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Unravelling the genetic bases of the sense of smell: clinical and molecular characterisation of a large cohort of COVID-19 patients with persistent olfactory dysfunction

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Introduction

The olfactory system plays an essential role in modulating human behaviour, survival, and interaction with the environment. Despite the strenuous research efforts aimed at better characterising the molecular mechanisms that regulate olfactory function, a full and thorough insight is still far from being achieved. Furthermore, studying the pathophysiological mechanisms underlying the various causes of olfactory dysfunction has proven equally demanding, leaving a significant knowledge gap. In particular, among the different types of smell alteration, the recent COVID-19 pandemic has risen considerable awareness towards a very common, yet underestimated condition, namely post-viral olfactory dysfunction (PVOD). As there are remarkable differences in the development of this disorder between people affected even by the same strain of SARS-CoV-2, it is believed that host genetic factors and, specifically, those that regulate the host immune response, are involved in its etiopathogenesis.

Aim of the study

In order to investigate the host genetic factors that might be implicated in COVID-19-related PVOD, the goal of this investigation has been to analyse the genetic landscape of rare and common variants within genes involved in antiviral response regulation in a cohort of 153 deeply clinically and molecularly characterised patients previously affected by SARS-CoV-2 infection and presenting with long-term olfactory dysfunction.

Materials and Methods

Concerning the clinical characterisation, a detailed personal anamnesis was collected for all participants and specific questionnaires were administered with the aim of both recollecting COVID-19-related symptoms experienced during the acute phase of the infection and reporting the symptoms sustained at the moment of the evaluation. In particular, patients were asked to report on the perceived severity of smell dysfunction through Visual Analogue Scales (VAS). A psychophysical evaluation has been carried out to objectively assess participants' smell alterations: specifically, orthonasal olfactory function was evaluated through the extended Sniffin' Sticks test battery (Threshold – Identification - Discrimination scores and combined TDI). Concerning the genetic analyses, Whole Genome Sequencing (WGS) was performed for all recruited subjects and all coding variants within a manually curated shortlist of 298 genes involved in antiviral response regulation were extracted. For each gene and for each individual, a gene score, defined as the ratio of the number of homozygous alternative variants within the gene to its length, was calculated. The association between the gene score and VAS or TDI was tested with linear regression models, adjusted for sex and age. All analyses were performed on the entire cohort and on women and men separately; for X-linked genes, the analyses were performed only on women and men separately.

Results

A total of 475 biallelic variants in 177 genes were extracted and classified according to variant frequency into rare (Minor Allele Frequency - MAF - <1%), low-frequency (MAF between 1% and 5%), common (MAF between 5% and 10%), and very common (MAF≥10%). The gene score was calculated, for each participant and for each gene, taking into account variant frequency. The association between the gene score and self-perceived smell alteration was evaluated considering the scores attributed by the participants to the Smell function VAS with reference to the moment of the psychophysical evaluation. In this case, no statistically significant association was highlighted in any of the tested MAF groups. Conversely, the association between the gene score and the objectively assessed olfactory performance was evaluated considering the scores obtained by the enrolled subjects at the combined TDI test and at the Threshold, Discrimination, and Identification subtests considered separately. The following statistically significant results have been obtained:

- Considering rare variants (MAF<1%), an increased gene score within the *ACE2* gene resulted associated with a worsen olfactory performance in females (combined TDI *p-value* = 0.03; Discrimination subtest *p-value* = 0.03).
- Regarding common variants (MAF≥10%), an increased gene score within the *IFI44* gene resulted associated with a better smell function in males (Discrimination subtest *p-value* = 0.01) and a similar outcome has been highlighted for an increased gene score within the *NDUFAF4* gene (Discrimination subtest *p-value* = 0.004).

Taking into account the physiological role of the proteins encoded by these genes and literature data, it can be hypothesised that an altered expression of *ACE2* may be associated with a protracted and severe inflammatory response in the olfactory epithelium, thus worsening patients' smell abilities. Conversely, an increased gene score in *IFI44* and

NDUFAF4 might be associated with a decreased inflammatory response, thus correlating with a better olfactory performance.

Conclusions

This study has allowed the identification of new host genetic factors that may play a pivotal role in determining the interindividual variability of COVID-19-related PVOD. These results help to shed light on the genetic architecture of long-lasting smell loss and open new possibilities to better clarify the molecular mechanisms underlying the complex physiology of the olfactory system.

ABSTRACT - ITALIAN

Introduzione

Il sistema olfattivo riveste un ruolo essenziale non solo nella modulazione dei comportamenti umani e nell'interazione con l'ambiente ma è anche un senso fondamentale per la sopravvivenza del genere umano. Nonostante vi sia un'intensa attività di ricerca volta ad identificare i meccanismi molecolari che regolano la funzione olfattiva, una conoscenza completa ed approfondita di questo organo di senso non è stata ancora raggiunta. Analogamente, lo studio dei meccanismi fisiopatologici responsabili delle diverse forme di disfunzione olfattiva si è rivelato altrettanto complesso, presentando tuttora significative lacune. In particolare, tra le varie tipologie di alterazione olfattiva, la recente pandemia da COVID-19 ha incrementato l'attenzione mediatica e scientifica nei confronti di una condizione piuttosto comune, anche se sottostimata, definita disfunzione olfattiva post-virale. Considerando come vi siano notevoli differenze nello sviluppo di questa patologia anche tra individui con infezione dovuta al medesimo ceppo di SARS-CoV-2, attualmente si ritiene che la variabilità genetica interindividuale, ed in particolare la presenza di varianti in geni coinvolti nella risposta immunitaria, possa contribuire in modo decisivo all'eziopatogenesi della disfunzione olfattiva post-virale.

Scopo dello studio

Al fine di approfondire il ruolo della variabilità genetica interindividuale nell'eziopatogenesi della disfunzione olfattiva post-virale, lo scopo di questo studio è stato quello di analizzare la presenza di varianti rare e comuni nei geni implicati nella regolazione della risposta immunitaria antivirale. Tale analisi è stata svolta in 153 soggetti, caratterizzati in modo accurato sia dal punto di vista clinico che molecolare, con storia di pregressa infezione da SARS-CoV-2 e presenza di disfunzione olfattiva a lungo termine.

Materiali e Metodi

Per quanto riguarda la caratterizzazione clinica, oltre alla raccolta di un'accurata anamnesi, ad ogni soggetto arruolato nello studio sono stati somministrati specifici questionari con lo scopo di ottenere informazioni in merito alla sintomatologia COVID-19-correlata presente sia durante la fase acuta dell'infezione che al momento della valutazione clinica. Nello specifico, ad ogni partecipante è stato richiesto di valutare la propria percezione della disfunzione olfattiva tramite scale VAS (Visual Analogue Scales). Inoltre, una valutazione oggettiva delle abilità olfattive di ogni soggetto è stata effettuata tramite il test esteso degli Sniffin' Sticks, che permette di esaminare la soglia olfattiva e le capacità di identificazione e discriminazione degli odori, sia in maniera indipendente che ai fini di calcolare uno score combinato definito TDI. Per quanto riguarda le analisi genetiche, per ogni paziente è stata eseguita l'analisi di sequenziamento dell'intero genoma al fine di estrarre tutte le varianti codificanti presenti in una lista curata manualmente di 298 geni coinvolti nella regolazione della risposta antivirale. Per ogni gene e per ogni soggetto è stato calcolato un *gene score*, definito come il rapporto tra il numero di varianti omozigoti alternative presenti in ogni gene e la sua lunghezza. Successivamente, l'associazione tra il *gene score* e il punteggio della scala VAS o del TDI è stata testata tramite modelli di

regressione lineare, aggiustati per sesso ed età. Tutte le analisi sono state eseguite sia sull'intera coorte che per maschi e femmine separatamente; per quanto concerne i geni localizzati sul cromosoma X, le analisi sono state eseguite unicamente considerando i due sessi separatamente.

Risultati

Dai dati di sequenziamento dell'intero genoma è stato possibile identificare 475 varianti bialleliche in 177 geni; le varianti sono state classificate secondo la loro frequenza in rare (frequenza dell'allele minore - MAF - <1%), a bassa frequenza (MAF compreso tra 1% e 5%), comuni (MAF compreso tra 5% e 10%) e molto comuni (MAF≥10%). Il *gene score* è stato calcolato per ogni soggetto e per ogni gene tenendo in considerazione la diversa frequenza delle varianti. L'analisi di associazione tra il *gene score* e i punteggi attribuiti dai soggetti alla scala VAS relativa alla funzione olfattiva riferita al momento della valutazione psicofisica non ha evidenziato alcun risultato statisticamente significativo. D'altra parte, l'analisi di associazione tra il *gene score* e l'esito della valutazione oggettiva delle performance olfattive espresso in termini di punteggio TDI combinato e di punteggio ottenuto ai singoli test di valutazione di soglia, identificazione e discriminazione degli odori ha permesso di identificare i seguenti risultati statisticamente significativi:

- Per quanto riguarda le varianti rare (MAF<1%), un *gene score* elevato nel gene *ACE2* è risultato essere associato ad una peggiore performance olfattiva nei soggetti di sesso femminile (test TDI combinato: *p-value* = 0.03; test di discriminazione degli odori: *p-value* = 0.03).
- Per quanto riguarda le varianti comuni (MAF≥10%), un *gene score* elevato nel gene *IFI44* è risultato essere associato ad una migliore performance olfattiva nei soggetti di sesso maschile (test di discriminazione degli odori: *p-value* = 0.01). Un risultato

analogo è emerso in relazione alla presenza di un *gene score* elevato nel gene *NDUFAF4* (test di discriminazione degli odori: *p-value* = 0.004).

Considerando il ruolo fisiologico delle proteine codificate dai geni soprariportati ed i dati pubblicati nella letteratura scientifica, è possibile ipotizzare come un'alterata espressione del gene *ACE2* possa essere associata alla presenza di una risposta infiammatoria prolungata e severa a livello dell'epitelio olfattivo, con conseguente peggioramento delle abilità olfattive dei pazienti. D'altra parte, un *gene score* elevato nei geni *IFI44* e *NDUFAF4* potrebbe invece essere associato ad una diminuita risposta infiammatoria, portando quindi ad un miglioramento della performance olfattiva.

Conclusioni

Questo studio ha permesso di identificare nuovi fattori genetici dell'ospite che potrebbero svolgere un ruolo fondamentale nella determinazione della variabilità interindividuale che caratterizza la disfunzione olfattiva post-virale COVID-19-correlata. Questi risultati incrementano le conoscenze scientifiche inerenti alle basi genetiche della disfunzione olfattiva persistente, aprendo nuovi orizzonti nello studio dei meccanismi molecolari che regolano la complessa fisiopatologia del sistema olfattivo.

1. INTRODUCTION

The olfactory system, from an evolutionary perspective, is one of the most ancient sensory systems, and is essential for human behaviour and interaction with the environment (1). However, despite its archaic origins, still little is known about the pathophysiological and molecular mechanisms that regulate smell function, as it was well underlined by the doctor and poet Lewis Thomas in 1990: "*I should think we might fairly gauge the future of biological science, centuries ahead, by estimating the time it will take to reach a complete, comprehensive understanding of odor*" (Thomas, L. (1990). *A Long Line of Cells: Collected Essays*. New York: Book-of-the-Month-Club). Even if more than thirty years have passed, this remark is still extremely factual.

1.1 The olfactory system: macroscopic and microscopic anatomy

The maturation of the olfactory structures begins early during embryonic and foetal development, as it has been demonstrated that primordial olfactory bulbs appear at 41 days of gestation (2). Afterwards, they become visible on foetal Magnetic Resonance Imaging (MRI) around 28-30 weeks of gestation, which is the exact time when foetal responses to odorants remarkably begin (3). Indeed, chemical odorants resulting from maternal exposure and

circulating in the amniotic fluid have been demonstrated to elicit specific responses from the foetus, as changes in foetal heart rate, movements, swallowing rates, and facial grimacing. Notably, these early responses are not accompanied by a full maturation of neuroanatomical structures, which, in terms of synaptogenesis and myelination, reach completeness after birth (4).

From an anatomical point of view, the olfactory system may be divided into a peripheral and a central portion. The peripheral section is formed by the nasal cavity, where the olfactory mucosa may be found, whereas the central olfactory structures comprise the olfactory bulb, the olfactory tract and the central nervous system (CNS) structures that receive and elaborate olfactory stimuli, as the olfactory cortex of the temporal lobe, the hippocampus, the amygdala, and the hypothalamus.

The nasal cavity presents an anterior portion, which is constituted by the nostrils and the vestibule, and a wider posterior section, where, from the lateral nasal wall, three osseous projections (conchae) covered in mucosa, defined as superior, middle, and inferior nasal turbinates, extend into the nasal passages (5). A fraction of individuals possesses an additional supreme turbinate, which may be unilateral or bilateral (6). All turbinates act as air passageways and are covered in nasal mucosa which has three main functions, namely humidification, clearing from airborne particles, and temperature conditioning of the incoming air (7). The lower and middle turbinates are covered by respiratory mucosa, while the supreme and superior turbinates together with the posterior portion of the middle turbinate are covered by olfactory mucosa (5). It has to be noted that the olfactory mucosa is not uniformly distributed, as patches of respiratory mucosa may be found amid it, and, with aging, they may even substitute it, thus making macroscopic morphological evaluations extremely challenging (8). Overall, the extension of the olfactory mucosa is highly specific for each species and few

studies have attempted to estimate its extension in humans. For instance, Salazar *et al*. have calculated that it covers approximately 3-5% of the nasal cavity (9). However, it has to be noted that the olfactory mucosa is not flat: indeed, it is characterised by the presence of olfactory pits, invaginations into the connective tissue of 150-200µm which not only extend its surface but also trap odorous molecules in order to prolong their interaction with the olfactory receptors (OR) (10).

The olfactory mucosa consists of three different layers: the olfactory epithelium (OE), the basal lamina, and the lamina propria (**Figure 1**).

