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Anosmia and ageusia in COVID-19 patients: the cytokines profile in upper airways

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ABSTRACT

OBJECTIVE

COVID-19 is a systemic disease affecting multiple organs, beyond the respiratory system. Although sensorineural symptoms, including anosmia and ageusia, have been frequently associated with milder forms of SARS-CoV-2 infection, the pathogenic mechanism and its relation with oromucosal immunomodulation remains poorly understood. Therefore, the main objective of this pilot study was to provide some pieces of evidence concerning the cytokines pattern in the mucosa of the upper airways in COVID-19 patients with and without anosmia and/or ageusia.

METHODS

Samples of nasopharyngeal/oropharyngeal swabs were obtained from 12 patients diagnosed with COVID-19. The patients were separated into two groups: with anosmia and/or ageusia (A/A,n=4), and a control group with patients without anosmia and/or ageusia (NA/A,n=8).

RESULTS

Mucosal cytokines concentration was determined by ELISA. Levels of IFN-γ, IL-6, and IL-10 in the mucosa of the upper airways were similar between the groups. In addition, no significant differences were found in the ratio of IFN-γ/IL-6, IFN-γ/IL-10, and IL-10/IL-6 between the two groups. However, using Pearson's correlation coefficient we found a significant positive correlations between IL-6 and IFN-γ, IL-10 and IFN-γ, and also IL-10 and IL-6 in the control group (NA/A), whereas a significant negative correlation between IL-10 and IFN-γ was found in A/A group.

CONCLUSIONS

Our results suggest that patients with mild forms of COVID-19, presenting anosmia and/or ageusia, have an altered mucosal immune response in the upper airways.

DESCRIPTORS

SARS-Cov-2. Sensorineural symptoms. IL-6. IL-10. IFN-gama.

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INTRODUCTION

In December, 2019 the Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2), a novel coronavirus, emerged in China, associated with reported clusters of patients with pneumonia in the city of Wuhan, Hubei Province1. The coronavirus disease 2019 (COVID-19) has spread globally, becoming the first-ever global pandemic caused by a coronavirus. As of September 07, 2020, COVID-19 has affected almost all countries and regions, with 27,500,000 confirmed cases and 895,000 deaths, representing one of the most serious and challenging public health crisis faced by this generation[1, 2]

It is widely accepted that COVID-19 is a systemic disease affecting multiple organs and systems, beyond the respiratory system. In this context, olfactory as well as gustatory dysfunction, including anosmia and dysgeusia, were found to be common symptoms, either isolated or with other manifestations, strongly associated with COVID-19. Analysis found a significantly higher rate of olfactory dysfunction using quantitative evaluations comparing to self-reported smell loss[3]. Interestingly, preliminary studies found that COVID-19-related anosmia was associated with milder forms of the disease [4-6].

Based on the study of Lechien et al. [7] , among 417 patients with COVID-19 presenting non-severe manifestations of this disease, 86% had a loss or evident decrease in smell and 88% had taste disorders. In addition, the same authors also reported that these two disorders occurred, in a general way, concomitantly with the most common clinical symptoms observed in the patients with COVID-19 (loss of appetite, cough, fever, and muscle pain), as well as with otorhinolaryngological symptoms (stuffy nose and facial pain) also characteristic of the SARS-CoV-2 infection (5). It is also noteworthy to point out that it has been demonstrated that some patients presented the occurrence of anosmia and ageusia before (12% of cases) or after (23% of cases) the onset of other symptoms and that almost half of the patients recovered their sense of smell within two weeks of other symptoms disappearing [7, 8].

Although the pathogenesis behind olfactory dysfunction remains unclear, it is plausible that SARS-CoV-2 presents a similar behavior to other respiratory viruses, triggering an innate immune response in upper airways, which includes production and releasing of secretory immunoglobulin A and also cytokines. In this regard, different cytokine patterns, which are related to distinct immune responses, such as Th1, Th2, and Th17, can be elicited by respiratory infections, in order to generate efficient mucosal immunity against these pathogens. Therefore, the possibility to determine a cytokine pattern predictive of severe outcomes, as well as identify a specific definition of the type of immune response elicited by the different respiratory virus infections could have significant implications in the clinical management of the patients.. Therefore, the possibility to determine a cytokine pattern predictive of severe outcomes, as well as identify a specific definition of the type of immune response elicited by the different respiratory virus infections could have significant implications in the clinical management of the patients.

