

## RESEARCH

# Detection of SARS-CoV-2 infection in thyroid follicular cells from a COVID-19 autopsy series

Sofia Macedo<sup>1,2,3,4</sup>, Ana Pestana<sup>1,2,4</sup>, Liliana Santos<sup>2,4,5</sup>, Celestino Neves<sup>4,6</sup>, Susana Guimarães<sup>4,6</sup>, Amaro Duarte-Neto<sup>7</sup>, Marisa Dolhnikoff<sup>7</sup>, Paulo Saldiva<sup>7</sup>, Georgina Alves<sup>1</sup>, Rute Oliveira<sup>1</sup>, Didier Cabanes<sup>1</sup>, Fátima Carneiro<sup>1,2,4,6</sup>, Manuel Sobrinho-Simões<sup>2,4,6</sup> and Paula Soares<sup>1,2,4</sup>

<sup>1</sup>Institute for Research & Innovation in Health, University of Porto, Porto, Portugal

<sup>2</sup>Institute of Molecular Pathology and Immunology of the University of Porto, Porto, Portugal

<sup>3</sup>Institute of Biomedical Sciences Abel Salazar, University of Porto, Porto, Portugal

<sup>4</sup>Faculty of Medicine, University of Porto, Porto, Portugal

<sup>5</sup>North Lisbon University Hospital Center, Lisbon, Portugal

<sup>6</sup>University Hospital Center of São João, Porto, Portugal

<sup>7</sup>Department of Pathology, Faculty of Medicine, University of São Paulo, São Paulo, Brazil

Correspondence should be addressed to P Soares: [psoares@ipatimup.pt](mailto:psoares@ipatimup.pt)

## Abstract

**Objective:** To understand whether thyroid cells can be directly infected by the SARS-CoV-2 virus and to establish a putative correlation with the expression of the host entry machinery: ACE-2, TMPRSS2, and furin.

**Methods:** We assessed the presence of SARS-CoV-2 virus at the gene level by RT-PCR, viral RNA transcripts localization by *in situ* hybridization, and by detecting viral proteins by immunohistochemistry for the nucleocapsid and the spike proteins. Furthermore, we also described the immunorexpression of key host factors for virus entry in the COVID-19 thyroid samples.

**Results:** We performed RT-PCR for SARS-CoV-2 in all autopsy specimens and detected viral genome positivity in 13 of 15 thyroid tissues and in a lung specimen. In 9 of the 14 positive samples, we were also able to confirm SARS-CoV-2 signal by *in situ* hybridization. Immunohistochemistry for the viral nucleocapsid and spike protein was also positive for ten and nine of the RT-PCR-positive cases, respectively, but revealed a lower sensitivity. We also described, for the first time in a COVID-19 series, the immunohistochemical expression of ACE-2, TMPRSS2, and furin in the thyroid.

**Conclusions:** Our results obtained in thyroid specimens from deceased COVID-19 patients indicate that thyrocytes can be directly infected by SARS-CoV-2 since we detected the presence of SARS-CoV-2 genome in follicular cells. Nevertheless, we did not find a clear correlation between the presence of viral genome and the expression of the host factors for virus entry, namely ACE-2, TMPRSS2, and furin.

## Key Words

- ▶ SARS-CoV-2
- ▶ thyroid
- ▶ ACE-2
- ▶ TMPRSS2
- ▶ furin

## Introduction

Corona virus disease (COVID-19) dissemination first took place in 2019; in March 2020 the World Health Organization (WHO) officially declared it a pandemic. The severe acute respiratory syndrome coronavirus 2

(SARS-CoV-2) is the virus causing the disease and is highly infectious, having already been responsible for more than 6 million deaths worldwide (<https://coronavirus.jhu.edu/>). COVID-19 disease mainly affects the respiratory tract,

yet, like other coronaviruses, it can also affect endocrine organs, such as the thyroid gland (1, 2).