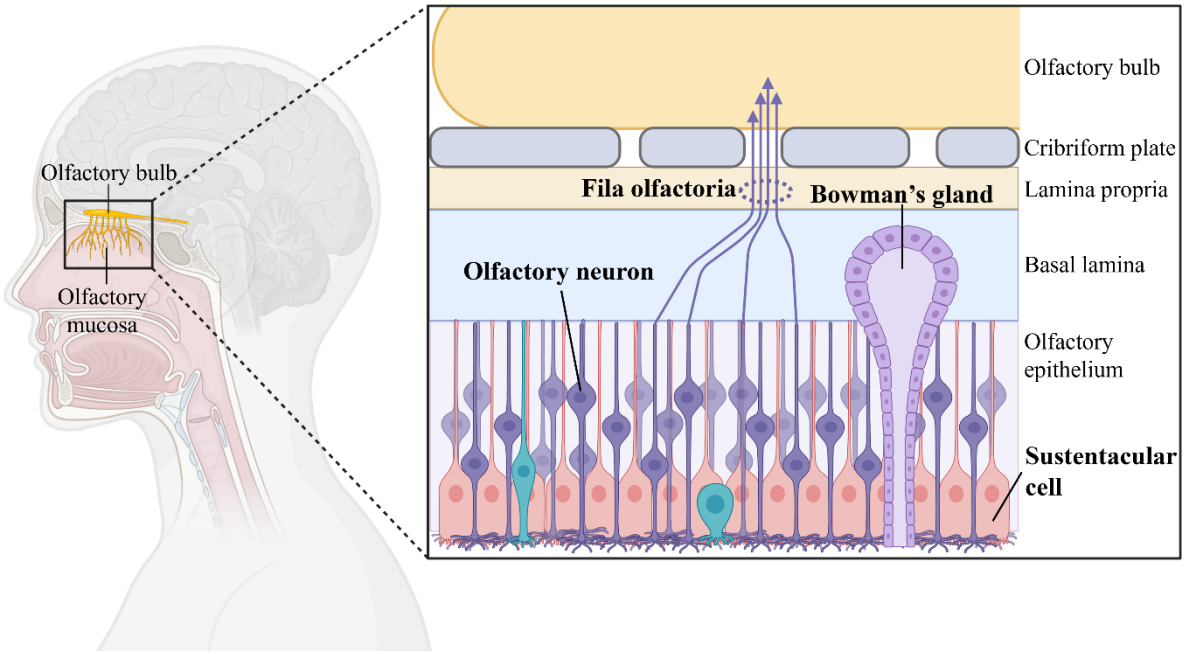

Figure 1. Olfactory mucosa and olfactory bulb anatomical organisation. *The olfactory mucosa comprises the olfactory epithelium, where olfactory neurons and sustentacular cells can be found, the basal lamina, and the lamina propria, that contains Bowman's glands. Unmyelinated axons departing from the ONs form the fila olfactoria, that cross the cribriform plate of the ethmoid bone to reach the olfactory bulb. Created with BioRender.com.*

The OE is a pseudostratified columnar epithelium that contains different types of cells, as olfactory neurons (ONs), sustentacular cells, microvillar cells, and basal cells (7). ONs are

sensory bipolar neuroepithelial cells specialised in odorant detection: it is estimated that in a healthy adult there are about ten million mature ONs (11). Each ON projects a single dendrite that present an apical swelling, defined as the olfactory knob, from which approximately ten immotile cilia originate and extend into the thick layer of mucus that covers the surface of the OE. Cilia are microtubule-based organelles, with nine doublets of microtubules arranged around a central core, where another pair of microtubules is found (9+2 configuration) (12); ORs are localised on the cilia and are responsible for the interaction with odorants. On the proximal end, ONs have a thin unmyelinated axon that projects towards the olfactory bulb (OB), in order to convey the impulse generated by odorous stimuli towards the central structures involved in smell perception (13). Sustentacular cells are supporting cells characterised by the presence of microvilli that are essential for the homeostasis and proliferation of ONs, with whom they are connected through tight junctions (8). The supranuclear compartment of sustentacular cells extends towards the surface of the OE, where they not only play a role in the metabolic transformation and removal of odorants, but also protect ONs from toxic agents (13). Conversely, the basal compartment of sustentacular cells lies alongside ONs and basal cells until the basal lamina. Microvillar cells are non-neuronal chemoresponsive cells that present on one extremity a bundle of microvilli that extends towards the mucus layer of the OE and on the other extremity a thin axon-like cytoplasmatic projection towards the OB. Their exact role is currently poorly elucidated but they are believed to play a modulatory function for the surrounding cells of the OE (14). Finally, basal cells are located above the basal membrane and are the stem cells of the OE, from which both ONs and sustentacular cells originate. There are two types of basal cells, horizontal and globose: the latter are they main source of ONs regeneration, both during physiological turnover and after OE injury (15). The ability to regenerate ONs is one of the peculiarities of the olfactory system;

however, the exact molecular mechanisms underlying this process have still not been thoroughly decoded.

The basal lamina separates the OE from the lamina propria, which contains connective tissue cells, capillaries, immune cells as macrophages and lymphocytes, ensheathing cells, and Bowman glands. Ensheathing cells are specialised glial cells that surround ONs axons after they join together to form fascicles that project towards the OB. Whenever the olfactory nerve is injured, they migrate to the injury site where they have an active role in debris clearance and promotion of neural regeneration (16). Bowman glands are branched tubuloalveolar glands that have narrow excretory ducts that extend across the OE towards its surface, where a thick mucus is discharged (17).

As previously mentioned, ONs project their unmyelinated axons towards the OB: they are covered by ensheathing cells and grouped together in small nerve bundles known as *fila olfactoria* (18). There are around 15-20 bundles on each side of the nasal cavity: all together they constitute the cranial nerve I, which crosses the cribriform plate of the ethmoid bone through its multiple foramina to ipsilaterally reach the OB (19). The OB is the first central relay of the olfactory system and it is located underneath the frontal cerebral lobe in the anterior cranial fossa, in a depression defined olfactory fossa (20). The OB is organised in multiple layers, that include the olfactory nerve layer, the glomerular layer, the external plexiform layer, the mitral cell layer, the internal plexiform layer, and the granular cell layer. ONs axons enter the OB forming the olfactory nerve layer and synapse with the dendrites of secondary olfactory neurons in the glomerular layer. Secondary olfactory neurons comprise mitral, tufted, and periglomerular cells: mitral and tufted cells present a solitary principal dendrite that is projected towards a glomerulus, several secondary dendrites that form the external plexiform layer, and a solitary axon that is projected towards the olfactory tract, whereas periglomerular cells act as

interneurons within the OB (21). The olfactory tract originates at the posterior margin of the OB and represents its major output towards the cerebral cortex. At the olfactory trigone, each olfactory tract is divided into a medial and a lateral olfactory stria. The medial olfactory stria is smaller and sends projections towards the ipsilateral olfactory nucleus and the contralateral olfactory bulb, where it is involved in the autonomic responses associated with olfaction (22). The lateral olfactory stria is responsible for conveying smell impulses towards the primary olfactory cortex, the main site of olfactory perception processing. The primary olfactory cortex is located at the base of the frontal lobe and inferior surface of the temporal lobe and is constituted by several cortical areas that include the olfactory tubercle, the piriform cortex, the amygdala, and the entorhinal cortex. All these regions are involved in behavioural and emotional response to odorous stimuli, participating in learning and memory of odours (i.e. quality, identity, familiarity, and hedonics) and in multisensory integration (23). The primary olfactory cortex is directly connected to the secondary olfactory cortex, which, in humans, is represented by the orbitofrontal cortex, that combines taste and olfactory stimuli, together with visual information to regulate flavour perception (24).

1.2 The sense of smell: physiology and genetics

The sense of smell is elicited by airborne chemicals, which are volatile, light, and usually hydrophobic compounds that are capable of binding the ORs localised on the ONs surface. ORs belong to the superfamily of Guanine nucleotide protein (G-protein)-coupled receptors (GPCRs), a wide family of membrane proteins consisting of seven hydrophobic transmembrane segments that are activated by a number of extracellular ligands (25). Upon binding with odorants, olfactory GPCRs transduce the signal by activating the heterometric G_{olf} protein that has intrinsic GTPase activity (26). Its main target is represented by adenylyl cyclase III (AC3) which catalyses the conversion of ATP into cyclic adenosine monophosphate

(cAMP). Its increased intracellular concentration leads to cyclic nucleotide gated channels opening, with a subsequent increase in intracellular cations that cause the depolarisation of ONs membrane (27).

Scattered across the human genome there are approximately 390 putatively functional genes encoding ORs and more than 460 OR pseudogenes, thus making OR the largest gene family in the entire genome (28). OR coding genes are localised in clusters on all chromosomes, with the exception of chromosome 20 and Y, and are organised in 18 families and 300 subfamilies, which may contain from a single gene or pseudogene to several ones (29). OR genes are highly polymorphic intron-less genes, with a coding region extending for approximately 1Kb and several equally polymorphic upstream regulatory motifs (30). Each ON expresses a single OR gene and, specifically, only its maternal or paternal allele; however, despite it seems to happen in a stochastic way, the exact molecular mechanisms underlying this event are still poorly elucidated (31).

The huge number of OR coding genes together with the "one neuron – one receptor" rule are not enough to explain the great variety of odorants and mixtures that the human brain is able to recognise. Indeed, it has been proposed that the olfactory system is based on combinatorial coding, as one receptor can bind several odorants, possibly sharing similar chemical properties, and one odorant can bind several receptors, being the combination of OR that recognises a single odorant unique (31,32). All ONs expressing the same OR converge in the OB in the same glomerulus and a given odorous stimulus is recognised through a specific combination of activated glomeruli. This creates a sort of map (i.e. chemotopic representation) in the OB that allows odorant discrimination based on the spatial coordinates of the activated glomeruli (32).

Considering the huge number of ORs, their high genetic variability, and the stochastic choice behind their expression in ONs, it is reasonable to believe that not all human beings

perceive odorants in the same way. Indeed, OR polymorphic nature constitutes the molecular basis for specific interindividual variations in threshold, identification, intensity, and pleasantness perception (33). For instance, it has been demonstrated that polymorphisms in the *OR7D4* gene are associated with a different perception of androstenone and androstadienone (34), whereas hyperosmia to isovaleric acid is due to polymorphisms in the *OR11H7P* gene (35). However, despite the huge efforts of the last decades, an exhaustive knowledge of all the ligands of each OR and of the combination of OR that is capable of recognising a specific odour is far from being achieved. To date, only 48 ORs have been attributed known ligands and the specificity and functional properties of about 90% of human ORs remain unknown (33).

Additionally, the exact molecular mechanisms that regulate odour recognition in the CNS are even less understood. Indeed, odorant identification requires higher cognitive functions, as training, memory, and experience, and involves a powerful interaction between emotion and cognition.

1.3 Olfactory dysfunction

During evolution, a correct identification of odorants has proven essential both for survival (i.e. recognition of potential poisons, spoiled foods, or smoke) and overall well-being (i.e. successful social relationships and mating) (36). Therefore, the alteration of the ability to smell poses a significant threat to health and quality of life.

The term “olfactory dysfunction” comprehends every alteration of the perception of odours and it includes the following conditions (37):

- Anosmia: complete loss of the sense of smell.
- Hyposmia: marked reduction of the sense of smell.

- Specific anosmia/hyposmia: selective alteration of the ability to perceive a single odorant.
- Phantosmia (olfactory hallucination): perception of an odorous stimulus in the absence of an odour.
- Parosmia: distorted perception of odours.

Anosmia and hyposmia represent quantitative alterations of the sense of smell, whereas phantosmia and parosmia are qualitative modifications of smell perception.

Overall, estimates of population prevalence of olfactory dysfunction are extremely variable, as studies have shown that this disorder can affect between 2.7% and 24.5% of people, depending on the considered age range and other specific features of the analysed group of subjects (38). It has also been demonstrated that up to 75% of affected patients are not aware of smell alterations until they are objectively tested (39), and studies are widely concordant in affirming that self-reports generally underestimate the magnitude of olfactory impairment (40).

Olfactory dysfunction is classified into three main categories, according to the aetiology and the anatomical location of damage, namely sensorineural, conductive, and mixed olfactory impairment (41). Sensorineural smell alterations are caused by direct damage to the peripheral or central structures involved in smell perception; conductive dysosmia is due to obstruction of nasal aeration that prevents odorants from reaching the OE; mixed olfactory dysfunction is characterised by both the above-mentioned components. Additionally, olfactory alterations can be primary disorders or secondary to a number of conditions and events.

1.3.1 Primary olfactory dysfunction

Congenital anosmia is an extremely rare primary disease affecting approximately 1:10.000 individuals and defined as a complete absence of the sense of smell from birth; it

might be an isolated finding or a feature of a syndromic condition (42). The genetic bases of isolated congenital anosmia are still poorly elucidated and, so far, only few cases have been reported, where causative variants in the *KCNA3*, *OBPIIA, CNGA2*, and *TENM1* genes have been identified (43–46). Conversely, common multisystemic genetic conditions that feature congenital anosmia and whose molecular bases are well characterised include Kallmann syndrome, congenital insensitivity to pain, and ciliopathies as Bardet-Biedl syndrome (47–49). According to a recent review, 71 different genes have so far been associated with syndromic olfactory impairment, being 48 of them causative of Kallmann syndrome (37). Furthermore, few genes are responsible of both isolated and syndromic forms of congenital olfactory impairment, as *PROK2*, *PROKR2*, *CHD7*, *FGF8*, *FGFR1*, and *SEMA3A* (50–53).

1.3.2 Secondary olfactory dysfunction

Secondary olfactory dysfunction is extremely common and might be attributed to several factors, including aging, environmental exposure, head trauma, brain tumours and surgery, neurodegenerative disorders, and infections.

It has been shown that the ability to detect, differentiate, and identify odours sharply declines after the age of 50 (54), affecting up to 60% of the population in the age group 80-97 (40). Biopsies of the olfactory mucosa have shown progressive substitution of the OE with respiratory epithelium and degenerative changes of the OE, with a marked decrease in basal cell proliferation and therefore a reduction of ONs physiological turnover (55).

Environmental exposure has long been known to potentially cause olfactory dysfunction and it can be divided into two different possible scenarios: on the one hand, industrial or occupational exposure of few individuals to high concentrations of a given airborne chemical compound (i.e. cadmium, chromium, manganese, lead, acrylates, styrene, solvent mixtures) (56), and, on the other hand, exposure of the entire population to air pollution

in industrialised cities (57). Several studies have demonstrated how environmental exposure to airborne pollutants causes morphological alterations of the OE, including basal cells hyperplasia and squamous metaplasia, and upper respiratory tract obstruction, thus ultimately being associated with a reduction in smell perception (58).

Concerning head trauma, injury at any level of the olfactory pathway, from the nasal cavity to the brain, can cause smell impairment. In these cases, sensorineural smell loss is more common but also mixed olfactory dysfunction can arise; notably, sensorineural alterations present an extremely complex pathophysiology and are often characterised by poor prognosis and low recovery rates (59). Opposite to the acute onset of olfactory dysfunction due to head trauma is the chronic smell alteration caused by the slow growth of brain tumours. They can directly arise from olfactory structures, as the esthesioneuroblastoma (60), or from other brain structures in close proximity, as the olfactory grove meningioma (61) or glioblastomas (62); in these cases, smell impairment may not only be due to the presence of the tumour itself but also arise as a consequence of brain surgery or radiotherapy.

Another intriguing occurrence is represented by the correlation between olfactory alterations and neurodegenerative disorders. Indeed, in the last decade, it has been highlighted how smell impairment can be considered an early hallmark of conditions as Alzheimer's and Parkinson's diseases, Lewy body dementia, and Huntington's disease, preceding by years the onset of cognitive and motor symptoms (63). Alzheimer's disease is the most common cause of dementia and is characterised by two main neuropathological markers, namely the extracellular deposition of amyloid-β plaques and the intracellular aggregation of hyperphosphorylated tau protein in neurofibrillary tangles (64). In postmortem tissues from Alzheimer's disease patients, both amyloid-β plaques and tau protein tangles can be found in the OE and OB, and it has been shown that their presence is associated with increased apoptosis of ONs and sustentacular cells (65). Parkinson's disease is characterised by progressive and

preferential loss of dopaminergic neurons in the substantia nigra that causes the appearance of motor symptoms. The main prodromal non-motor symptoms in affected patients are represented by alterations in odour discrimination, identification, and detection threshold, which can be highlighted in approximately 90% of patients (66). It has been demonstrated that smell alteration correlates with progressive deposition of Lewy bodies composed by misfolded alpha-synuclein in the OB, which seems one of the earliest affected brain regions (63).