In this context, a better understanding of the modulation of cytokines, both pro- and anti-inflammatory, present in the mucosa of the upper airways from COVID-19 patients appears to be also of paramount importance. To the best of our knowledge, data showing the cytokines pattern in the mucosa of the upper airways of patients with COVID-19 presenting olfactory dysfunction, including anosmia and/or ageusia, in the literature is very scarce. Therefore, the main objective of this pilot study was to provide some pieces of evidence on the cytokines pattern in the mucosa of the upper airways between COVID-19 patients presenting or not anosmia and/or ageusia.

METHODS

Subjects of the study

The present study was performed using samples for the RT-PCR SARS-CoV-2 test collected via nasopharyngeal or oropharyngeal swab at one of three hospitals in the city of São Paulo: 1. University Hospital (HU-USP); 2. Santa Casa de São Paulo Hospital (HSCM); 3. Hospital Municipal Infantil Menino Jesus (HMIMJ). The nasopharyngeal/oropharyngeal swabs samples were obtained within 5 days after onset of symptoms onset and it was maintained in a buffer conservative during the transport of the sample until the laboratory. All patients were classified as mild cases and based on the presence of anosmia and/or ageusia they were divided in two groups: the group of patients presenting anosmia and/or ageusia (A/A, n=4), and the control group with patients without anosmia and/or ageusia (NA/A, n=8). The definition of anosmia and/or ageusia occurrence was based on the clinical evaluation in the patients reporting the loss of smell and/or taste. Demographic and clinical characteristics are presented in Table 1.

All the subjects enrolled in this study signed the informed consent form previously approved by the Ethics Committee of the University of São Paulo and by the National Research Ethics Committee (number CAAE: 36011220.0.0000.0081). It is noteworthy to highlight that both the study and all experiments were performed in accordance with the Declaration of Helsinki.

Table 1. Demographic and clinical characteristics presented by the COVID-19 patients with anosmia and/or ageusia (A/A) and the control group (NA/A).evel of significance was established at 5% (*p < 0 05).

Characteristics	Patients (n=12)		
	NA/AG (n=8)	A/AG (n=4)	p value
Age (year)	38.5 (14.2)	39 (14.9)	ns
Women (n)	6	3	ns
Men (n)	2	1	ns
W/M ratio	3:1	3:1	ns
Clinical symptoms (n)#			
Anosmia	0	4	<0.05
Ageusia	0	1	ns
Fever	2	1	ns
Cough	2	2	ns
Coryza	5	2	ns
Sore throat	1	0	ns
Dyspnea	1	1	ns
Fatigue	1	0	ns
Body pain	1	2	ns
Myalgia	2	1	ns
Headache	1	1	ns
Chill	1	1	ns

#number of individuals. Ns: non-significant. A/AG: group of COVID-19 patients with anosmia and/or ageusia. NA/AG: Control group of COVID-19 patients (without anosmia and/or ageusia)

RNA extraction and determination of virus infection by qRT-PCR

The total acid nucleic were extracted on the NUCLISENS® easyMag platform (bioMérieux, Massachusetts, USA) and the real-time PCR for 15 seasonal virus and Sars.CoV-2 was on ABI 7500 real-time PCR machine, utilizing the AgPath-ID One-Step RT-PCR master mix kit (both Applied Biosystems Inc., USA). All extracts were tested for human RNase P gene by RT-PCR to confirm sample quality. The protocols used for SARS-CoV-2[14], Respiratory Syncytial Virus (RSV-A and -B), Human Metapneumovirus (hMPV), Influenza virus (Flu-A and -B), Parainfluenza virus (hPIV-1, -2, -3 and -4), Rhinovirus (RV), Enterovirus (EV), pan-Adenovirus (AdV) and Coronavirus sazonal in Brazil (CoV-229E, -OC43, -NL63, -HKU1) was previously published [15-17]. We selected positive samples for SARS.CoV-2 without coinfections with another virus tested.

Determination of cytokines

Cytokine concentrations were determined in the oro/nasopharyngeal swab samples by ELISA test. Biomarkers were: interferon (IFN)-γ (PeproTech, NJ, USA), interleukin (IL)-6, and IL-10 (Invitrogen by Thermo Fisher Scientific, Vienna, Austria), following the manufacturer's instructions. The concentration of these cytokines was calculated using appropriate standard curves, which showed the correlation coefficients range of 0.95 to 0.99, besides the intra-assay coefficients of variance between 3–5%, and interassay coefficients of variance between 8–10%. %. In addition, the lower limit of detection for each cytokine was calculate by comparing to the reference value provided into the kit's manufacturer over 2.5 standard deviations from these values. After carried out each cytokine determination, its concentration values were normalized by the total protein concentration determined by the Bradford method 18.