Angiotensin-converting enzyme 2 (ACE-2) receptor was reported as the functional receptor of the SARS-CoV virus (3). Recent studies reported that SARS-CoV-2 also employs the same receptor (4, 5), with a much higher affinity in comparison with previous SARS-CoV virus (6), probably related to the higher spreading of the disease (6). ACE-2 is a metallo-carboxypeptidase type I transmembrane protein encoded by a gene localized in Xp22.2 (7) and is responsible for the cleavage of angiotensin (Ang) I and Ang II to generate Ang 1–9 and Ang 1–7, respectively (8). Interestingly, the thyroid gland constitutes one of the top ten organs expressing ACE-2 mRNA (9), and its expression in thyroid cells has been recently reported to be modulated by pro-inflammatory cytokines which ultimately can facilitate the entering of the virus into the host cells (10). Hence, it is reasonable to advance that the thyroid gland may represent a possible target for SARS-CoV-2 infection. Since the pandemic outbreak, some studies have been reporting, in COVID-19 patients', alterations in thyroid hormone levels (11, 12), thyrotoxicosis (13, 14), and subacute thyroiditis (2, 15). These effects are mostly transient during the infection period, but when comparing severe COVID-19 patients with severe non-COVID-19 pneumonia patients, it has been reported a significantly higher decrease in TSH serum levels for COVID-19 patients, reinforcing the putative (direct or indirect) role of SARS-Cov-2 on thyroid hormones' regulation (16).

In order for SARS-CoV-2 to infect the host cell, other key players besides ACE-2 are required. Furin and the transmembrane protease serine 2 (TMPRSS2) are two proteases involved in SARS-CoV-2 spike protein processing in the host cell, ensuring an efficient infection. The coronavirus is constituted by four main structural proteins: nucleocapsid (N), membrane (M), envelope (E), and spike (S) (17). The entry of the virus into the cells encompasses the cleavage of the subunit S1 of the S protein by the convertase furin, leading to the binding of S1 to the ACE-2 receptor. On the other hand, the S2 subunit connects the S protein to the membrane in order for fusion to occur (5). As reviewed by Jackson *et al.*, an additional trigger for SARS-CoV-2 glycoprotein entry is the cleavage of an additional internal site of the S2 subunit, the S2', that is cleaved at the cell surface by the TMPRSS2 protease, which is exposed when the binding to ACE-2 occurs (17). Interestingly, both TMPRSS2 and furin are thought to be highly expressed in the thyroid gland (18).

In this article, we aimed to understand if the thyroid cells could be directly infected by SARS-CoV-2 by detecting

viral RNA molecules in the thyroid tissues from a series of autopsies of COVID-19 patients. Also, we intended to characterize protein expression of the cellular targets for virus entry, ACE-2, TMPRSS2, and furin, in the same series of thyroid tissues from patients deceased by COVID-19.

## Materials and methods

### Patients' collection and clinicopathological data

In collaboration with the University of São Paulo, in Brazil, and Hospital São João, in Portugal, we were able to obtain a series of thyroid specimens from autopsies of patients who died from COVID-19 in 2020. The series from Brazil was composed of 13 thyroids and 1 lung specimen ( $n = 14$ ). The cases from Portugal were six thyroid samples from two different patients. Therefore, a total of 20 samples (including the lung) from 16 patients were obtained. All the slides were stained for hematoxylin and eosin (H&E) and reviewed by an experienced thyroid pathologist (M S-S). Clinicopathological data of the patients were collected (Table 1), and each of these cases had either a *pre-mortem* or a *post-mortem* nasopharyngeal swab performed. The lung specimen was used as a positive control for the experiments in the thyroid series. The period of sample collection occurred between March and September 2020, for both series. The Research Ethics Committee CHUSJ, Portugal, approved this study under the number 272/2020 under the project entitled 'Is thyroid gland a target of SARS-CoV-2 infection? Early identification and follow-up of thyroid dysfunction in COVID-19 patients', and for Brazil series, the study was approved under the project 'Studies of the fatal COVID-19 through-guided ultrasound minimal invasive autopsy', process number #4.813.199, March 28, 2020.