Finally, the most common cause of secondary smell impairment is represented by upper respiratory tract viral infections, which can cause both an acute smell impairment, due to nasal obstruction, increased mucus production, and mucosal oedema, and a persistent condition known as post-viral olfactory dysfunction (PVOD) (67). In most cases PVOD is transient and between 66% and 94% of patients experience a spontaneous improvement of the olfactory function (67). However, whenever the condition is permanent, it has a significant impact on patients' daily living and psychological well-being, thus representing a public health concern (68). Several viruses can cause PVOD, including Rhinoviruses, Influenza and Parainfluenza viruses, Respiratory Syncytial Virus, Adenoviruses, and Herpesviruses (69). PVOD recognises different pathophysiological mechanisms: for instance, the Parainfluenza viruses have been shown to increase the production of pro-inflammatory cytokines that promote damage to the OE (70), while the Respiratory Syncytial Virus can directly infect ONs and transcriptomic studies have demonstrated how olfactory signalling is among the altered pathway in patients infected by this pathogen (71). Overall, three different degrees of severity of OE damage have been identified: decreased number of ONs with maintenance of their cellular structure; complete depletion of ONs in the presence of normal basal and sustentacular cells; OE destruction with squamous epithelium metaplasia (72). Additionally, it has been postulated that PVOD might be due to damage to the central olfactory pathways leading to retrograde

degeneration, as the OE is directly connected to the brain and thus might provide a direct route for viruses to penetrate the CNS (73).

In the last years, PVOD has been gaining increasing attention from the medical community in consideration of its extraordinary high frequency among patients affected by Coronavirus disease 19 (COVID-19), caused by the Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) (74).

1.3.2.1 Olfactory dysfunction in COVID-19

COVID-19 related olfactory impairment may be divided into two distinct conditions, namely acute loss of the sense of smell and persistent PVOD. Acute olfactory dysfunction has been estimated to present a global prevalence of 47.85%, with significant differences according to COVID-19 clinical severity, geographical area, sex, and viral variant (75). Acute smell loss typically arises around the fourth day of infection and, in most cases, improves spontaneously within two weeks after the resolution of upper respiratory tract symptoms (76). Since the beginning of the COVID-19 pandemic, in early 2020, several hypotheses have been suggested to explain the pathophysiology of acute olfactory impairment and some of them have easily been discarded. For instance, in the early stages, it was speculated that, in accordance with the events that follow other upper respiratory tract infections, congestion of the nasal mucosa due to swelling and obstruction of the olfactory cleft could explain the transient nature of smell loss. However, it was soon proved that most patients with COVID-19 do not present rhinorrhoea, thus conductive olfactory impairment does not seem to affect patients (77). Conversely, sensorineural smell alteration seems a much more plausible hypothesis and different molecular mechanisms may contribute to this occurrence. The main receptor that mediates SARS-CoV-2 entry into host cells is the Angiotensin-Converting Enzyme 2 (ACE2): the receptor-binding domain of the Spike (S) protein of the virus engages ACE2 and exploits

the Transmembrane Serine Protease 2 (TMPRSS2) for S protein priming, in order to achieve viral and cellular membranes fusion (78). ACE2 and TMPRSS2 are highly expressed in the OE and, specifically, immunostaining and single-cell sequencing studies confirmed that they are expressed in sustentacular cells, Bowman gland cells, perivascular cells, and stem cells, rather than in ONs (79). This discovery has allowed to discard the hypothesis of SARS-CoV-2 neurotropic potential and direct ONs damage, highlighting instead how the depletion of supporting cells may be a crucial event. Firstly, SARS-CoV-2 infection and disruption of sustentacular cells and Bowman gland cells causes a decrease in the production of mucus that physiologically covers the OE (80). Mucus alterations, both in quantity and in chemical composition, impedes the diffusion of odorant molecules towards the ONs, thus interfering with the processing and signal transduction induced by odorants (81). Secondly, sustentacular cells are fundamental for ON metabolism. Specifically, the olfactory knob of ONs lacks a sufficient number of mitochondria to supply enough energy for signal transduction and thus generates ATP by glycolysis (82). Glucose transport in the OE is finely regulated by sustentacular cells, which uptake glucose from the capillaries of the basal lamina and secrete it at their apical surface in the mucus. ONs uptake glucose through glucose transporters (GLUT3) expressed in the cilia and employ it to maintain the energy-dependent signal transduction cascade that follows ORs binding with odorants (83). It has been demonstrated that reduction in ONs glucose supplement could be due to two different mechanisms: on the one hand, in the earliest stage of infection, SARS-CoV-2 redirects glucose utilisation in infected sustentacular cells towards viral replication, and on the other hand, in later stages, supporting cells death completely depletes glucose supplies in the mucus (74). Thirdly, sustentacular cells are essential for ONs cilia development and maintenance. Upon loss of contact between ONs and supporting cells, the olfactory knob of ONs swells and degenerates, thus losing its cilia; in animal models, ONs cilia depletion has been demonstrated to occur as early as two days after

SARS-CoV-2 infection (84). Cilia retraction is associated with a complete abolishment of the olfactory transduction signal cascade (84). All these mechanisms efficiently explain acute smell loss in COVID-19 patients, as they are compatible with the transient nature of this event and the rapid recovery observed. Indeed, smell recovery can be appreciated when approximately 5-10% of ONs have regained their functionality. This figure, corresponding to about 500.000 to one million ONs, can be quickly achieved through both maturation of immature ONs that are still present in the OE and by regrowth of cilia on surviving ONs after the regeneration of supporting cells, which requires approximately 4-8 days (74).

However, these hypotheses fail to clarify why a significant fraction of patients develop persistent PVOD, which is defined as the persistence of the symptom more than 12 weeks after the infection (85). In this light, recent studies have raised attention towards the possible involvement of the host immune response in modulating ONs function (**Figure 2**).

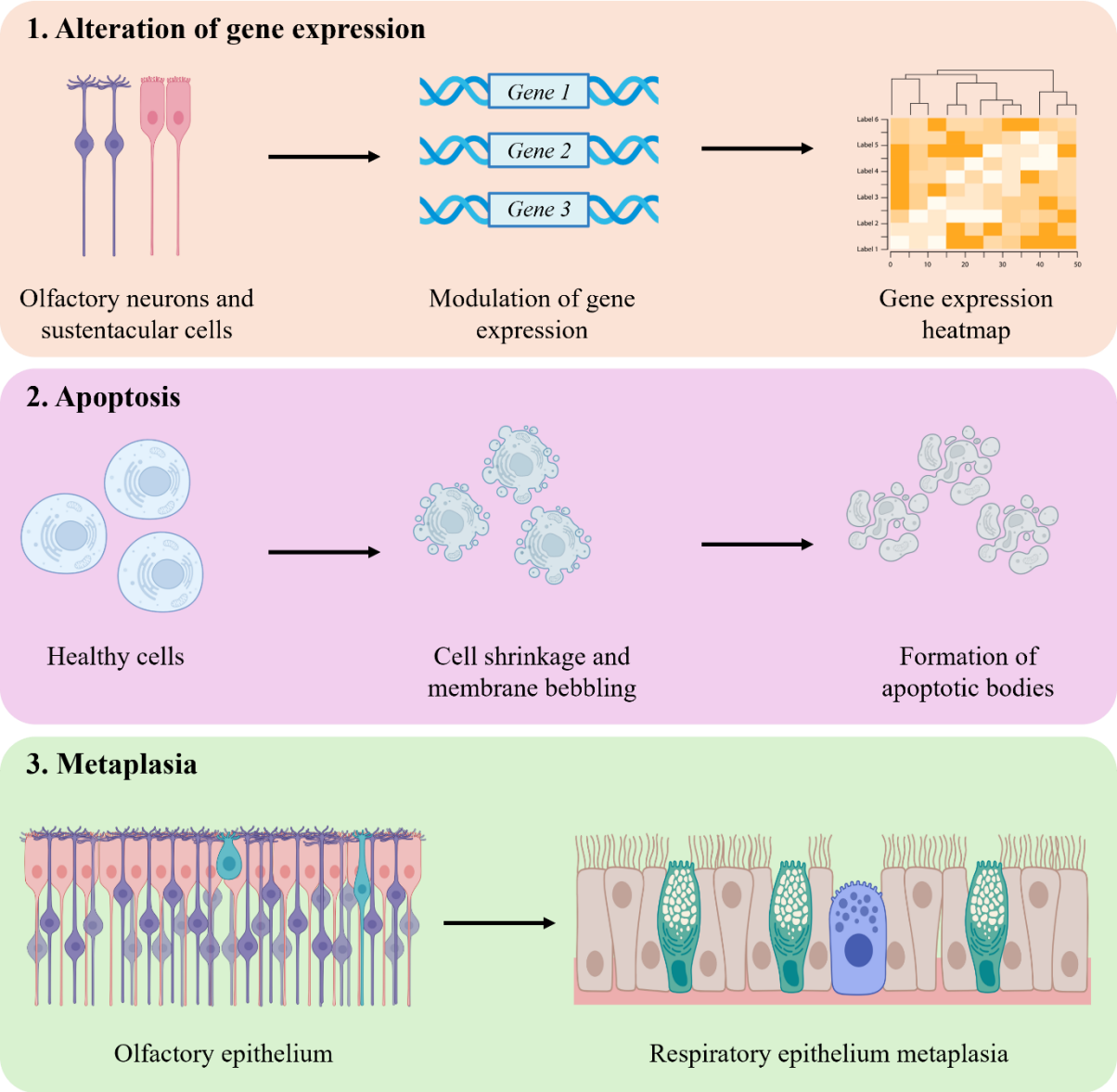

Figure 2. Consequences of OE chronic inflammation. *1. Alteration of gene expression: chronic activation of the immune system modulates the gene expression of both ONs and sustentacular cells, thus causing an overall impairment of the molecular pathways that are physiologically activated by odorants. 2. Apoptosis: chronic inflammation causes massive apoptosis of all cell types of the OE, with a significant delay in its recovery rates. 3. Metaplasia: chronic inflammation could also cause a complete substitution of the OE with ciliated respiratory epithelium, similarly as seen in age-related olfactory dysfunction. Created with BioRender.com.*

Indeed, inflammation modulates gene expression in the OE and it has been shown that it can downregulate the expression of OR genes and other genes involved in olfactory signal transduction, as *ADCY3* (86). This modulation may be a consequence of deciliation, which leads ONs back to a less mature state when OR assembly and trafficking to the cilia are not yet

fully developed (87). Additionally, the host immune-response can induce massive apoptosis of OE cells, including ONs. In this case, especially if the inflammatory response becomes chronic, OE recovery might be extremely delayed or not happen at all, with a substitution of the olfactory mucosa with respiratory epithelium or squamous metaplasia (88). However plausible these hypotheses may seem, they do not explain while there is considerable interindividual variability in persistent smell loss onset, progression, and resolution timing. In this light, it seems reasonable to take into account host genetic factors that may be crucially involved in determining these discrepancies, as they have already proven to play a central role in determining interindividual variability in SARS-CoV-2 infection-related acute anosmia. Indeed, a Genome-Wide Association Study (GWAS) has highlighted how polymorphisms in the *UGT2A1/UGT2A2* locus are associated with acute smell alterations in COVID-19 patients. Both genes encode for glucuronosyltransferases that metabolise odorants and that are expressed by sustentacular cells, thus underlining once more their role in COVID-19-related acute olfactory impairment (89). Nevertheless, to date, no host genetic factor has been linked to persistent smell alterations after SARS-CoV-2 infection, posing a significant knowledge-gap towards the understanding of this intriguing condition.

2. AIM OF THE STUDY

Despite the strenuous research efforts of the last decades aimed at better characterising the molecular mechanisms that regulate olfactory function, a full and thorough knowledge is still far from being achieved. Indeed, both the peripheral and the central portions of the olfactory system present peculiar anatomical and functional features that make their investigation extremely challenging. In this context, studying the pathophysiological mechanisms underlying the different causes of olfactory dysfunction has proven equally demanding, leaving a significant knowledge-gap.

In particular, the recent COVID-19 pandemic has risen awareness towards a very common, yet underestimated and understudied condition, namely PVOD. Despite several studies have been conducted to analyse the epidemiology, timing of onset, progression, and resolution, still little is known about its underlying molecular mechanisms. Specifically, the causes behind the acute loss of smell perception have been fairly understood and some host genetic factors that can modulate this event have already been identified, as polymorphisms in the *UGT2A1/UGT2A2* locus. Conversely, there are still few hypotheses that might explain the persistent alteration of olfactory function that affects only a fraction of patients and, so far, they are focused on the possible alteration of the host immune response. However, to date, no host

genetic factor that could explain the interindividual variability of this condition has been recognised yet.

In order to further investigate the occurrence of PVOD in COVID-19 patients, the aim of this study has been to identify possible host genetic factors that could determine the interindividual variability of this disorder. In this light, the goal of this investigation has been to apply an approach that has already proven effective in the identification of genes associated with the host response to COVID-19 (90,91), namely the analysis of the genetic landscape of rare and common variants, in a cohort of 153 patients previously affected by COVID-19 and presenting with long-term olfactory dysfunction. Specifically, the purposes of our study have been organised as follows:

1. Carry out an accurate clinical characterisation of all subjects, through a detailed anamnesis and an evaluation of both their perception of olfactory alteration achieved via self-administered questionnaires and their olfactory abilities achieved through objective smell tests.
2. Perform Whole Genome Sequencing (WGS) analysis on all patients and identify in all of them coding biallelic variants in genes involved in the antiviral response regulation pathway.
3. Calculate, for each gene and for each individual, a gene score defined as the ratio of the number of homozygous alternative variants within the gene to its length, in order to evaluate the association between this parameter and olfactory function, both as self-reported alteration perception and objectively tested ability. The final goal has been to assess the possible contribution of biallelic variants in genes involved in the immune response in modulating olfactory ability.

The identification of host genetic factors possibly involved in the pathogenesis of persistent smell loss may help to better stratify affected patients, thus providing them with the best standards of care, and potentially allowing for a more personalised and appropriate clinical management.

3. MATERIALS AND METHODS

3.1 Ethical statement

Written informed consent was obtained from all participants for their participation in the study and the collection of biological samples for research purposes. The study was conducted in accordance with the tenets of the Helsinki Declaration and was approved by the Ethics Committee of Friuli-Venezia Giulia Region (Application No. CEUR-2020-Os-156).

3.2 Study cohort and clinical evaluations

One hundred and fifty-three patients were recruited in collaboration with the Division of Otorhinolaryngology and Neck Surgery of Cattinara Hospital (Trieste, Italy) between September 2021 and November 2022. All patients were referred to an otorhinolaryngology consultation due to the presence of long-lasting smell loss, defined as the persistence of the symptom more than 12 weeks after SARS-CoV-2 infection (85).

COVID-19 was previously confirmed for all patients through Reverse Transcription Polymerase Chain Reaction (RT-PCR) test for SARS-CoV-2 RNA performed on nasopharyngeal swab, according to international guidelines (92). During the clinical evaluation, baseline demographic data, dates of first positive and negative COVID-19 test, date

of onset of chemosensory alteration, and, when applicable, information concerning the type and date of vaccine administration were collected. A brief anamnesis was compiled, with specific attention to physiological information (i.e. weight and height that were employed to subsequently calculate the Body Mass Index (BMI)), personal habits (i.e. smoking and alcohol consumption), and past medical history (i.e. previous history of hypertension, cardiovascular diseases, diabetes, chronic respiratory diseases, liver diseases, present history of cancer). Furthermore, specific information regarding COVID-19 was collected through self-administered questionnaires and olfactory function was assessed through a detailed psychophysical evaluation obtained by means of objective tests and procedures.

3.2.1 Self-administered questionnaires

At the moment of the clinical evaluation, all patients were required to fill in specific questionnaires aimed both at recollecting COVID-19-related symptoms experienced during the acute phase of the infection and at reporting the symptoms presently sustained. In particular, patients were firstly asked whether they had suffered from COVID-19 pneumonia, to immediately identify subjects with a severe condition. Afterwards, patients were required to complete a specific item of the standardised Sino-Nasal Outcome Test-22 items (SNOT-22) questionnaire, the Acute Respiratory Tract Infection Questionnaire (ARTIQ), and the World Health Organisation-Five Well-Being Index (WHO-5); additionally they were asked to report on the perceived severity of chemosensory dysfunction through Visual Analogue Scales (VAS).

