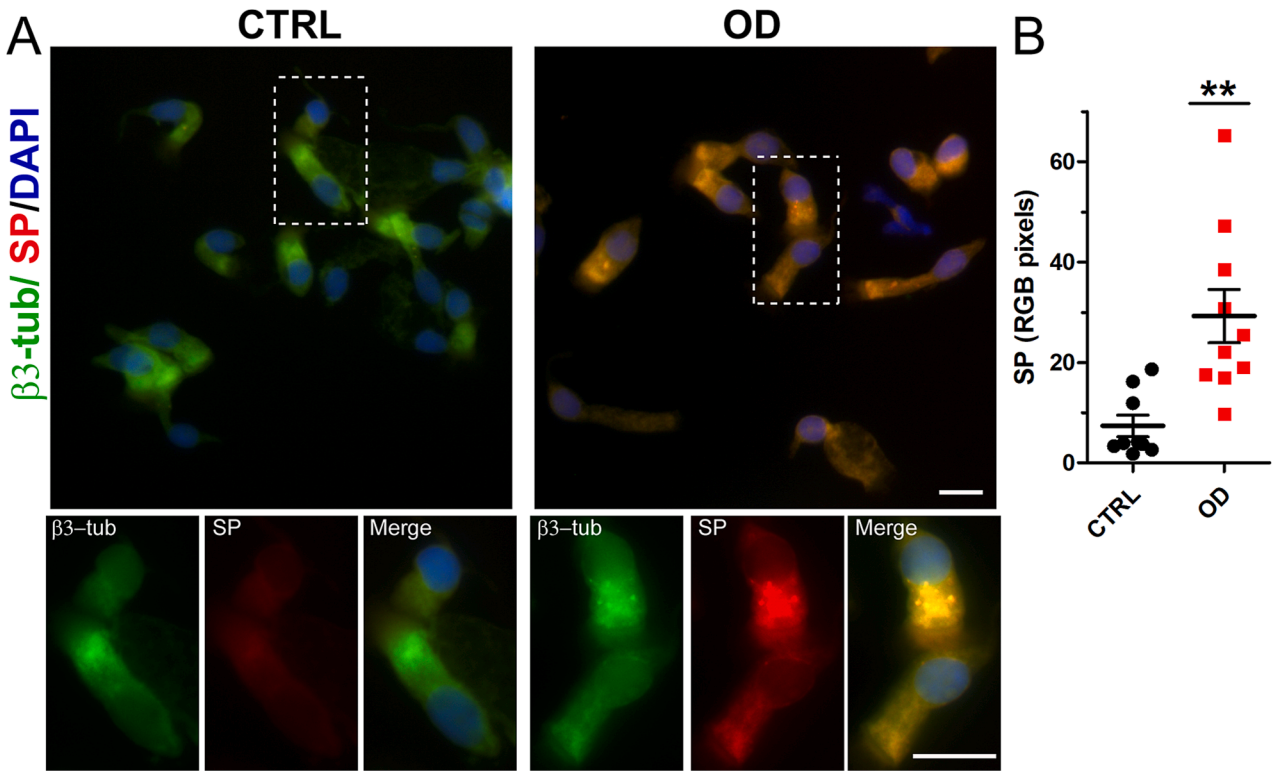


**Fig. 3.** Immunofluorescence images of SP protein in ONs from OD and control groups. (A) SP (red) and β3-tubulin (green). Cell nuclei were counterstained with DAPI (blue). Scale bar: 10 μm in all panels (B) SP fluorescence quantification by RGB analysis (data-points = immunofluorescence quantification value; bars: mean value ± SEM) in CTRLs (n = 10) and OD patients (n = 10). **p < 0.01 OD vs CTRLs.