### Real-time reverse transcriptase-polymerase chain reaction

Total RNA was extracted from two slides of 10  $\mu$ m thick formalin-fixed paraffin-embedded (FFPE) sections for each case, in an automatic extractor Maxwell<sup>®</sup> CSC RNA FFPE Kit, from Promega (AS1360). RT-PCR for SARS-CoV-2 gene amplification was performed with two different kits: Fosun COVID-19 RT-PCR Detection Kit (Cat. #PCSYHF03-a) and TaqPath<sup>™</sup> COVID19 CEIVD RT-PCR Kit (Cat. #A48067). The run method was the same for both and started with a cycle of 15 min at 50°C and a cycle of 3 min at 95°C. The PCR program was 5 cycles of 5 s at 95°C and 40 s at 60°C,

**Table 1** COVID-19 patients' demographic and clinical data.

Case	Tissue	Series	Date of death	Days until post-mortem examination	Age	Sex	BMI	Interval of days from symptoms to death	Comorbidities
1	Lung	Brazil	n.a.	March 2020	83	F	24.56	5-10	-
2	Thyroid	Portugal	June 2020	+3 days	44	F	<25	10-20	N
3	Thyroid	Portugal	April 2020	+1 day	70	F	>25-30	n.a.	HT
4	Thyroid	Brazil	April 2020	+1 day	71	F	28.31	10-20	DM, HT, CP, A
5	Thyroid	Brazil	May 2020	+1 day	45	M	23.25	10-20	DM, HT, CKD
6	Thyroid	Brazil	May 2020	Same day	32	M	29.39	>30	-
7	Thyroid	Brazil	May 2020	+1 day	34	F	30.32	10-20	N
8	Thyroid	Brazil	May 2020	+1 day	38	F	20.42	10-20	N, CP
9	Thyroid	Brazil	May 2020	Same day	22	M	24.69	10-20	-
10	Thyroid	Brazil	July 2020	+1 day	51	F	29.41	<5	N
11	Thyroid	Brazil	July 2020	Same day	24	F	29.41	>30	-
12	Thyroid	Brazil	July 2020	Same day	49	F	35.91	20-30	HT
13	Thyroid	Brazil	July 2020	Same day	26	F	25.76	>30	-
14	Thyroid	Brazil	September 2020	+1 day	63	M	29.41	20-30	-
15	Thyroid	Brazil	August 2020	+1 day	66	M	24.09	10-20	DM, HT, CP
16	Thyroid	Brazil	August 2020	+1 day	70	M	24.08	<5	DM, HT

-, no major comorbidities reported; A, asthma; CKD, chronic renal disease; CP, cardiopathology; DM, diabetes mellitus; F, female; HT, hypertension; M, male; N, neoplasia; n.a., non-available.

followed by 40 cycles of 5 s at 95°C and 40 s at 60°C. The genes amplified in the Fosun kit were ORF1ab that encodes for orf1ab polyproteins (representing two-thirds of the virus genome (19)) and gene N and gene E that encode for nucleocapsid and envelope proteins, respectively. In the TaqPath™ kit, the genes amplified were ORF1ab, gene N, and gene S (that encodes for the viral spike protein). The human RnaseP (hRnaseP) was used as an endogenous control in both kits. About 50 ng of RNA was used for each RT-PCR test, and in three of the cases, a second RNA extraction was needed. Samples were considered positive when at least two of the four SARS-CoV-2 genes were amplified at maximum at cycle threshold (Ct) 37 (Supplementary Fig. 1A and B, see section on [supplementary materials](#) given at the end of this article). Samples were considered inconclusive when only one viral gene was amplified or there was amplification after Ct 37. A case was considered negative when hRnaseP was positive but none of the viral genes were amplified (Supplementary Fig. 1C). The reaction was only considered valid when hRnaseP control was amplified. RT-PCR was performed for all 16 patients plus 2 pre-COVID FFPE controls (Table 2) and 10 pre-COVID thyroid frozen samples (data not shown).