SNOT-22

The SNOT-22 questionnaire is a patient-reported tool implemented to evaluate symptoms and quality of life in patients affected by chronic rhinosinusitis with or without nasal

polyposis; in time, its use has spread to evaluate the perception of patients affected by several other conditions, included hereditary haemorrhagic telangiectasia, Wegener's granulomatosis and Chronic obstructive pulmonary disease, as well as their outcome after surgical procedures, as septoplasty (93). Among the 22 different items, in this study only the question regarding the "Loss of taste and smell" was taken into account. The scoring system is based on a Likert scale where 0 = "No problem", 1 = "Very mild problem", 2 = "Mild or slight problem", 3 = "Moderate problem", 4 = "Severe problem", and 5 = "Problem as bad as it can be." Higher scores therefore indicate the presence of more severe symptoms (94). Patients were asked to rate their chemosensory dysfunction both during the acute phase of the infection and at the moment of the psychophysical evaluation.

ARTIQ

The ARTIQ is a patient-reported outcome measure to assess the severity and functional impacts of acute respiratory tract infections. The questionnaire is focused on the main symptoms that affected patients usually experience, and the items included comprised: dry cough, coughing up mucus, blocked nose, rhinorrhoea, sneezing, lacrimation, raucousness, fever, sweating, chills, headache, sore throat, muscle pain, joint pain, chest pain, sinonasal pain, neck tumefaction, loss of appetite, problems breathing, and shortness of breath (95). Furthermore, specific *ad hoc* questions were added to the standard ones in order to further evaluate specific symptoms that are associated with COVID-19, as the presence of tiredness, dizziness, diarrhoea, nausea, vomit, and abdominal pain (96). The scoring system is based on a Likert scale where 0 = "No symptom", 1 = "Mild symptom", 2 = "Severe symptom". Additionally, a specific question aimed at evaluating the possible presence of chemosensory dysfunction is included in the ARTIQ; in this case the scoring system is the following: 0 = "No alteration", 1 = "Smell loss only", 2 = "Taste loss only", 3 = "Smell and taste loss" (95).

Patients were asked to rate their symptoms both recalling the acute phase of the infection and considering the moment of the psychophysical evaluation.

VAS

VAS aim at measuring subjective or behavioural experiences and psychosocial responses to health problems and consist in 10cm lines with well-defined descriptors at each end (97). Patients are asked to place a mark on the point of the scale that best fits their feelings in relation to the descriptors; the placement of the mark is measured and it can both be used to consider the analysed variable as continuous or, if the line is broken into segments, as discrete. VAS have long been used in several different contexts and their application in the medical field has thoroughly been studied especially to assess patients' perception of pain (98). In this study, VAS were employed to evaluate patients' perception of nasal congestion, olfactory and gustatory alteration, and flavour perception, with reference both to the acute phase of COVID-19 and to the moment of the psychophysical evaluation. All scales ranged to 0 (completely altered perception of the analysed item) to 10 (physiological perception of the considered item). During the evaluation, patients were additionally asked to mark a VAS on trigeminal function: in this case, patients were asked to smell 70% acetic acid and label the scale according to the perceived intensity level.

WHO-5

The WHO-5 is a measure of general subjective psychological well-being and is one of the most widely employed questionnaires aimed at evaluating patient self-perception of current feelings (99). It is organised in five different items, namely 1) "I have felt cheerful and in good spirits", 2) "I have felt calm and relaxed", 3) "I have felt active and vigorous", 4) "I woke up feeling fresh and rested" and 5) "My daily life has been filled with things that interest me".

Each patient had to rate how well every sentence described their feelings on a scale from 0 (none of the time) to 5 (all the time) with reference to the two weeks preceding the moment of the psychophysical evaluation.

3.2.2 Psychophysical evaluation

The psychophysical evaluation consisted in objective tests aimed at evaluating patients' chemosensory dysfunction. In particular, regarding the sense of smell, orthonasal olfactory function was assessed; furthermore, the Peak Nasal Inspiratory Flow (PNIF) was measured and a direct inspection of the olfactory cleft was achieved through nasal fibroscopy.

Orthonasal olfactory function evaluation

The orthonasal olfactory function was assessed through the validated extended Sniffin Stick's Test battery (Burghart Messtechnik, Wedel, Germany), which consists of three separate tests that allow to determine odour threshold (T), discrimination (D), and identification (I) abilities. The examination is based on pen-like odorous devices that were placed at a distance of approximately two centimetres from the patient's nostrils for about three seconds. In the threshold test, 16 different dilutions of a specific odorant (i.e. phenyl ethyl alcohol) were tested: for each concentration, patients were presented with three felt-tip pens, one containing the odorant and the other two containing a solvent (propylene glycol). The assessment began with the lowest odorant concentration, thus proceeding with increased concentrations; whenever a patient could identify the correct pen in each triplet twice in a row, the test was repeated lowering the concentration until the subject could not perceive the odorant anymore. In the discrimination test, 16 pen triplets were presented to patients; in each triplet, two pens contained the same odorant and the third released a different odorous molecule that the subject was supposed to identify. Finally, in the identification test, patients were asked to smell 16 pens

emanating different odours. Upon smelling, subjects had to indicate which object better described the scented smell choosing among four different possible items written on flash cards (100). Each separate test was attributed a score that ranged from 0 to 16 and results were summed to obtain the combined TDI score (range: 0-48). Overall results allow to classify olfactory dysfunction in three different categories of increasing severity:

- TDI ≤ 16: anosmia.
- TDI between 16.25 and 30.5: hyposmia.
- TDI ≥ 30.75: normosmia.

PNIF

The PNIF is a measure of nasal patency and evaluates the maximum airflow that is produced during forced nasal inspiration (101). The evaluation has been repeated three times for each patient and the average of the values was considered informative. It has to be noted that, to date, there is no shared consensus regarding the cut-off value that marks altered nasal patency; however, it has been shown that a cut-off of 90mL/min shows a high sensitivity and negative predictive value (102).

Fibroscopy

Nasal fibroscopy was employed to visually inspect the olfactory cleft, with the final goal of determining the presence of oedema and secretions. According to their clinical presentation, patients were attributed a score of 0 if the appearance of the olfactory cleft was physiological, a score of 1 if they presented either oedema or secretions, and a score of 2 if both signs were present.

3.3 Whole Genome Sequencing (WGS) analysis

3.3.1 DNA extraction and quality control

A peripheral blood sample was collected from every patient and genomic DNA was extracted using the QIAsymphony® SP instrument with the QIAsymphony® Midi Kit (Qiagen, Venlo, The Netherlands), following manufacturer's instructions. DNA quality was verified through 1% agarose gel electrophoresis and final concentration was assessed employing the Nanodrop ND 1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE, USA) and the Qubit DNA Broad Range kit (Thermo Fisher Scientific, Waltham, MA, USA).

3.3.2 DNA library preparation

DNA libraries were prepared using the Illumina DNA Prep Kit (Illumina, San Diego, CA, USA), which requires a minimum DNA input of 100-500ng and grants DNA tagmentation, amplification, and pooling (https://support.illumina.com/content/dam/illumina-support/documents/documentation/chemistry_documentation/illumina_prep/illumina-dna-prep-reference-guide-1000000025416-10.pdf).

DNA tagmentation is a transposome-mediated reaction that is performed to simultaneously enzymatically fragment DNA and tag it with adapter sequences. Bead-linked transposomes (BLT) technology involves the presence of an engineered transposase pre-loaded with double-stranded sequencing adapters; genomic DNA is captured by BLTs and, upon fragmentation, tagged with partial adapter sequences. BLTs saturation with DNA allows for the normalisation of higher DNA inputs, as once the beads are saturated, no more tagmentation can happen. After tagmentation, two rounds of clean-up washes were performed before proceeding to the amplification step.

Amplification was achieved using a limited-cycle PCR program, where the adapter sequences added during the tagmentation process act as complementary sequences for the PCR

primers. This step also allowed not only for the amplification of DNA fragments but also for the addition of Index 1 (i7) and Index 2 (i5) adapters together with additional sequences required for sequencing cluster generation. Afterwards, double-sided bead purification was employed to purify the amplified libraries.

Finally, library pooling was performed by mixing an equal volume of 24 different libraries. After all steps of DNA library preparation were completed, the pool was quantified using the Qubit and its quality was checked using the Advanced Analytical Fragment Analyzer with the HS-NGS High Sensitivity 474 kit (Agilent, Santa Clara, CA, USA). The last step of library preparation required dilution of the pool to the needed concentration for the sequencing procedure.

3.3.3 DNA sequencing

DNA sequencing was performed on an Illumina NovaSeq6000 instrument (Illumina, San Diego, CA, USA). Illumina sequencing is based on the sequencing-by-synthesis method, that uses fluorescent-labelled nucleotides to identify single bases as they are added to DNA strands. The process is divided in three phases (https://www.illumina.com/):

- Loading: DNA fragments are loaded onto a flow cell which is covered in forward and reverse oligos complementary to the adapter sequences of the fragments. Double-strand DNA is denatured into single-strand fragments that hybridise to the flow-cell oligos.
- Clustering: oligos are used as primers and a polymerase synthesises a complement of each hybridised fragment, thus forming new double-strand DNA molecules, which are denatured in order to wash away the original template fragments. Single-strand DNA molecules remain attached to the flow cell and are clonally amplified through bridge amplification that generates clusters of identical DNA molecules. After amplification,

the reverse strands are cleaved and washed off, and the 3'-end of all forward strands is blocked to prevent further amplification.

- Sequencing-by-synthesis: a primer binds the forward strand primer binding site and a polymerase adds fluorescently labelled nucleotides (deoxynucleotides triphosphates - dNTPs) to the DNA strand. The fluorophore acts as a terminator for polymerisation and only one nucleotide at a time can be incorporated. Each of the four bases has its specific colour and, after every dNTP incorporation, the emitted fluorescence is recorded. Afterwards, the fluorophore is enzymatically removed and the following nucleotide can be incorporated in the elongating DNA strand. The analysis of the emission wavelength together with the signal intensity determines the base call.

3.3.4 WGS data analysis

After sequencing, FASTQ files were generated and analysed following GATK best practices. This workflow comprises several steps, including:

- Quality control: it was performed with *Fastqc* software (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/, last accessed on July 19, 2024) and *Fastp* software (v0.21.0) (103), retaining only reads with Q>20.
- Sequence alignment to Human Genome Reference build 38 (GRCh38p.13) using *BWA-mem* (v2.1) (104).
- PCR-duplicates removal and Base Quality Score Recalibration: this step was achieved using *Sambamba* (v1) (105) and *GATK* (v4.1.9.0) (106), respectively.

Joint variant calling was performed using GATK *haplotype caller* and *GenomicsDBImport* (106) generating gVCF files. Afterwards, a quality check was performed applying *Variant Quality Score Recalibration* with *GATK* and the following filter thresholds:

Hardy–Weinberg equilibrium (p-value < 10^{-8}), missing rate (missing rate > 0.05), heterozygosity rate (p-value < 10^{-8}), coverage (coverage pre variant calling ≥ 20), and singletons distribution (singletons distribution mean >3 standard deviations). Finally, data was phased using *Eagle* (v2.4.1) (107) and annotated using the *Variant Effect Predictor* (VEP) (v.106) (108).

3.3.5 Genes and variants selection

The Ingenuity Pathway Analysis software (Ingenuity System Inc., Redwood City, CA, USA; https://digitalinsights.qiagen.com/products-overview/discovery-insights-portfolio/analysis-and-visualization/qiagen-ipa/) was employed to extract a list of genes involved in the antiviral response regulation. The list was further manually curated in order to select only the genes described in the Online Mendelian Inheritance in Man® (OMIM®) free-access catalogue of human genes and genetic disorders (https://www.omim.org/), finally retaining 298 genes (See **Supplementary Table 1**). For each gene, all biallelic coding variants were extracted from WGS data.

For each gene, the start and end position were extracted using BioMart (GRCh38.p12, Ensembl) (109) and, subtracting these two values, each gene's length was calculated.

3.4 Statistical analyses

Concerning the phenotypic characterisation, Wilcoxon signed-rank test for paired samples was employed to compare SNOT-22, ARTIQ, and VAS scores attributed by patients with reference to the acute phase of the infection and at the moment of the psychophysical evaluation. A *p-value* of <0.05 was considered statistically significant.

In order to verify the possible association between host genetic factors and smell function, the following approach has been implemented: firstly, we subdivided all biallelic

variants into bins according to their Minor Allele Frequency (MAF): 1) MAF<1% (rare variants); 2) MAF comprised between 1% and 5% (low-frequency variants); 3) MAF comprised between 5% and 10% (common variants); 4) MAF≥10% (very common variants). Genotypes were coded 1 for alternative homozygotes and 0 for reference homozygotes and heterozygotes. Afterwards, for each individual and for each gene, a gene score was calculated defined as the ratio of the number of homozygous alternative variants within the gene to its length calculated in kilobases. Finally, for each gene, the association between the gene score (independent variable) and smell alteration VAS at the moment of the psychophysical evaluation or orthonasal olfactory function tests results (dependent variables) was tested with linear regression models. All models were adjusted for sex and age. All analyses were performed on the entire cohort and on women and men separately; for X-linked genes, the analyses were performed only on women and men separately. In each MAF bin, all *p-values* were adjusted using the Benjamini-Hochberg method. An adjusted *p-value* of <0.05 was considered statistically significant. All statistical analyses were performed using the R software version 4.1.2 (R Foundation for Statistical Computing, Vienna, Austria).

4. RESULTS

4.1 Clinical characterisation of the cohort

One hundred and fifty-three subjects presenting persistent olfactory dysfunction after mild-to-moderate SARS-CoV-2 infection were enrolled in this study. The majority of the study sample was composed by female individuals, who represented 72.5% of the cohort. Baseline demographic data of the participants are reported in **Table 1**.

Age	**Total** (N = 153)	**Females** (N = 111)	**Males** (N = 42)
Mean (SD)	49.2 (14.3)	51.1 (14.0)	44.2 (14.2)
Range	18.0, 90.0	18.0, 90.0	20.0, 76.0

Table 1. Baseline demographic data. *The table reports the age of the enrolled subjects, expressed as mean, standard deviation, and age range; details are reported for the entire cohort and for females and males separately.*

4.1.1 Personal habits and past medical history

Concerning the personal habits, smoking and alcohol consumption were considered, dividing the enrolled subjects in three different categories, according to whether they had never presented the specific bad habit, they presented it in the past or at the moment of the psychophysical evaluation (**Figure 3**). Regarding smoking, 130 subjects (86.1% of the cohort) declared to have never smoked, 12 (7.9%) reported to have been smokers in the past, and nine

(6.0%) revealed to currently be smokers. The information was missing for two patients. With reference to alcohol consumption, 129 patients (85.4%) reported to have never been alcohol consumers, 19 (12.6%) notified to have consumed alcohol in the past, while three (2.0%) disclosed to be current alcohol consumers. The information was missing for two subjects.