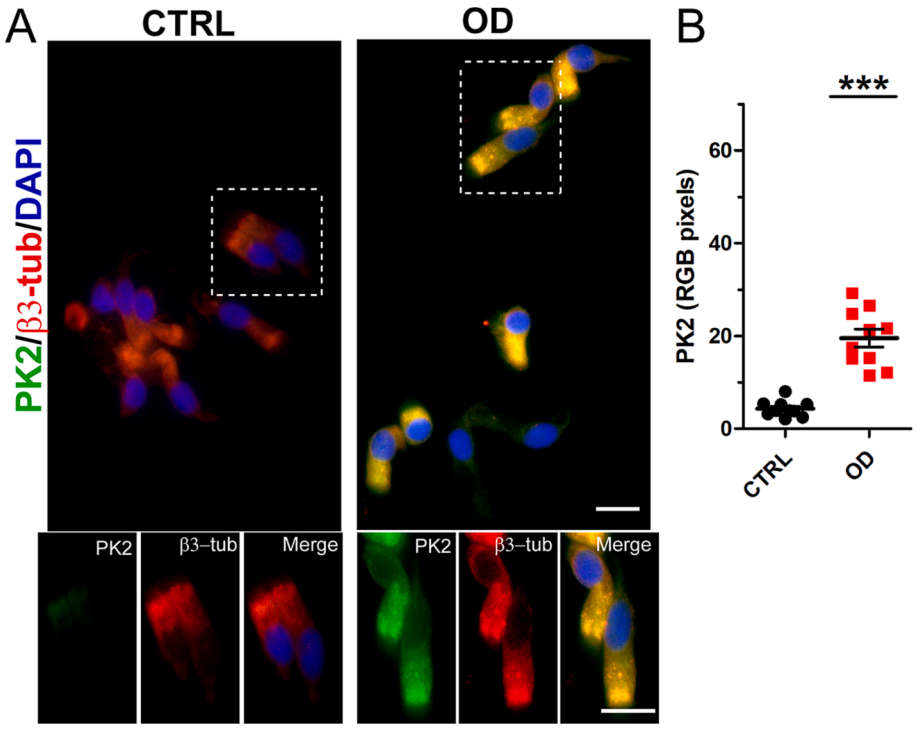


**Fig. 4.** Immunofluorescence images of PK2 protein in ONs from OD and control groups. (A) PK2 (green) and β3-tubulin (red). Cell nuclei were counterstained with DAPI (blue). Scale bar: 10 μm in all panels. (B) PK2 fluorescence quantification by RGB analysis (data-points = immunofluorescence quantification value; bars: mean value ± SEM). ***p < 0.001 OD vs CTRLs.

inflammatory state able to affect the olfactory signalling. In addition, we found that PK2 was overexpressed in ONs, probably in a compensatory way because of the direct association between the levels and the residual olfaction. Therefore, we could hypothesize two distinct, probably opposite, roles for SP and PK2 in persistent post-COVID-19 OD, with the first involved in the pathogenesis, and the latter in the recovery. Definitely, dedicated experiments are needed to establish the exact mechanisms by which SP and PK2 directly operate on olfactory signalling. Since OD prevalence also changes depending on the virus variants (Hintschich et al., 2022; Vaira et al., 2022), different molecular responses to various virus strains should be considered.

ONs are now recognized as a reliable model for brain neuropathology, since many of neurodegenerative diseases' pathological hallmarks could be found in ONs (Brozzetti et al., 2020; Stefani et al., 2021). Accordingly, we might argue that persistent inflammatory changes observed in the olfactory mucosa of post-COVID-19 OD patients could also affect the brain, accounting for some structural or functional changes. Indeed, a brain connectivity rearrangement within the olfactory functional network has been observed in patients with persistent post-COVID-19 hyposmia (Esposito et al., 2022).

To reduce the theoretical risk for long-term neurological sequelae and alleviate complaints of patients with post-COVID-19 OD an effective therapy is wandered (Noce et al., 2021). Therefore, evidence for the involvement of SP and PK2 could be useful in designing precise therapeutic interventions. The modulation of chronic inflammation with the SP/NK1R axis antagonism has already been postulated as a potential treatment either for the acute or the successive phases of COVID-19 (Mehboob, 2021). In particular, aprepitant, an NK1R inhibitor, showed exciting effects in a single case of "long COVID" and in small pilot study on critical COVID-19 patients (Mehboob et al., 2020; Reinoso-Arija et al., 2021).

Our data support this possibility, but especially suggest PK2 as an alternative therapeutic target for post-COVID-19 OD, which might lead to smell improvement via restoring mitochondrial functioning and the resolution of inflammation.

## 5. Conclusions

This work provided unprecedented, preliminary evidence that SP and PK2 pathways may play a role in persistent post-COVID-19 OD, emerging both as candidate targets for therapeutic interventions. Overall, further confirmatory studies on larger replication cohorts are now needed, possibly including post-COVID-19 patients without OD.

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## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data availability

Data will be made available on request.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbi.2022.12.017.

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