### In situ hybridization (ISH)

RNAscope® ISH was performed with RNAscope 2.5 HD Reagent kit - Brown, (Advanced Cell Diagnostics, Inc., Newark, CA, USA) (20). Three different probes were used for each sample: SARS-CoV-2 spike probe (V-nCoV2019-S), DapB probe that targets the bacterium gene (negative

control probe), and Ubiquitin C (UBC) (positive control probe) to evaluate RNA detection and integrity. Four-micrometer sections of FFPE blocks were deparaffinized and subjected to antigen retrieval for 20 min with a 1× target retrieval solution. The slides were then incubated with protease plus and treated for 20 min at 40°C using the HyBEZ™ oven (Advanced Cell Diagnostics, Inc). Subsequently, the slides were incubated for 2 h at 40°C in the oven with the target probe. Finally, the signal was amplified using a series of the six amplifiers recommended by the manufacturer. The chromogen detection was performed with DAB for 10 min at room temperature and counterstained with Gill's hematoxylin.

### Immunohistochemistry

Immunohistochemistry (IHC) was performed in all the FFPE specimens. Dewaxing and rehydration, followed by 45 min of heat-induced antigen retrieval with EDTA buffer (pH 9.0) in the steamer, were performed. The IHC protocol including the peroxidase and protein block, antibody amplifier, and polymer incubations was performed with the Ultravision Quanto Detection System HRP, Epredia®, TL-125-QHL, according to the manufacturer's instructions. The antibodies used were: mouse monoclonal anti-SARS/SARS-CoV-2 coronavirus nucleocapsid antibody (Invitrogen, MA1-7404, 1:100); mouse monoclonal SARS-CoV/SARS-CoV-2 spike antibody ((1A9), GTX632604, Genetex (Irvine, CA, USA), 1:200); mouse monoclonal anti-ACE-2 (Invitrogen, MA5-31395, 1:2500); rabbit monoclonal

**Table 2** RT-PCR for amplification of SARS-CoV-2 genes and RNAscope results.

Case	Tissue	Status	Amplification Cts					RNAscope
			Gene Orf1ab	Gene N	Gene E	Gene S	hRNAP	
1	Lung	Positive	25	23	24	22	19	++
2	Thyroid	Positive	No amplification	33	32	32	22	+
3	Thyroid	Positive	32	33	No amplification	No amplification	27	–
4	Thyroid	Positive	34	32	No amplification	36	20	+ <sup>a</sup>
5	Thyroid	Positive	34	31	33	No amplification	21	–
6	Thyroid	Positive	No amplification	31	33	No amplification	20	–
7	Thyroid	Positive	33	30	30	32	20	+ <sup>a</sup>
8	Thyroid	Positive	34	33	35	No amplification	20	–
9	Thyroid	Positive	32	33	No amplification	No amplification	19	+++ <sup>a</sup>
10	Thyroid	Positive	32	30	30	28	20	–
11	Thyroid	Inconclusive <sup>b</sup>	No amplification	34	33	No amplification	22	–
12	Thyroid	Negative	No amplification	No amplification	No amplification	No amplification	21	–
13	Thyroid	Positive	35	35	No amplification	No amplification	20	+ <sup>a</sup>
14	Thyroid	Positive	No amplification	31	33	No amplification	19	+
15	Thyroid	Positive	32	30	31	33	20	+
16	Thyroid	Positive	No amplification	32	32	No amplification	20	+
Control 1	Thyroid	Negative	No amplification	No amplification	No amplification	No amplification	22	ND
Control 2	Thyroid	Negative	No amplification	No amplification	No amplification	No amplification	23	ND

<sup>a</sup>RNAscope positivity in the adjacent tissue; <sup>b</sup>Case positive for the Fosun Kit in the first RNA extraction and negative in the second extraction for both Fosun and TaqPath kits.

–, no signal detected; +, at least one focal area with signal; ++, several focal areas with signal; ND, not done.

anti-TMPRSS2 antibody (EPR3862), Abcam, ab109131, 1:1000); rabbit polyclonal anti-furin (Invitrogen, PA5-96680; 1:200); and rabbit monoclonal anti-cleaved caspase-3 (Asp175, Cell Signalling, 9664, 1:1000). The detection was performed with DAB chromogen for all antibodies (Epedria®, TA-125-QHDX), with the exception of spike and furin which detection was performed with HIGHDEF® Red chromogen (Enzo Life Sciences (Farmingdale, NY, USA), NC1609433). All slides were counterstained with Gill's hematoxylin.