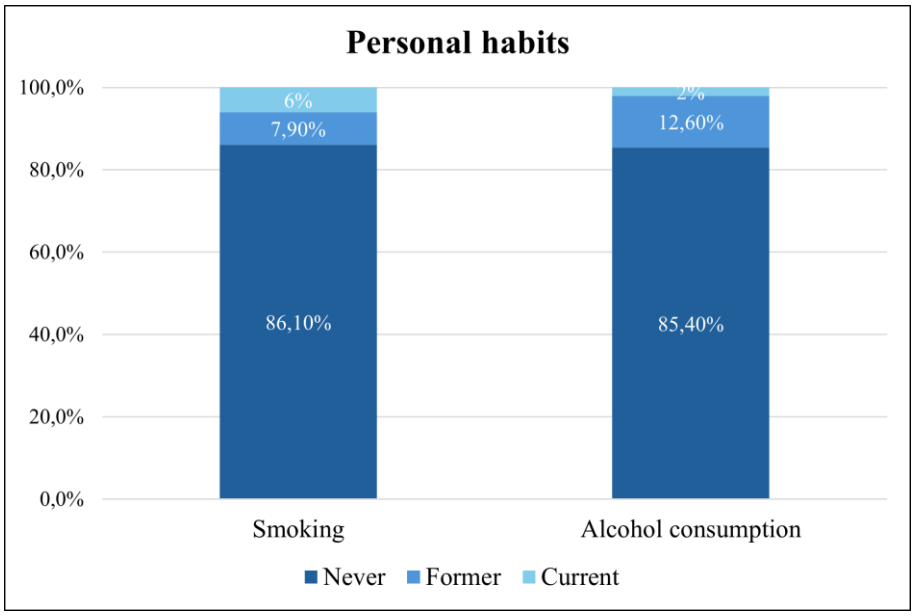

Figure 3. Stacked bar chart of the distribution of personal habits. *The two categories of personal habits are reported on the X axis. The percentages of subjects that have never presented the specific habit, that presented it in the past, or at the moment of the psychophysical evaluation are reported on the Y axis.*

Patients' past medical history was briefly collected, in order to assess the possible presence of factors that could have an impact on the clinical course after SARS-CoV-2 infection. Specifically, the presence of hypertension, cardiovascular diseases, diabetes, chronic respiratory diseases, liver diseases, and cancer was assessed (**Figure 4**). Overall, only few patients reported the presence of comorbidities and cardiovascular diseases and hypertension were the most frequently reported conditions. Indeed, two male (4.9%) and six female (5.5%) individuals presented a history of chronic heart disease, while five men and 14 women declared to suffer from increased blood pressure. Additionally, anamnestic data revealed that two male (4.9%) and one female (0.9%) subjects displayed chronic respiratory diseases. Interestingly,

none of the male patients disclosed a history of diabetes or active cancer, while four women (3.6%) suffered from the former condition and one (0.9%) from the latter.

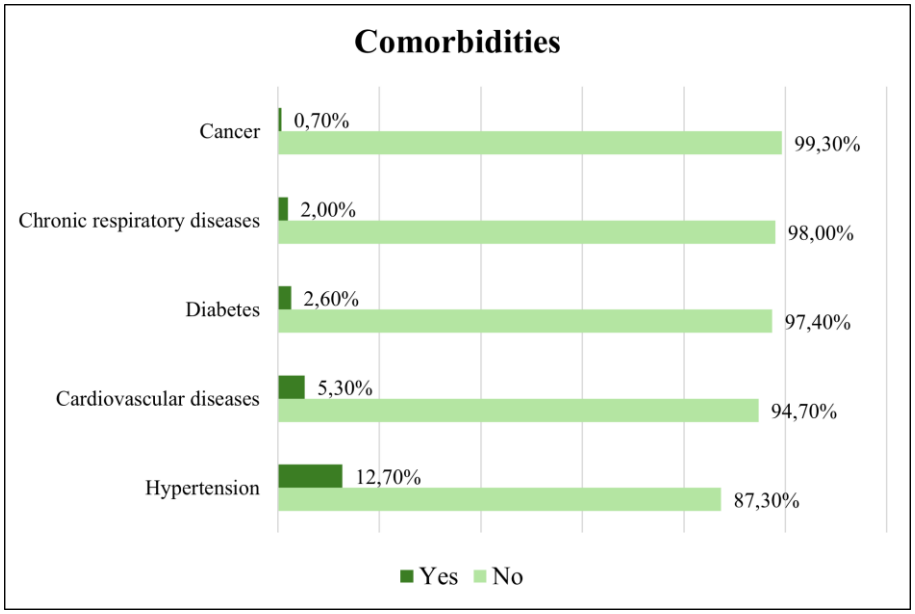

***Figure 4. Horizontal grouped bar chart of participants' comorbidities**. Active cancer, chronic respiratory diseases, and diabetes were rarely reported by the enrolled subjects, affecting 0.70%, 2.00%, and 2.60% of the entire cohort, respectively. Cardiovascular diseases and arterial hypertension were reported more frequently, affecting 5.30% and 12.70% of the entire cohort.*

Patients' heigh and weigh measurements were registered in order to assess the Body Mass Index (BMI) distribution across the cohort; however, it has to be noted that these information was missing for 76 individuals. Considering the available data, the mean BMI was 25.6±6.2kg/m^2; participants were subsequently subdivided into BMI classes, as reported in **Figure 5**, underlining how normal weight subjects represented the majority of enrolled individuals (54.40%), followed by overweigh patients (22.10%).

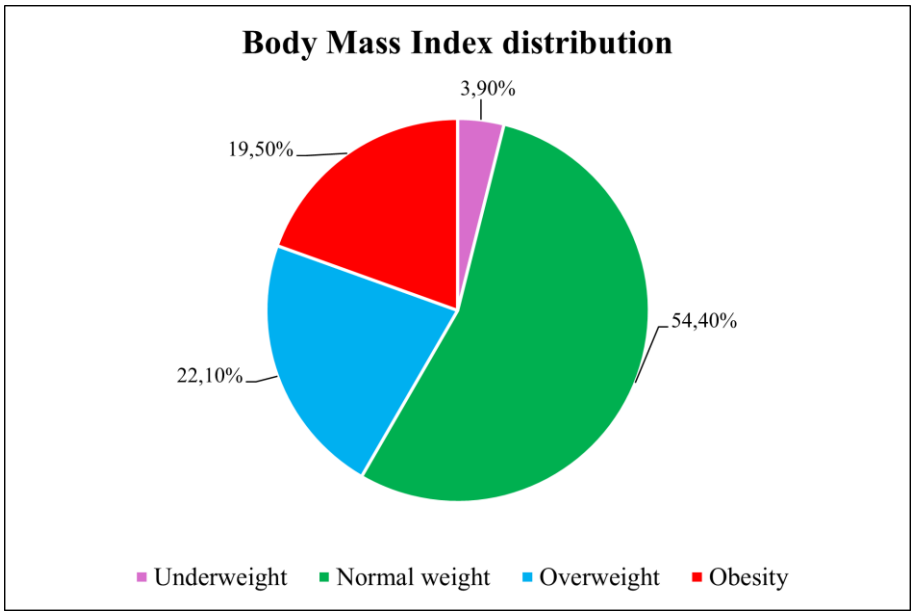

Figure 5. Pie chart of Body Mass Index distribution*. 3.90% of the patients presented a BMI ≤18.5 and were therefore classified as underweight. Normal weight patients represented the majority of all enrolled subjects and displayed a BMI comprised between 18.5 and 24.9. The presence of a BMI comprised between 25 and 30 allowed to classify 22.10% of participants as overweight, while 19.5% of the cohort showed a BMI higher than 30, thus presenting a clinical diagnosis of obesity.*

4.1.2 Self-administered questionnaires

Enrolled subjects were asked to fill in specific questionnaires aimed at evaluating their perception of the chemosensory dysfunction both with reference to the acute phase of SARS-CoV-2 infection and to the moment of the psychophysical evaluation. Additionally, the WHO-5 index was presented in order to assess participants' overall well-being.

SNOT-22

For the purposes of this study, only the item of the SNOT-22 questionnaire regarding the "Loss of taste and smell" was taken into account and patients were required to confer a score between 0, corresponding to "No problem", to 5, corresponding to "Problem as bad as it can be" (**Figure 6**). Concerning the acute phase of the infection, the median values reported by the recruited subjects was 5.0 (Q1, Q3: 4.0, 5.0), while at the moment of the psychophysical

evaluation, the median registered value was 4.0 (Q1, Q3: 3.0, 4.0). A statistically significant difference was detected between the scores attributed with reference to the two phases (*p-value* <0.001).

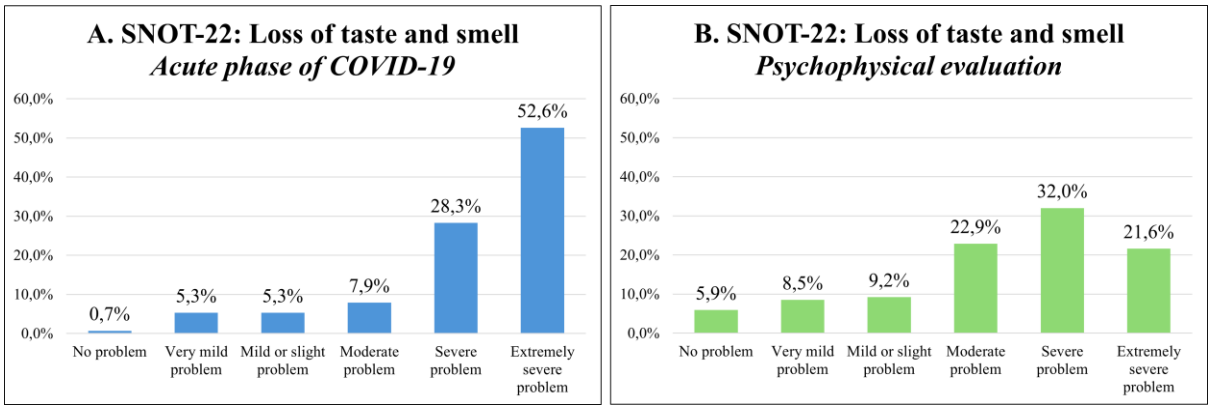

Figure 6. Bar plot of the scores attributed by participants to the "Loss of taste and smell" item of the SNOT-22. ***A****. Scores attributed by the participants recalling their symptoms during the acute phase of SARS-CoV-2 infection. Only one patient declared not to have suffered from smell and taste alterations (0.7% of the cohort): however, this subject reported an extremely severe alteration at the moment of the psychophysical evaluation. The majority of subjects reported a severe problem (n = 43; 28.3% of the cohort) or an extremely severe alteration (n = 80; 52.6% of the cohort).* ***B****. Scores attributed by the participants at the moment of the psychophysical evaluation. Despite patients reported a statistically significant improvement, the majority of participants still reported a moderate (n = 35; 22.9% of the cohort), severe (n = 49; 32.0% of the cohort), or extremely severe alteration (; n = 33; 21.6% of the cohort). Interestingly, nine subjects (5.9% of the cohort) declared not to suffer anymore from smell and taste alterations, however they all resulted hyposmic upon objective testing.*

ARTIQ

In this study, only the question of the ARTIQ focused on chemosensory dysfunction was analysed and patients were required to report the presence of smell, taste, or both senses alterations (**Figure 7**). Concerning the acute phase of SARS-CoV-2 infection, 83.0% of patients declared to have presented an alteration of both senses and 16.3% of participants recalled the presence of smell alteration alone; interestingly, only one patient (0.7% of the cohort) notified an isolated taste alteration. Similarly, also at the moment of the psychophysical evaluation, most patients still suffered from a dual chemosensory dysfunction (75.8%) or from

olfactory dysfunction alone (22.2%). A statistically significant difference was detected between the scores attributed with reference to the two phases (*p-value*: 0.006).

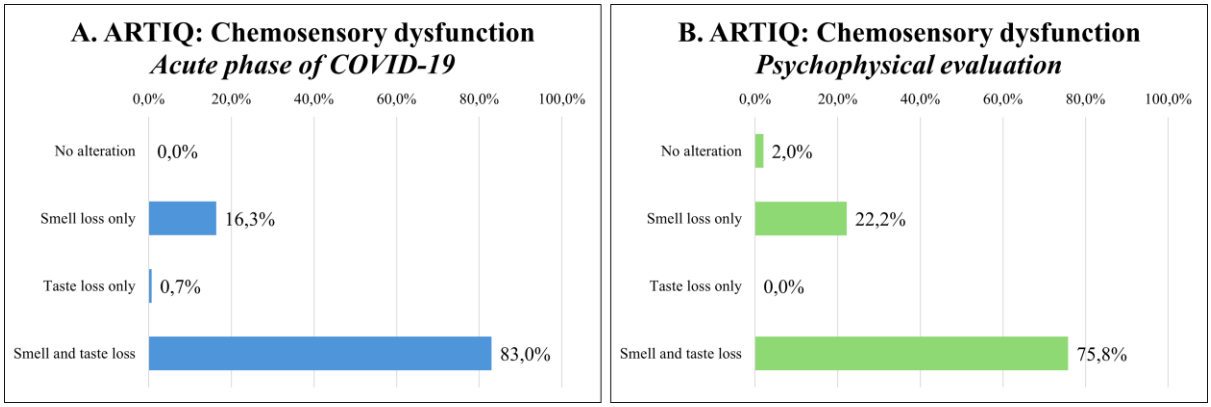

Figure 7. Horizontal bar plot of patients' answers to the "Chemosensory dysfunction" item of the ARTIQ. A*. Sensory alterations reported by participants with reference to the acute phase of SARS-CoV-2 infection. No patient recalled not to have suffered from taste or smell dysfunction (0.0% of the cohort) and only one patient reported to have experienced taste alteration alone (0.7% of the cohort)**;** conversely, 16.3% of the cohort (n = 25) suffered from smell alteration alone, while the majority of enrolled subjects (n = 127) reported a dual chemosensory alteration (83.0%). **B**. Sensory alterations reported by participants at the moment of the psychophysical evaluation. 2.0% of the participants (n = 3) disclosed a perception of complete recovery of chemosensory function; however, they all resulted hyposmic upon objective testing. None of the participants reported a persistent isolated taste alteration (0.0% of the cohort). 22.2% of the cohort (n = 34) still reported an isolated smell alteration, while the majority of the participants (n = 116) continued to suffer from dual chemosensory alteration (75.8%).*

VAS

For the purposes of this project, only the VAS aimed at the evaluation of participants' perception of nasal congestion and olfactory alteration were considered. Subjects were required both to recall their symptoms during the acute phase of COVID-19 and report on their symptoms at the moment of the psychophysical evaluation (**Table 2**, **Figure 8**).