Statistical analysis

Data obtained in this study were initially tested against to Normality hypothesis by the Shapiro-Wilk test procedure. The age variable is presented by mean and standard deviation values. Differences between the cytokine concentrations found in the patient groups were analyzed by the Student t-test, and Pearson product-moment correlation coefficient was applied to test measures of the statistical relationship between them. The absolute number and ratio of gender, as well as clinical symptoms, were analyzed by Fisher Exact test, with Bonferroni post-hoc correction. The adopted statistical significance was 5.0% level (p≤0.05), with statistical analyses performed by GraphPad Prism 8.1.2 software.

RESULTS

According to the data presented in Table 1, the mean age as well as the ratio of women and men in each group were comparable.

Similar levels of IFN-γ (Figure 1A), IL-6 (Figure 1B), and IL-10 (Figure 1C) were found in both groups.

As shown in Figure 2, no significant differences were found in the ratio of the three cytokines evaluated in this study (Figure 2A - IFN-g/IL-6, Figure 2B - IFN-g/IL-10, and Figure 2C - IL-10/IL-6) in both groups.

In order to improve the understanding of how these cytokines can impact the occurrence of anosmia and ageusia in COVID-19 patients, we carried out the correlation analysis by Pearson´s correlation coefficient method. As shown in Figure 3, whereas the control group of patients without anosmia and/or ageusia (NA/A group) showed significant positive correlations between IL-6 and IFN-γ (Figure 3A), Il-10 and IFN- γ (Figure 3C), and also between IL-10 and IL-6 (Figure 3E), the group of patients presenting anosmia and/or ageusia (A/A group) showed a significant negative correlation between the levels of IL-10 and IFN-γ (Figure 3B). No significant correlations were found between the levels of IL-10 and IFN-γ (Figure 3D) and between IL-10 and IL-6 (Figure 3F) in A/A group.

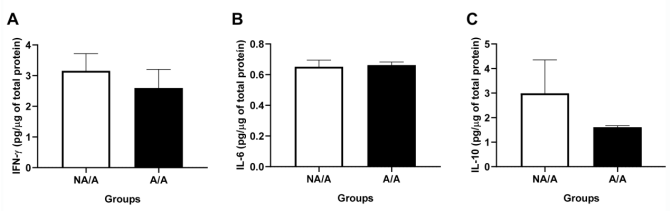

Figure 1. Comparison of IFN-γ (A), interleukin (IL)-6 (B), and IL-10 (C) levels between COVID-19 patients with anosmia and/or ageusia (A/A) and the control group (NA/A). Values are presented in mean and standard deviation. Level of significance was established at 5% ($p < 0.05$).

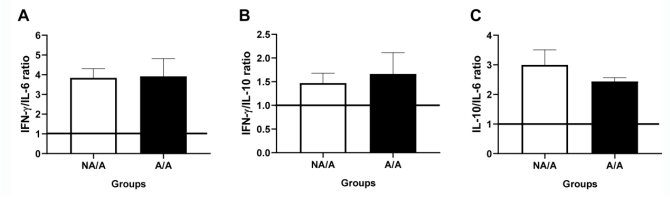

Figure 2. Comparison of the ratio of IFN-γ/IL-6 (A), IFN-γ/IL-10 (B), and IL-10/IL-6 (C) between COVID-19 patients with anosmia and/or ageusia (A/A) and the control group (NA/A).. Values are presented in mean and standard deviation (SD). Level of significance was established at 5% ($p < 0.05$).