## Results

### Patients' clinicopathological data

The age of the 16 patients varied from 22- to 83-year-old (Table 1), with a median age of 47 years. The population was composed of ten females (62.5%) and six males (37.5%), and females were younger than males (median age: 46.5 vs 54.0, respectively). BMI was available for the 16 patients, and 50% were overweight. From these, two female patients were obese (BMI > 30). Major comorbidities in this population were 3/16 (19%) patients that had neoplasia and 5/16 (31%) patients who had any type of cardiac pathology (Table 1). None of these patients had reports of a previous history of thyroid disease, and the thyroid function of these patients was not evaluated during hospital admission.

### Histology of the thyroid specimens

All the slides were evaluated by an experienced pathologist (MSS) and no major lymphocytic infiltrate or destruction was observed in the thyroid sections (Fig. 1A), besides expected from *post-mortem* autolysis. In some cases, we were able to identify slight follicle alterations (Fig. 1B). No signs of hyperplastic/neoplastic lesions were detected.

### RT-PCR

We performed RT-PCR in samples corresponding to the 16 patients. All the cases amplified for hRNaseP, the gene used for control of RNA integrity. Of the 16 cases, 14 were positive (88%) on the RT-PCR reaction since amplification of at least two of the four viral genes tested was observed before Ct 37 (Table 2 and Supplementary Fig. 1). Four cases showed amplification for all the viral genes (the lung sample and three thyroid samples; Table 2). In four cases, three of the viral genes gave positive signals and in six cases, two viral genes were positive (Table 2). One case was negative (6%), since none of the viral genes were amplified, only the positive control hRNaseP produced a signal (Table 2). Another case was considered inconclusive since the result of a second RNA extraction was negative for all genes after first extraction had been positive for two of the genes (Table 2). In order to exclude some potential non-specificity of the viral genes amplified in the RT-PCR, we have also performed the RT-PCR in two different FFPE

thyroids (control 1 and control 2; Table 2) plus a series of ten frozen samples of thyroid that were pre-pandemic samples (data not shown). No gene amplification was observed, except for the hRNAP gene.

## ISH

To confirm the presence of viral material in the tissues, we have performed ISH using RNAscope<sup>®</sup>. For control of our experiment, SARS-CoV-2-infected (Fig. 2A) and not infected (Fig. 2B) monkey VERO cells (ATCC, CCL-81) were used. The results from RNA-ISH obtained with the positive control probe (UBC) revealed expression in all samples (Fig. 2C), including the lung (Fig. 2F), indicating that all samples had preserved mRNA. To monitor for non-specific signals, we used a negative control probe, the DapB gene from the *Bacillus subtilis* bacterium that, as expected, was negative for all samples (Fig. 2D and G). In the samples that revealed positivity for the SARS-CoV-2, the signal detected was very discrete and focal, as previously described in the thyroid (21). In 9/20 (45%) of the samples, it was possible to detect RNA-ISH signals in the slides (Fig. 2E), including the lung (Fig. 2H). In four of the samples, we detected a signal in the thyroid follicles (Fig. 2E), whereas, in the additional five, the signal was observed in the adjacent tissue (adipose tissue and/or muscle) (data not shown).

## IHQ

### Expression of SARS-CoV-2 N and S proteins

The thyroid samples displayed a focal, cytoplasmic, and granular-like staining for SARS-CoV-2 nucleocapsid antibody (Fig. 3A). In 4 out of 20 samples (20%), we observed >25% of the tissue with the expression of the protein on the thyrocytes and was mostly cytoplasmic granular-like staining. Eleven out of 20 (55%) had <25% of the tissue expressing the protein, and 5/20 (25%) were completely negative. As for the spike protein, the staining was scarcer, with small, focal areas of cytoplasmic staining