	Nasal blockage VAS		Smell function VAS	
	Acute phase	**Psychophysical evaluation**	**Acute phase**	**Psychophysical evaluation**
Median (Q1, Q3)	65.0 (14.8, 94)	90.0 (62.3, 100)	0.8 (0, 12)	30.0 (13.0, 52)
Range	0, 100	1, 100	0, 100	0, 100
Unknown	5	3	7	5

Table 2. Nasal blockage and Smell function VAS. *Patients were required to evaluate both items with reference both to the acute phase of COVID-19 and to the moment of the psychophysical evaluation. Median (Q1, Q3) ad Range values are reported in the table. A statistically significant difference was identified between the acute phase and the moment of the psychophysical evaluation both for Nasal blockage (p-value <0.001) and for Smell function (p-value <0.001).*

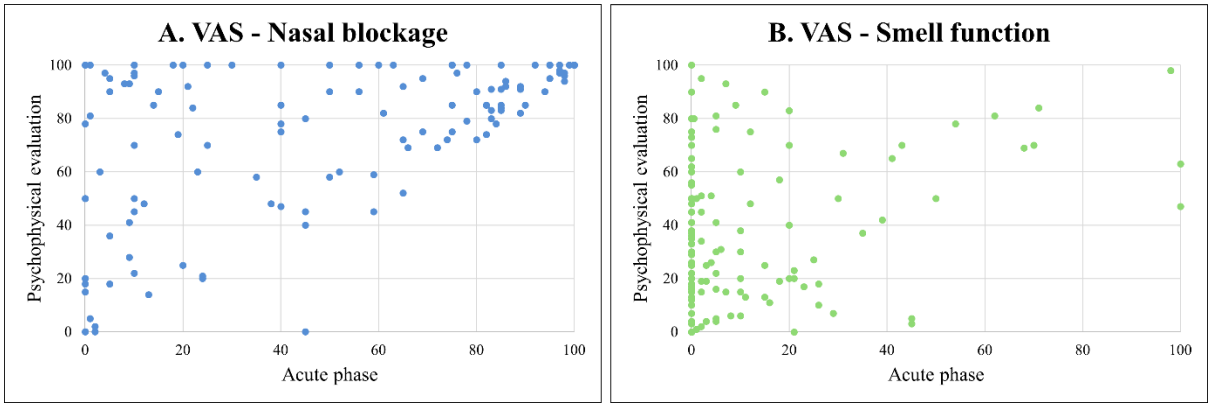

Figure 8. Scatter plots of VAS scores during the acute phase of COVID-19 and at the moment of the psychophysical evaluation. A. *Nasal Blockage VAS.* ***B****. Smell function VAS. A score of 0 corresponds to a 0 completely altered perception of the analysed item while a score of a 100 corresponds to a physiological perception of the considered item. A comparison of the distribution of the scores shows the presence of olfactory dysfunction during the acute phase of the infection despite the absence of significant nasal blockage.*

WHO-5

The WHO-5 aimed to broadly measure participants self-perception of psychological well-being with reference to the two weeks preceding the psychophysical evaluation, with the goal of assessing how persistent chemosensory dysfunction could affect their quality of life. Overall, participants' mean score was 50.9±28.2 and they could be divided into three separate groups, namely severely impaired well-being, poor well-being, and psychological well-being,

according to their scoring range (**Figure 9**). Approximately half of the cohort reported an overall psychological well-being, while, remarkably, slightly over than a quarter of the enrolled participants show severe psychological concern.

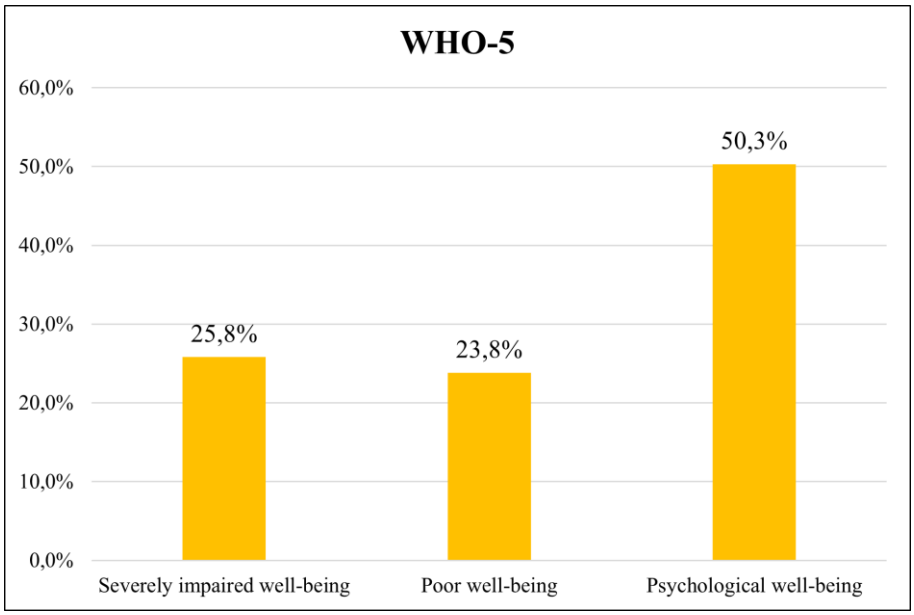

Figure 9. Bar plot of the scores of the WHO-5 index*. Group 1: 25.8% of participants (n = 39) attributed an overall score ≤28, which might be indicative of depression or severely impaired well-being. Group 2: 23.8% of patients (n = 36) achieved a total score between 28 and 50, indicative of a poor well-being. Group 3: 50.3% of participants (50.3) assigned a total score ≥50, which marks psychological well-being.*

4.1.3 Psychophysical evaluation

The psychophysical evaluation was aimed at objectively testing patients smell abilities and at performing an anatomical and functional evaluation of the olfactory cleft.

Orthonasal olfactory function evaluation

The assessment of the orthonasal olfactory function was performed using the validated extended Sniffin Stick's Test battery, which allowed to calculate both separate Threshold, Discrimination, and Identification scores and a combined TDI score. Results in the total cohort and stratified according to sex are reported in **Table 3**. Overall, 17% (26/153) of the total cohort

resulted anosmic, 70.6% (108/153) resulted hyposmic and, notably, 12.4% (19/153) obtained a final score within the normosmic range.

Orthonasal olfactory function	Total cohort N = 153	Females N = 111	Males N = 42
Threshold test			
Mean (SD)	4.5 (2.9)	4.8 (2.8)	3.9 (3.2)
Range	0.0, 11.3	0.0, 11.3	0.0, 10.5
Discrimination test			
Mean (SD)	9.4 (2.7)	9.5 (2.6)	9.1 (2.7)
Range	2.0, 14.0	2.0, 14.0	4.0, 14.0
Identification test			
Mean (SD)	9.4 (3.2)	9.5 (3.3)	9.0 (3.0)
Range	1.0, 16.0	1.0, 16.0	3.0, 15.0
Combined TDI score			
Mean (SD)	23.3 (7.0)	23.8 (6.9)	22.0 (7.1)
Range	4.0, 37.5	4.0, 35.5	7.0, 37.5

Table 3. Orthonasal olfactory function evaluation of patients presenting persistent smell loss. *The table summarises the results of the objective testing performed with the extended Sniffin Stick's Test battery, described as mean, standard deviation (SD), and score range.*

PNIF and Fibroscopy

An anatomical and functional evaluation of the olfactory cleft was performed to assess whether persistent olfactory alterations could be explained by established factors that play a very-well known role in PVOD, namely an altered nasal patency and the presence of olfactory mucosa oedema and secretions.

Nasal patency was measured with the PNIF and, overall, the mean registered value was 116.1±72.3mL/min (range: 27-350mL/min). For 64 participants, the mean of the three registered attempts to calculate the PNIF resulted <90mL/min. The anatomical evaluation of

the olfactory cleft was assessed through fibroscopy, which allows a direct visualisation of the olfactory mucosa. Data showed that, at the moment of the psychophysical evaluation, the majority of patients did not present any anatomical abnormalities (**Figure 10**).

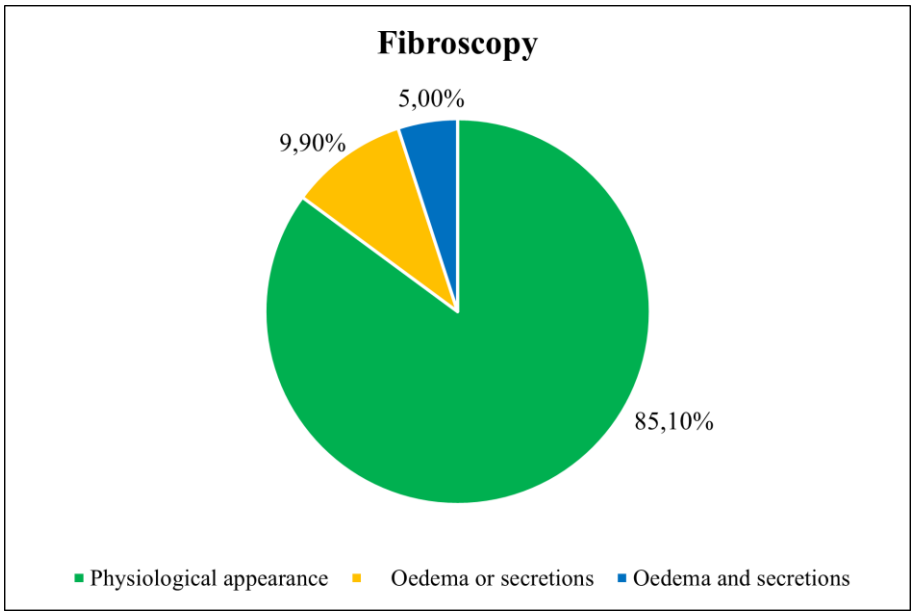

Figure 10. Pie chart of Fibroscopy results. *85% of participants (n = 120) did not present any oedema or secretions in the olfactory cleft; 9.90% of subjects (n = 14) showed oedema or secretions; 5.0% (n = 7) of patients displayed both oedema and secretions. The examination was not performed in 12 enrolled subjects.*

4.2 Biallelic variants identification

Starting from the list of 298 selected genes involved in antiviral response regulation, a total of 475 biallelic variants in 177 genes were extracted and subsequently classified according to variant frequency. **Figure 11** and **Table 4** display the consequence of the identified variants, both in the total 177 genes and divided by bins of MAF. Notably, in all MAF groups most variants were either missense or splicing, while frameshift, inframe indels, start loss, stop gained and stop loss were less frequently detected.

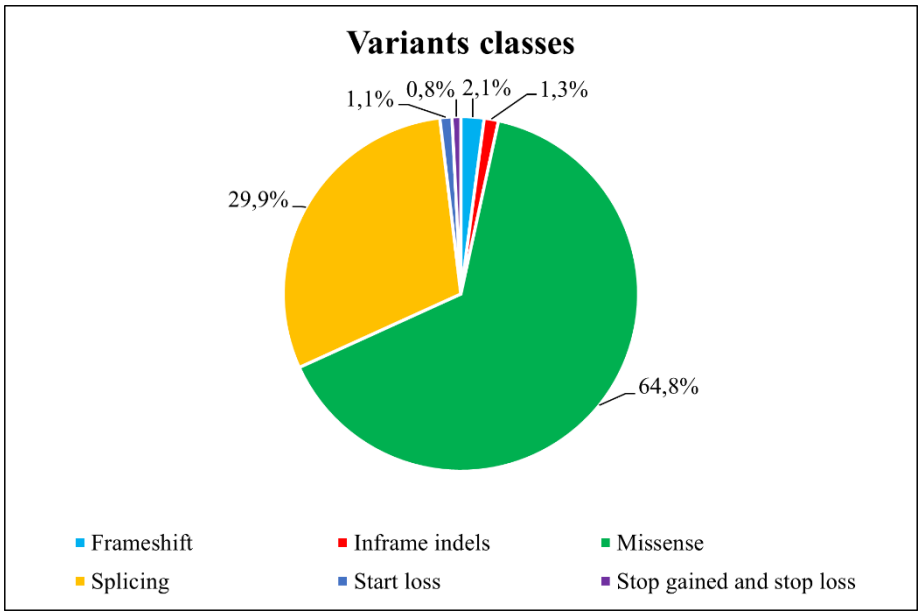

Figure 11. Pie chart of identified variants. *The most frequently identified variants are missense variations (64.8%, 308/475), followed by splicing variants (29.9%, 142/475); a minority of variants are represented by frameshift (2.1%, 10/475), inframe indels (1.3%, 6/475), start loss (1.1%, 5/475), and stop gained and stop loss variants (0.8%, 4/475).*

	MAF <1%		1%≤MAF<5%		5%≤MAF<10%		MAF≥10%	
	N	%	N	%	N	%	N	%
Missense	4	50	31	79.5	50	65.8	223	63.4
Splicing	4	50	7	17.9	22	28.9	109	31.0
Frameshift	0	0	1	2.6	0	0	9	2.6
Inframe indels	0	0	0	0	1	1.3	5	1.4
Start loss	0	0	0	0	2	2.6	3	0.9
Stop gained/stop loss	0	0	0	0	1	1.3	3	0.9

Table 4. Identified variants classified according to frequency*. Variants were divided into four different bins based on their MAF, namely MAF<1%, MAF between 1% and 5%, MAF between 5% and 10%, and MAF≥10%.*

4.3 Gene score calculation and association analyses with olfactory function

In order to evaluate the possible contribution of the identified variants in modulating COVID-19-related PVOD, a gene score was calculated for each participant and for each gene in every bin of MAF. Subsequently, the presence of an association between the gene score and both the self-perceived and objectively assessed olfactory performance was tested (**Figure 12**).

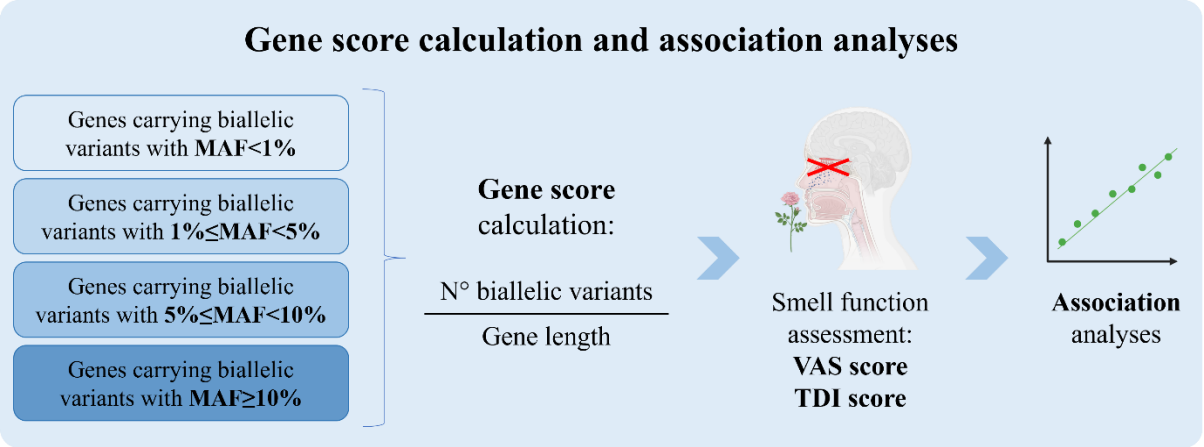

Figure 12. Genetic analyses workflow. *All the identified biallelic coding variants were grouped according to MAF into rare, low-frequency, common, and very common variants. Within each variant bin, for each gene and for each individual, a gene score was calculated. Linear regression models were employed to evaluate the association between the gene score and smell function, both in terms of self-perceived alteration (VAS score attributed by participants at the moment of the psychophysical evaluation) and of objectively-assessed olfactory performance (TDI score results).*

Specifically, the association between the gene score and the self-perceived smell alteration was evaluated considering the scores attributed by the participants to the Smell function VAS with reference to the moment of the psychophysical evaluation. In this case, no statistically significant association was highlighted in any of the tested MAF bins.

Conversely, the association between the gene score and the objectively assessed olfactory performance was evaluated considering the scores obtained by the enrolled subjects at the combined TDI test and at the Threshold, Discrimination, and Identification subtests

considered separately. In this case, statistically significant associations were identified in the MAF<1% and MAF≥10% bins and are discussed in detail in the following paragraphs.

4.3.1 Association analyses between rare variants and olfactory performance

The analysis of the impact of the gene score, calculated considering all biallelic variants with a MAF<1%, on participants olfactory performance highlighted a statistically significant association regarding the *ACE2* gene, encoding the Angiotensin-Converting Enzyme 2, in the female group. Specifically, an increased gene score within the *ACE2* gene resulted associated with a worsen olfactory performance, both at the combined TDI test (*p-value* = 0.03) and at the odour Discrimination subtest (*p-value* = 0.03) (**Table 5**). It has to be noted that, being *ACE2* an X-linked gene, the analysis has been performed for females and males separately, and not considering the entire cohort; no statistically significant associations have been identified in the male group.