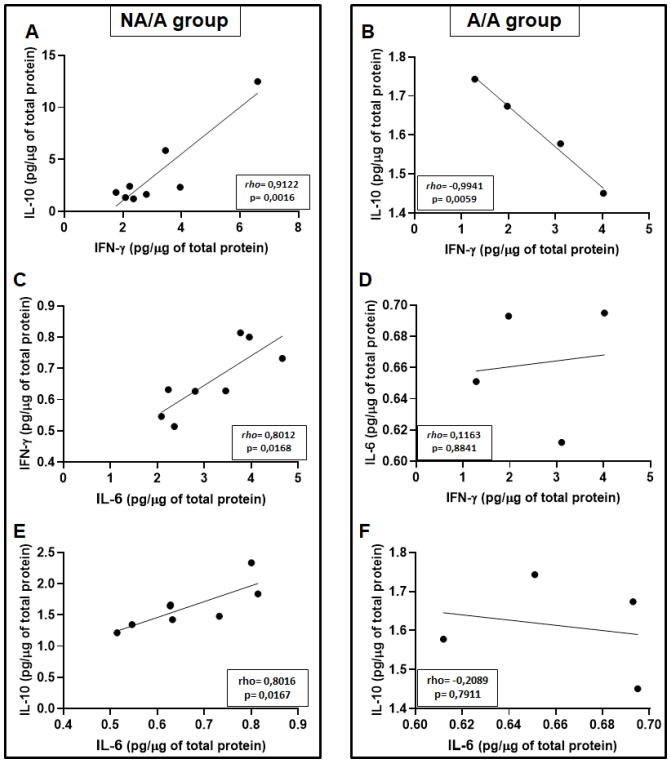

Figure 3. Pearson´s correlation coefficient (rho) between the levels of IFN-γ and IL-10 (A and B), IFN-γ and IL-6 (C and D), and also between IL-10 and IL-6 (E and F) in the group of COVID-19 patients with anosmia and/or ageusia (A/A) and the control group (NA/A). Level of significance was established at 5% ($p < 0.05$).

DISCUSSION

In this study, we were able to demonstrate that the patients with COVID-19 presenting anosmia and ageusia showed signif-

icant alteration in the upper airways mucosal cytokine pattern, particularly in terms of the correlation between IFN-γ and IL-10. In this regard, these patients presented a significant negative correlation, in opposition to the positive correlation found in the patients with COVID-19 non-presenting anosmia and ageusia.

Based on clinical reports, a high proportion of patients with SARS-CoV-2 infection have presented olfactory and gustatory dysfunctions, in a similar way to what was previously reported among patients with other coronavirus infections [5, 19, 20]. It has been also highlighted that the occurrence of anosmia and ageusia in COVID-19 patients is not related to nasal or rhinitis symptoms [5]. These findings suggest that the SARS-CoV-2 can induce direct damage to the olfactory and gustatory sensory neurons [5, 20]. Furthermore, the observation that the recovery from COVID-related anosmia, in a general way, occurs over weeks can suggest a specific pattern of SARS-CoV-2 infection, since the recovery from typical post-viral anosmia occurs over months [20]. Another point that renders to be highlighted is that the frequency of olfactory loss among COVD-19 patients is higher than in influenza patients [21, 22].

Although the mechanism elicited by SARS-CoV-2 to induce disturbances in the olfactory and gustatory sensory neurons is not fully understood, a recent study developed by Brann and colleagues [20] showed results that can "shed light" on the development of further studies. They were able to demonstrate that different non-neuronal cell types, such as supporting cells, stem cells, and perivascular cells, both in the olfactory epithelium and olfactory bulb, express the well-known receptor used by SARS-CoV-2 to entry in the cells, the ACE2 receptor. Thus, these authors suggest that the SARS-CoV-2 can putatively infect these non-neuronal cell types in the olfactory area and this fact could be closely related to the induction of anosmia in COVID-19 patients [20].

In association with this scenario, it is accepted that several viral infections (including coronavirus) are able to elicit a local inflammatory response, which may be involved in the transient alteration in olfactory perception [20]. Particularly in terms of SARS-CoV infection, Netland et al [23] demonstrated that mice infected by this type of virus presented prominent neuronal death. Interestingly, these authors did not observe any neuronal inflammatory infiltration and they postulated that the neuronal death by SARS-CoV infection was due to a local storm of cytokines, especially by IL-6 [23].

Although the occurrence of a significant systemic "cytokine storm" in COVID-19 patients with severe symptoms has been reported, it is very important to point out that, based on the literature, the systemic cytokine evaluation does not necessarily reflect cytokine concentration observed in the central nervous system (CNS)[24-26] or in the mucosa of upper airways[27, 28]

Despite the role of inflammation-inducing anosmia and ageusia in COVID-19 patients is still under debate, the data presented above can support the hypothesis that the primary infection of neuronal and/or different non-neuronal cell types by a coronavirus (SARS-CoV and SARS-CoV-2) may elicit a local inflammatory response and, both the cytokine levels and its pattern, can putatively affect the function of olfactory and gustatory sensory neurons [20, 21, 23]

The best proof for such a proposition is that respiratory infections can elicit different immune responses with the aim to generate a protective immunity [13].