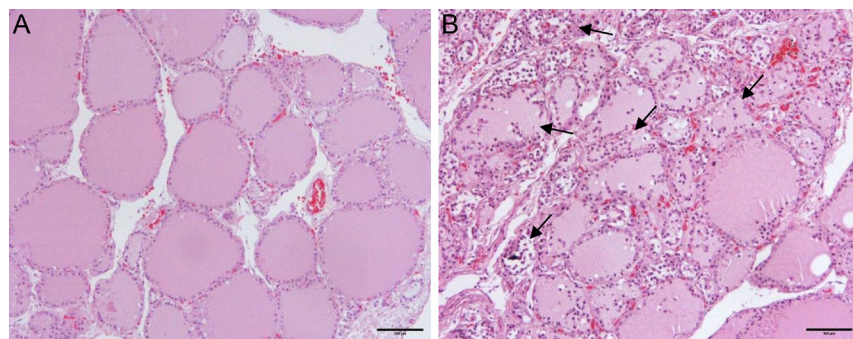
(Fig. 3B). In 2 out of 20 samples (10%), we observed >10% of the tissue staining and 4/20 (20%) had <10%. Five samples (25%) had only one or two focal areas and the remaining seven (35%) were totally negative. Both proteins displayed focal and cytoplasmic expression in the lung tissue (Fig. 3C and D) (see Supplementary Table 1).

### Expression of cleaved caspase-3

The cleaved caspase-3 antibody detects the endogenous levels of activated caspase-3 in the tissue. The staining for cleaved caspase-3 was mainly cytoplasmic, with a granular pattern in some cases, and its expression throughout the series was high, indicating a high level of apoptosis in these cases, including the lung (Fig. 4A and B). In 5/20 (25%) cases, we observed >75% of the tissue staining, 11/20 (55%) cases had 50–75% of positive cells, 2 cases had 25–50% of positive cells, and 2 cases (10%) had less than 25% of the cells with cleaved caspase-3 expression (see Supplementary Table 1).

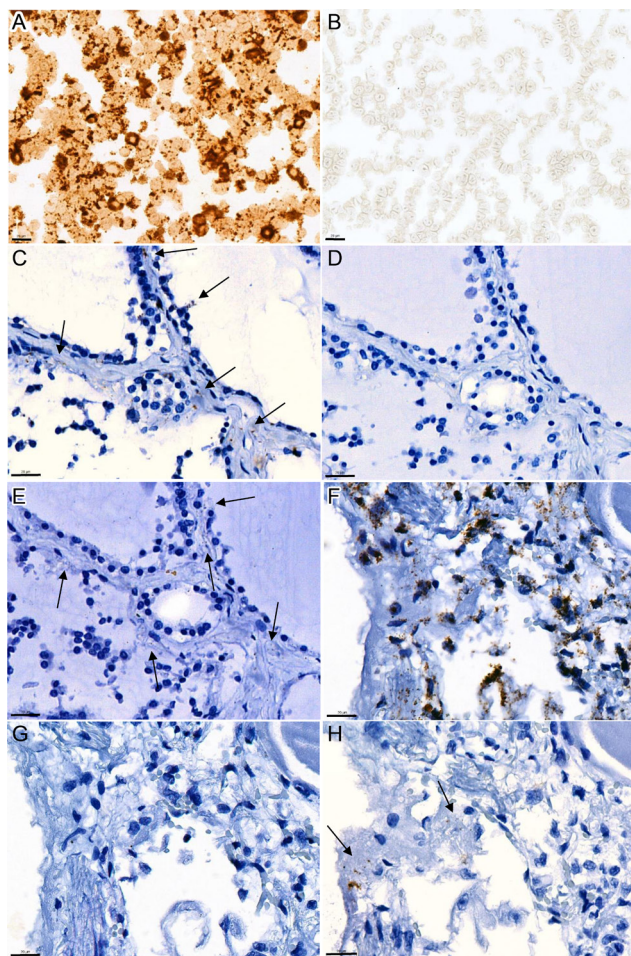
### Expression of ACE-2, TMPRSS2, and furin