Group	Olfactory function test	Gene	β Gene	Adjusted *p-value*
Female	Combined TDI score	*ACE2*	-21.70	0.03
Female	Odour Discrimination test	*ACE2*	-8.21	0.03

Table 5. Association between gene score and olfactory performance (MAF<1.0%). *The table reports the significant results of the association analyses. β Gene: effect size of the association. Adjusted p-value: p-value of the association adjusted using Benjamini-Hochberg method.*

Specifically, the association is driven by the presence of a biallelic missense variant (chrX:g.15600835T>C; rs4646116) detected in the homozygous state in a single female individual. The identified person is a 57-year-old woman with none of the comorbidities considered in this study and who declared to have never smoked or consumed alcohol. She reported a severe alteration of chemosensory function with taste and smell involvement both

with reference to the acute phase of COVID-19 and at the moment of the psychophysical evaluation ("Loss of taste and smell" item of the SNOT-22: score 4; "Chemosensory dysfunction" item of the ARTIQ: score 3). She totalled a score of 4 at the combined TDI test, therefore being the worst performer of the entire cohort. The variant has not been identified at the hemizygous state in any of the male subjects of the cohort.

4.3.2 Association analyses between very common variants and olfactory performance

The analysis of the impact of the gene score, calculated considering all biallelic variants with a MAF≥10%, on participants olfactory performance highlighted, in the male group, two statistically significant associations regarding the *IFI44* gene, coding the Interferon Induced Protein 44, and the *NDUFAF4* gene, coding the NADH:Ubiquinone Oxidoreductase Complex Assembly Factor 4. In particular, an increased gene score within the *IFI44* gene resulted associated with a better olfactory performance at the odour Discrimination subtest (*p-value* = 0.01); a similar effect is observed for homozygous alternative variants carriers in the *NDUFAF4* gene (*p-value* = 0.004) (**Table 6**). No statistically significant associations were detected for the total cohort and the female group.

Group	Olfactory function test	Gene	β Gene	Adjusted *p-value*
Male	Odour Discrimination test	*IFI44*	0.39	0.01
Male	Odour Discrimination test	*NDUFAF4*	0.26	0.004

Table 6. Association between gene score and olfactory performance (MAF≥10%). *The table reports the significant results of the association analyses. β Gene: effect size of the association. Adjusted p-value: p-value of the association adjusted using Benjamini-Hochberg method.*

Concerning the *IFI44* gene, the association is driven by the presence of a missense variant (chr1:g.78650220T>C; rs2070123) and of a frameshift variant (chr1:g.78662885G>GT; rs368139253).

The rs2070123 variant has been detected in the homozygous state in two individuals, a 28-year-old and a 42-year-old male. The youngest subject declared to be a former smoker and alcohol consumer, while the oldest participant reported to have never smoked or consumed alcohol; neither of them was affected by any of the comorbidities taken into account in this study. They both reported an extremely severe alteration of chemosensory dysfunction with reference to the acute phase of COVID-19 ("Loss of taste and smell" item of the SNOT-22: score 4 for both subjects) and a subsequent improvement of symptoms with time ("Loss of taste and smell" item of the SNOT-22: score 3 for one subject and 1 for the other). One of the patients declared an involvement of both smell and taste during the infection, whereas both of them still complained of olfactory dysfunction alone at the moment of the psychophysical evaluation. Interestingly, one of the these two participants totaled a score of 32.5 at the total TDI test, thus registering a value within the normosmic range; the other subject totaled a score of 27, therefore being in the upper range of the hyposmic range. Both patients achieved a high score at the Discrimination subtest, totaling 12 and 14 points, respectively.

The rs368139253 variant has been identified in the homozygous state in thirteen male individuals, whose mean age was 38.1±14.2 years. Only one of the subjects reported to currently smoke and one to be a former smokers; two of them reported previous alcohol consumption. Concerning comorbidities, one of the participants disclosed to be affected by arterial hypertension and cardiovascular disorders while another presented a history of chronic respiratory disease; all others were healthy. At the moment of the psychophysical evaluation, 8/13 of these patients reported a moderate to extremely severe chemosensory alteration ("Loss of taste and smell" item of the SNOT-22: scores 3, 4, 5); six of them declared an involvement

of olfactory function alone, whereas seven still disclosed and alteration of both smell and taste. Overall, their mean total TDI score was 24.3±7.3 while their mean odour Discrimination subtest score was 11.1±2.4. It has to be noted that two of the subjects carrying the biallelic rs368139253 variant also carry the rs2070123 variant.

Concerning the *NDUFAF4* gene, the association is driven by the presence of two splicing variants (chr6:g.96891399C>CA and chr6:g.96896083C>T (rs9386930)).

The chr6:g.96891399C>CA variant has been identified in the homozygous state in 23 male individuals, whose mean age was 42.4±13.9 years. Only one of the subjects reported to currently smoke and one to be a former smokers; four of them reported previous alcohol consumption, while one disclosed to be a current alcohol consumer. Concerning comorbidities, two of the participants disclosed to be affected by arterial hypertension and one by cardiovascular disorders while another presented a history of chronic respiratory disease; all others were healthy. At the moment of the psychophysical evaluation, 12/23 of these patients reported a moderate to extremely severe chemosensory alteration ("Loss of taste and smell" item of the SNOT-22: scores 3, 4, 5); 13 of them declared an involvement of olfactory function alone, whereas all the other still disclosed and alteration of both smell and taste. Overall, their mean total TDI score was 24.9±5.8 while their mean odour Discrimination subtest score was 10.7±2.1.

The chr6:g.96896083C>T variant has been detected in the homozygous state in a single 40-year-old men, whose personal anamnesis was not available. He reported a mild to moderate alteration of chemosensory function with smell involvement alone both with reference to the acute phase of COVID-19 and at the moment of the psychophysical evaluation. He totaled a score of 22.75 at the combined TDI test and obtained a score of 10 at the Discrimination subtest. It has to be noted that this participant also carries the biallelic chr6:g.96891399C>CA variant.

5. DISCUSSION

The COVID-19 pandemic has risen awareness towards an extremely fascinating, yet understudied condition, namely long-lasting olfactory dysfunction. Indeed, even if most patients that experience smell alteration during the acute phase of SARS-CoV-2 infection completely recover within a few weeks (110), a substantial fraction of individuals still present significant sensorial alterations months after the event.

Long-term COVID-19-related symptoms are collectively designated as "long COVID" or "post-acute sequelae of COVID-19" (PASC); they are defined as the continuation or development of new symptoms three months after the initial infection and these clinical manifestations need to last at least for two months (https://www.who.int/europe/news-room/fact-sheets/item/post-covid-19-condition). Considering the different conditions that can be classified as long-COVID (i.e. gastrointestinal, respiratory, and neurological symptoms, joint pain, fatigue, etc.), chemosensory alterations are among the most frequent and a recent systematic review has showed how they are reported in 11-13% of patients (111).

Despite great interest has developed towards studying and understanding the pathophysiological bases of COVID-19-related PVOD, full knowledge is still far from being reached and, in particular, researchers and clinicians still fail to precisely explain why not all

patients develop this condition. On the one hand, it has clearly been recognised that viral factors play a central role in this disorder; in particular, an association between the specific SARS-CoV-2 variant that caused the infection and smell outcome has been demonstrated. Indeed, the original untyped and Alpha variants caused both hyposmia and anosmia more frequently than the Delta or Omicron variants (40.1% and 47.6% *vs* 28.1% and 24.8%, respectively) (112). On the other hand, the involvement of several host risk factors have been considered, including a lower severity of the acute illness, increased age, and female gender (113). However, host genetic factors that might be associated with this condition have still been limitedly explored.

In this light, this study focused on a cohort of 153 subjects, deeply clinically and molecularly characterised, with the final goal of identifying novel host genetic factors associated with persistent post-COVID-19 olfactory dysfunction and possibly shedding some light on the overall pathophysiological mechanisms that regulate chemosensory alterations.

This study population mainly consisted in middle-aged female individuals, in line with previous studies that showed that increased age and female sex are associated with a lower likelihood of recovery from COVID-19-related smell loss (114). Concerning age, the mean age of the total study population was consistent with other works on post-COVID-19 smell loss, which showed that olfactory impairment is more frequent in middle-aged individuals and rarely occur in either extreme age (114,115).

Concerning sex, literature data show that women are more likely to develop PVOD than men (116) and a few hypotheses have been made to explain this event. For instance, it has been argued that women present a better olfactory performance at baseline, and this could lead to a greater sensitivity and a higher subjective feeling of impairment (117). Some authors have additionally endorsed the role of oestrogens: since long-lasting smell loss is more frequent in post-menopausal women, it has been hypothesised that they may have a protective role (118).

This has been further supported by a study where post-menopausal oestrogen replacement therapy was taken into account and that showed that women that were taking the therapy displayed a better olfactory performance (119). Furthermore, as the leading hypothesis underlying the etiopathogenesis of post-COVID-19 olfactory dysfunction concerns the involvement of the host immune system and the immune response to the viral insult, it has to be noted that immune-related X-linked genes present a different expression in immune cells of women in comparison to men (120). In this light, women might be more prone to develop a chronic inflammatory response that damages the olfactory mucosa.

As the role of smoking, alcohol consumption, and comorbidities have been evaluated in previous studies on persistent post-COVID-19 olfactory dysfunction and reported results are conflicting, these information have also been analysed in our cohort, in order to verify whether their prevalence in the study participants would differ from the general population. According to the most recent estimates of the Centers for Disease Control and Prevention (CDC), current smokers represent 11.5% of the population in the United States of America (https://www.cdc.gov/tobacco/data_statistics/fact_sheets/adult_data/cig_smoking/index.htm), while, as specified by the Istituto Superiore di Sanità (ISS) current smokers in Italy exceed 24% of the entire population (https://www.iss.it/en/-/no-tobacco-day-2022-iss-en). Conversely, among enrolled patients, only 6% of them reported a present smoking habit: in this light, despite smoking has been associated with a lower olfactory performance (121), it seems that our cohort is not enriched in this particular category of patients, thus refuting a contribution of smoking towards COVID-19-related PVOD. This further supports the findings of previous works that did not identify an association between smoking and COVID-19-related PVOD in patients with mild SARS-CoV-2 infection (122,123). Concerning alcohol consumption, according to Eurostat, the statistical office of the European Union, approximately 8% of the European population reports to daily have an alcoholic drink

(https://ec.europa.eu/eurostat/). On the other hand, only 2% of recruited subjects disclosed current alcohol consumption: also in this case, as it involves only a minor fraction of enrolled patients, this personal habit can hardly be considered one of the key risk factors for persistent smell loss, as already confirmed by previous studies (124). Furthermore, the presence of selected comorbidities has been studied in the enrolled participants and results show that the most frequent disorder is represented by arterial hypertension. This condition presents a European population prevalence of 22% (https://ec.europa.eu/eurostat/), while only 12.70% of the recruited subjects are affected by increased blood pressure. Despite substantial evidence depicts hypertension as a risk factor for severe acute COVID-19 (125), the evidence considering this disorder as a potential risk factor for long COVID and, particularly, for persistent smell loss is still insufficient. Furthermore, recent studies have suggested how arterial hypertension itself might arise as part of PASC (126): it is therefore still debatable if the two events could be related and, unfortunately, data on the onset of hypertension among recruited subjects was not available. Finally, patients' BMI was calculated, as a BMI in the obesity range has been proven to be an established risk factor for long-term sequelae after COVID-19 (127). Interestingly, despite there is no relationship between obesity and the development of chemosensory alterations (128), an association has been identified between persistent olfactory dysfunction and the increase of BMI in time (129). Indeed, smell alterations have a strong impact on nutrition and eating behaviour, as they might alter food preferences, reduce appetite, or decrease food enjoyment, which, in turn, might lead too malnutrition, not only in terms of reduced calories intake but also of overnutrition (130). It would therefore seem sensible to monitor the BMI of patients affected by long-lasting smell alteration in time, with the final goal of assessing whether chemosensory alterations would foster significant changes worthy of medical intervention.

Participants' personal history was further explored through validated questionnaires specifically aimed at evaluating the self-perception of chemosensory alterations. Recruited subjects were asked both to recall the severity of the symptoms during the acute phase of SARS-CoV-2 infection and to rate them at the moment of the psychophysical evaluation, with the final goal of identifying any possible differences. It has to be noted that both the "Loss of taste and smell" item of the SNOT-22 and the "Chemosensory dysfunction" item of the ARTIQ questionnaires take into account both olfactory and gustatory alterations and not a single sensorial deficit. Indeed, according to literature and consistently with our results, even if hyposmia and anosmia represent the most common findings, a co-occurrence of the two disorders is also recognised; conversely, gustatory dysfunction alone is seldom reported and presents a higher recovery rate (114). This might be due to a mischaracterisation of taste loss and retronasal olfactory dysfunction, which impairs flavour identification, and calls for an objective evaluation of all chemosensory performances in order to pinpoint the exact symptom of each patient (112). The presented results of the "Loss of taste and smell" item of the SNOT-22 and of the Smell function VAS underline how, despite registering a statistically significant difference in the severity of chemosensory alteration between the two assessed moments, more than 50% of participants still reported a severe or extremely severe chemosensory alteration. In this context, it has to be considered that, according to literature data, self-reported assessments of olfactory alterations generally register a worsen outcome in comparison to objective testing (131). Among different possible explanations, it can be argued that people with optimal olfactory performances might perceive as meaningful even slight changes in their ability, thus deeming the symptom extremely severe. In this light, a considerable limitation of the present study is represented by the absence of an objective evaluation of smell function at baseline, namely before SARS-CoV-2 infection, thus only relying on participants' confirmation of a physiological olfactory ability before the event.

Lastly, participants were administered the WHO-5 index, in order to assess their overall well-being, under the assumption that a marked change in olfactory function might have considerable psychological repercussions. Indeed, it has been shown that chemosensory dysfunction may have several social, psychological, and emotional consequences, including depression, anxiety, lack of self-esteem and self-confidence, social isolation, and alteration of eating behaviour (132). Approximately half of the participants reported concern about their overall well-being, while, according to literature data, up to 76% of patients disclose a deterioration of the overall aspects of quality of life after the onset of COVID-19-related smell loss and, with particular reference to the psychological well-being, almost 16% of patients declare that the main effect of the olfactory dysfunction has been the onset of depression (133). Interestingly, it has been demonstrated that episodes of depression involve 40-76% of patients suffering from olfactory dysfunction due a wide range of aetiologies (134), thus it is not a typical complication of COVID-19 alone. Nevertheless, these results point out the need for a careful and comprehensive clinical evaluation of patients affected by chemosensory dysfunction, as this condition has wider implications than those envisaged before the outburst of the pandemic.