Concerning the role of cytokines in upper airways mucosa, our group reported that the local higher expression of IL-6, a pro-inflammatory cytokine, was associated with the occurrence of different symptoms in the upper airways [28]. Furthermore, we also reported that the presence of IL-10, a classical anti-inflammatory cytokine, in the mucosa of the upper airways was able to modulate the IL-6 releasing and, in this way, inhibited the manifestation of upper airway symptoms [28, 29]. Even though the levels of IL-6 and IL-10 were not different between the groups with and without anosmia and/or ageusia, the results of IL-10/IL-6 ratio showed that all the patients in both groups were able to release more IL-10 than IL-6, which could help to modulate the mucosal inflammatory response, in order to avoid an unbalanced pro-inflammatory response between these cytokines in patients with mild COVID-19. Corroborating this observation that a controlled inflammatory response was present in the mucosa of the upper airways, the patients in the group without anosmia and/or ageusia showed a positive correlation between the IL-10 and IL-6 levels. However, in the group with these olfactory dysfunctions a correlation was not observed, and this dysregulated local immune response may have impacted on the development of anosmia and ageusia.

Beyond these cytokines, we also evaluated the type II interferon (IFN-γ) levels in the mucosa of the upper airways since it was reported that the levels of this cytokine were increased after infections caused by the other zoonotic coronavirus, MERS-CoV and SARS-CoV[30]. It is well-known that interferon is a classical cytokine associated with the Th1 immune response profile and also that it is closely involved in the immune response against virus infections due to its strong capacity to interfere with virus replication, including coronaviruses [30-32].

Regarding the IFN-γ levels, in a similar way to the observed for IL-6 and IL-10, no differences were found in its levels as well as in the ratio between the levels of IFN-γ and IL-6 or IL-10. However, it is noteworthy to point out that the data related to the ratio between the cytokines showed that the IFN-γ levels were higher than the other two cytokines indicating that the SARS-CoV-2 infection elicited a Th1 immune response in the mucosa of the upper airways, which could be helpful to minimize the severity of COVID-19 in these patients. Moreover, it is important to highlight that the correlation analysis between IFN-γ and IL-6 or IL-10 showed very interesting results in the two groups, mainly in terms of the levels of IL-10 and IFN-γ. In this regard, a significant negative correlation was observed between the levels of IFN-γ and IL-10 in the group of patients with olfactory dysfunction. Taken together, these findings allow us to suggest that the occurrence of anosmia and ageusia could be influenced by a dysregulated pattern of inflammatory modulation.

In spite of one limitation of this study is the small number of patients with olfactory dysfunction included in the analysis, and consequently, the statistical conclusion can generate some uncertainty, our results can shed a light on the possibility of the occurrence of these outcomes in COVID-19 patients. This pilot study is not underpowered by the statistical results either by the type II error adopted for such, underestimating the conclusion about the cytokine profile[33]. In accordance with this, although there may be uncertainties, the significant correlations verified between the levels of IFN-γ and IL-10 in the patient's group can putatively mitigate this drawback.

Furthermore, it is paramount to clarify that, in accordance with the literature, higher systemic levels of IL-6 and IFN-γ are related to the development of the hyperinflammatory state named "cytokine storm", which can be found particularly in patients with severe COVID-19, as well as in children with the recently described multisystem inflammatory syndrome (MIS-C) [34, 35]. Nevertheless, studies that aimed to evaluate not only the levels of these pro-inflammatory cytokines in the mucosa of the upper airways but also its consequences in COVID-19 patients are very scarce in the literature. Therefore, the results presented in this study can improve the knowledge about the COVID-19, especially by pointing out some relevant aspects of the mucosal immune response in the upper airways elicited by SARS-CoV-2 infection. In addition, it can also drive

other researchers to develop studies focusing on the mucosal immune responses in COVID-19 patients presenting anosmia and ageusia.

In conclusion, we were able to show that patients with mild COVID-19 presenting anosmia and ageusia have an altered mucosal immune response in the upper airways as compared to the control group of COVID-19 patients not presenting olfactory dysfunction. Further studies, including a larger number of patients from different age groups, should be performed to reproduce our preliminary findings in order to evaluate whether this dysregulated scenario is associated with the occurrence of anosmia and ageusia in COVID-19 patients.

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