Concerning the psychophysical evaluation, all recruited subjects underwent an accurate objective assessment of their orthonasal olfactory function through the validated extended Sniffin Stick's Test battery. The majority of patients achieved a combined TDI score that allows to classify them as hyposmic, while only a fraction of patients resulted completely anosmic. This result is in line with a published work on patients with self-reported olfactory dysfunction that were evaluated with the same test battery, which highlighted how, two years after the acute infection, 61.1% of them still have reduced smell abilities, while only approximately 5% continue to experience complete anosmia (135). In another study, similar

results were achieved, as baseline evaluation upon COVID-19 diagnosis highlighted how 61.8% of patients were hyposmic and 12.7% were anosmic while the 12 months follow-up showed how 51.0% pf subjects were still hyposmic and 4.1% anosmic (136). Overall, it is now widely confirmed how, despite the high prevalence of long-term chemosensory alterations, total anosmia is a rare occurrence (81). Interestingly, 12.4% of the subjects enrolled in our study resulted normosmic at the TDI evaluation. As already mentioned with reference to the self-administered questionnaires, it could be argued that patients with a smell performance towards the upper limit of the normal range might consider disproportionately severe even a slight decrease in their abilities and therefore still complain about a significant dysfunction. In this light, it is necessary to mention that a limitation of this study might be represented by the absence of an objective evaluation of participants' smell performances before the onset of COVID-19-related smell loss. Indeed, previous assessments would have been invaluable to better explore also this circumstance and provide more detailed insight. Additionally, previous works have shown how in about 8% of patients olfactory abilities can fluctuate, thus indicating that continuous monitoring of smell function might be indicated (137). Finally, a functional and an anatomical evaluation of the olfactory cleft have been performed, in order to exclude possible objective alterations that could sustain smell dysfunction. Indeed, findings of the present investigation show that only a minor fraction of patients present olfactory cleft oedema or serous secretions and that less than half of the participants have a decreased PNIF, thus refuting a significant level of obstruction. These results are in line with previous evidence that has discarded conductive deficit involvement in the etiopathogenesis of persistent smell loss (138,139).

Concerning the genetic analyses, the availability of WGS data allowed the simultaneous selection and separate analysis of all biallelic coding variants in 298 genes involved in antiviral

response regulation. An innovative approach was employed to verify possible associations between the identified variants and smell function, namely the calculation of a gene score. This strategy proved powerful as it provides a complete overview of the host genetic architecture, not being limited to a single analysis of rare or common variants. Additionally, for the first time to our knowledge, it assesses the impact of biallelic variant accumulation. The rationale of this approach resides in the fact that the analysis of variant accumulation has been increasingly applied to explore the genetic landscape of multifactorial diseases, leading to tremendous insight in their etiopathogenesis. Indeed, several studies have demonstrated how rare and low-frequency variants may have an impact on disease predisposition, onset, and progression (140–142), piling up on the solid knowledge concerning the contribution of common variants to multifactorial conditions pathophysiology, mainly derived from Genome-Wide Association Studies (143–145). Additionally, the most recent evidence from the scientific literature suggests how combining the contribution of both rare and common variants extracted from Next Generation Sequencing data could highlight their additive role to the phenotype of interest (146). In this context, we propose to take a further step forward in this type of analyses by considering biallelic variants, under the hypothesis that they might additionally contribute to gene function modulation.

Overall, we highlighted statistically significant associations between rare and very common variants and olfactory performance evaluated through the objective TDI test. Considering the gene score calculated analysing variants with a MAF<1%, an increased gene score related to the *ACE2* gene was associated with a worsen olfactory performance in females. The *ACE2* gene encodes the Angiotensin-Converting Enzyme 2, a dipeptidyl carboxydipeptidase that cleavages angiotensin I and angiotensin II into their active forms (147). It has extensively been linked to SARS-CoV-2 infection, as viral engagement of ACE2 is essential for its entry into the host cells (148). Interestingly, the specific variant identified in

this study cohort (rs464611) has been shown to be involved in a modulation of host-virus interaction, as it acts as a boost of ACE2 binding to the S viral protein (149,150). Alteration of *ACE2* expression has been associated with COVID-19 severity: in particular, virus-mediated down-regulation of ACE2 fosters a pro-inflammatory response which, in the acute phase of the infection, might lead to a more severe illness (151). Additionally, its sustained dysregulation in several tissues has already been implicated in long COVID pathogenesis (152,153). Conversely, previous studies have demonstrated how, physiologically, ACE2 has an immune regulatory function, as, by reducing angiotensin II, it decreases monocyte and endothelial cell activation (154), and, by producing Angiotensin-(1-7), it exerts powerful anti-inflammatory effects (155). Overall, the expression pattern of *ACE2* might be critical not only for COVID-19 susceptibility, but also for the overall patients outcome in terms of possibility to develop PASC. In this light, despite ACE2 dysregulation has not yet been directly associated with long-term smell loss, based on the above-mentioned data, it can be argued that individuals with a higher presence of rare biallelic variants in the *ACE2* gene may develop a protracted and more severe inflammatory response in the OE. The inflammatory microenvironment has already been shown to undermine the regeneration rate of the OE, which, in some patients, may not occur at all (88,113), thus leading to persistent olfactory alteration.

Furthermore, as far as it concerns the gene score calculated considering variants with a MAF≥10%, our study highlighted how an increased gene score in the *IFI44* and *NDUFAF4* genes was associated with a better olfactory performance. The *IFI44* gene encodes the Interferon Induced Protein 44, which is particularly expressed in the lymphoid tissue and in the bone marrow (https://www.proteinatlas.org/ENSG00000137965-IFI44). Despite this gene has been implicated in the regulation of the interferon signalling pathway, little is known about its precise physiological function. The gene has already been linked to the host response to viral infections, as it was shown that *IFI44* silencing inhibits the replication of multiple viruses,

whereas its overexpression inhibits the antiviral response due to negative regulation of the interferon pathway (156). Concerning COVID-19, a recent work has demonstrated its upregulation in patients with severe SARS-CoV-2 acute infection, together with several interferon, cytokine, and immune-related genes (157). Additionally, it has been reported that, through an involvement of dendritic cells, it is implicated in SARS-CoV-2 replication and immune escape in patients affected by rheumatoid arthritis (158). The *NDUFAF4* gene encodes the NADH:Ubiquinone Oxidoreductase Complex Assembly Factor 4, an ubiquitously expressed assembly factor of the mitochondrial respiratory chain (159). The role of this gene in the host viral response is still poorly elucidated; however, the mitochondrial respiratory chain has been shown to affect the replication of many viruses (160). Regarding the possible association with COVID-19, recent data show that NDUFAF4 is differentially regulated in the immune cells of patients affected by long COVID in comparison with healthy controls (161). Neither *IFI44* nor *NDUFAF4* have already been linked with persistent smell alteration and none of the biallelic variants that drive the associations in our cohort have been described in literature either in relationship with COVID-19 or olfactory abilities. However, considering literature data, it may be hypothesised that the accumulation of biallelic variants in both genes may modulate their function, thus promoting a decreased inflammatory response in the OE that thus correlates with a better outcome of the olfactory performance.

Overall, this study highlighted, for the first time, a statistically significant association between host genetic factors, expressed as an increased gene score, and smell abilities in patients affected by COVID-19-related PVOD, thus underlining how the host genetic architecture may play a pivotal role in explaining the marked interindividual variability that characterises this occurrence.

6. CONCLUSIONS AND FUTURE PERSPECTIVES

Olfaction is a key component of chemosensory perception and the capacity to discriminate odorants is the earliest sense that developed in the evolution of animal life (4). It is often believed that humans possess olfactory abilities that are less sophisticated than other mammals; however, recent evidence shows that human subjects are as capable as animals to successfully employ their sense of smell in a wide variety of behavioural contexts, as food selection, environment assessment and spatial orientation, social communication, reproduction, learning, and memory (162). In this context, it is clear that any disruption of the physiological mechanisms that regulate olfactory abilities may have tremendous impact, not only in terms of increased personal and social burden (i.e. loss of appetite, body weight changes, personal hygiene concerns, depression, and decrease in social and professional interactions), but also with potential harmful effects on the overall individual health, as, for instance, it causes the inability to recognise spoiled food or gas leaks, with a subsequent feeling of decreased safety (163–166).

Despite no effort has been spared by the international scientific community to better delineate the etiopathogenetic bases of smell function and astounding discoveries have been

made especially in the neuroanatomical field, a full knowledge about the exact molecular mechanisms that regulate olfaction is still far from being achieved. On the same wavelength, understanding the pathophysiological mechanisms underlying the different causes of olfactory dysfunction has proven equally demanding and this significant knowledge-gap has fiercely emerged in the last years, following the COVID-19 pandemic, that has been characterised by a massive increase in the number of patients presenting PVOD.

Many different factors have been implied in the aetiology of COVID-19-related PVOD, and the ability of SARS-CoV-2 to cause an extensive and prolonged inflammatory response in the OE promoted by the innate immune system seems to be one of the most endorsed hypotheses. As a matter of fact, a chronic activation of the immune system is associated with an alteration of the gene expression pattern of both ONs and sustentacular cells, with an overall disruption of the odorous stimulus signal transduction cascade (86). Furthermore, chronic inflammation may cause massive apoptosis of all cell types within the OE, thus significantly delaying the recovery of smell abilities, which, in some patients may not happen at all, also due to respiratory metaplasia of the OE (88). Nevertheless, none of these mechanisms are able to explain the marked interindividual differences in the development of chemosensory dysfunction: indeed, not every COVID-19 patient develops persistent smell loss. In this context, this study has permitted the identification of new host genetic factors that may play a pivotal role in determining this heterogeneity, showing, once again, how complex the interaction between molecular and environmental factors is, even in conditions that are far from presenting strong genetic bases. These findings, by highlighting novel pathophysiological insights, may help to better stratify affected patients, with the final goal of providing them with the best standards of care. In this light, *in vitro* and *in vivo* functional studies would be essential to further confirm the role of the identified genes and better elucidate their biological

mechanisms of action. Additionally, in order to gain considerable insight on this genotype-phenotype correlation, it would be of paramount importance to perform a clinical follow-up for all enrolled participants, to verify whether there have been further changes in their olfactory abilities and to assess whether subjects with an increased gene score in the identified genes show a different evolution of symptoms in time in comparison with the other patients.

In conclusion, the results of this study contributed to deepen our knowledge on the genetic architecture of long-lasting smell loss, opening new possibilities to better clarify the molecular mechanisms underlying the complex physiology of the olfactory system, and potentially allowing for a more personalised and appropriate clinical management of affected subjects.

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APPENDIX

ABCC9	*CCR7*	*HYAL3*	*IL6*	*OAS3*	*SPRY4*
ACE2	*CD207*	*IFI16*	*IRF1*	*OASL*	*STAT1*
ACOD1	*CD40*	*IFI27*	*IRF2*	*OPRK1*	*STAT2*
ADAR	*CD86*	*IFI44*	*IRF3*	*OSM*	*STING1*
AGBL4	*CD8A*	*IFI44L*	*IRF5*	*OTUB1*	*STK4*
AGBL5	*CGAS*	*IFI6*	*IRF7*	*OTUB2*	*TAGAP*
AIM2	*CHUK*	*IFIH1*	*IRF9*	*PARP9*	*TBK1*
APOBEC1	*CNOT7*	*IFIT1*	*ISG15*	*PCBP2*	*TICAM1*
APOBEC3A	*CREBZF*	*IFIT1B*	*ISG20*	*PCIF1*	*TICAM2*
APOBEC3B	*CXCL10*	*IFIT2*	*ITCH*	*PDE12*	*TLR2*
APOBEC3D	*CXCL12*	*IFIT3*	*ITGAX*	*PFDN6*	*TLR3*
APOBEC3F	*CXCR4*	*IFIT5*	*ITGB6*	*PLA2G10*	*TLR7*
APOBEC3G	*DDIT4*	*IFITM1*	*ITGB8*	*PLAAT4*	*TLR8*
APOBEC3H	*DDX1*	*IFITM2*	*IVNS1ABP*	*PLP1*	*TLR9*
ARMC5	*DDX21*	*IFITM3*	*JAK1*	*PLSCR1*	*TNF*
ATG16L1	*DDX3X*	*IFNA1*	*KCNJ8*	*PMAIP1*	*TNFRSF9*
ATG7	*DDX41*	*IFNA14*	*LAMTOR5*	*PPIA*	*TNFSF4*
AUP1	*DDX56*	*IFNA17*	*LCN2*	*PQBP1*	*TOMM70*

AZU1	*DDX60*	*IFNA2*	*LILRB1*	*PRF1*	*TRAF3IP1*
BANF1	*DHX15*	*IFNA4*	*LSM14A*	*PRKRA*	*TRAF3IP2*
BATF3	*DHX36*	*IFNA5*	*LYST*	*PTPN22*	*TREX1*
BCL2	*DHX58*	*IFNA7*	*MAP3K14*	*PTPRC*	*TRIM22*
BCL2L1	*DHX9*	*IFNA8*	*MAPKAPK2*	*PYCARD*	*TRIM31*
BCL3	*DICER1*	*IFNAR1*	*MAVS*	*RAB2B*	*TRIM41*
BIRC2	*DTX3L*	*IFNAR2*	*MBL2*	*RICTOR*	*TRIM44*
BIRC3	*DUOX2*	*IFNB1*	*MFN1*	*RIGI*	*TRIM5*
BNIP3	*EGFR*	*IFNE*	*MICA*	*RIPK3*	*TRIM52*
BNIP3L	*EIF2AK2*	*IFNG*	*MLKL*	*RNASE2*	*TRIM56*
BST2	*EIF2AK4*	*IFNGR1*	*MLST8*	*RNASEL*	*TRIM6*
C1R	*EXOC2*	*IFNGR2*	*MMP12*	*RNF185*	*TTC4*
C2	*EXOSC4*	*IFNK*	*MOV10*	*RNF216*	*TYK2*
C2CD4B	*EXOSC5*	*IFNL1*	*MPO*	*RPS15A*	*UNC13D*
C3	*EXT1*	*IFNL3*	*MSR1*	*RSAD2*	*URI1*
C4A	*F2RL1*	*IFNL4*	*MST1R*	*RTP4*	*USP17L2*
C4B	*FADD*	*IFNLR1*	*MX1*	*SAMHD1*	*USP21*
C4BPB	*FCGR1A*	*IFNW1*	*MX2*	*SELENOK*	*USP25*
C5	*FCN3*	*IKBKB*	*MYD88*	*SERINC3*	*USP27X*
C6	*FGR*	*IKBKG*	*NCBP1*	*SERINC5*	*VAV1*
C7	*FN1*	*IL12B*	*NCBP3*	*SETD2*	*XCL1*
C8A	*FOSL1*	*IL12RB1*	*NCR1*	*SHFL*	*XPR1*
C8G	*G3BP1*	*IL15*	*NDUFAF4*	*SIN3A*	*ZBP1*
CARD8	*GARIN5A*	*IL17C*	*NLRP1*	*SKP2*	*ZC3H12A*
CARD9	*GBP2*	*IL17RA*	*NLRP3*	*SLFN11*	*ZC3HAV1*
CC2D1A	*GBP5*	*IL1B*	*NLRP6*	*SMPD1*	*ZCCHC3*
CCL11	*GBP7*	*IL21*	*NLRP9*	*SOCS1*	*ZDHHC1*
CCL19	*GPAM*	*IL23A*	*NLRX1*	*SOCS3*	*ZDHHC11*
CCL22	*HSP90AA1*	*IL23R*	*NMI*	*SPN*	*ZMYND11*

CCL4	*HSPA8*	*IL27*	*NT5C3A*	*SPON2*	*ZNFX1*
CCL5	*HYAL1*	*IL33*	*OAS1*	*SPRY1*	
CCL8	*HYAL2*	*IL4*	*OAS2*	*SPRY2*	

Supplementary Table 1. List of 298 genes involved in antiviral response regulation. *The Ingenuity Pathway Analysis software was employed to extract a list of 298 genes involved in the antiviral response regulation. The list was further manually curated in order to select only the genes described in the Online Mendelian Inheritance in Man® (OMIM®) free-access catalogue of human genes and genetic disorders. All downstream analyses have been performed on the retained genes.*