ACE-2 immunohistochemistry displayed mostly basal staining that seems to co-localize with the endothelial cells (Fig. 5A). No expression of ACE-2 was observed in the cytoplasm or apical membrane of the thyrocytes (Fig. 5A). The expression was variable among the cases and 6/19 (32%), including the lung tissue, had less than 10% of the tissue staining (Fig. 5B). The majority of the samples, 13/19 (68%), presented ACE-2 expression in >25% of the tissue, and two of those samples had >75% of the tissue expressing ACE-2. TMPRSS2 protein expression was very high in the thyroid specimens. Eleven out of the 20 samples (55%) had >75% of the tissue expressing cytoplasmic staining and occasionally, nuclear staining for TMPRSS2 was observed as well (Fig. 5C and D). Furin protein expression was generally low, and 5/19 (26%) of the samples had up to 25% of the tissue expressing cytoplasmic staining with a low to moderate intensity. Four samples (21%) showed positivity in up to 50% of



**Figure 1**

(A) H&E of thyroid specimens of COVID-19 patients: neither lymphocytic infiltration nor thyroid wide-spread alterations were evident; (B) in some cases, there were focal areas of follicle alterations (arrows). Scale bars: 100  $\mu$ m.



**Figure 2**

(A) Positive RNA-ISH signal in VERO cell lines infected with SARS-CoV-2; (B) negative RNA-ISH signal in VERO cell lines without infection of SARS-CoV-2; (C) representative section of a thyroid sample showing positivity for control UBC probe (arrows); (D) the respective negative control for DapB probe; (E) thyroid sample showing positivity for SARS-CoV-2 probe in the thyroid follicles (arrows); (F) lung tissue showing positive signal for control with UBC probe and (G) the negative control with DapB probe; (H) the lung for the SARS-CoV-2 probe (arrows). Scale bars: 20  $\mu$ m.

the tissue, presenting moderate staining. The remaining samples, 10/19 (53%), were negative or with less than 10% of the cells staining (see Supplementary Table 1).

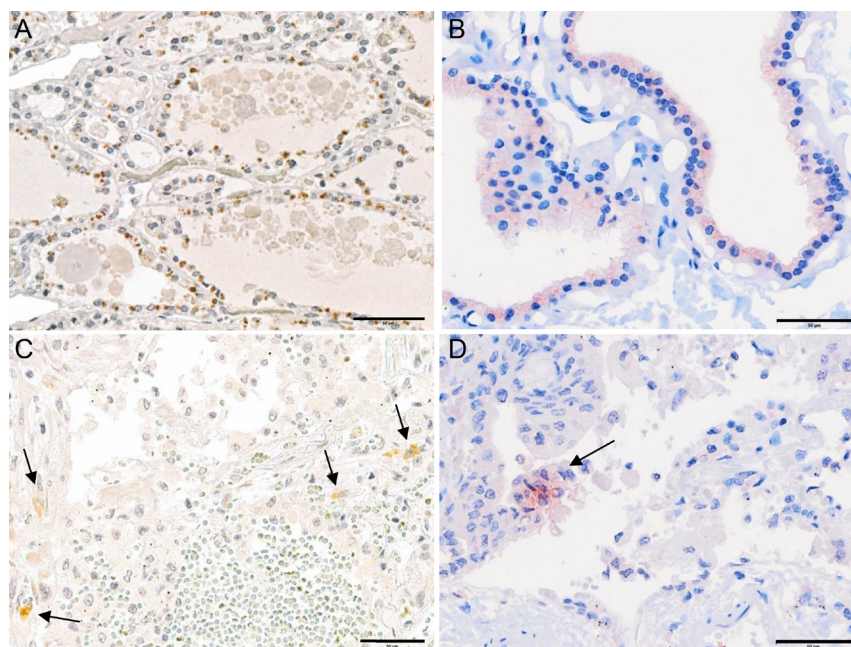
## Discussion

COVID-19 infection has been revealed as a multisystemic disease, presenting several endocrine repercussions. In this study, we aimed to clarify if the thyroid cells could be directly infected by the SARS-CoV-2 through the analysis of a series of thyroid specimens from patients deceased by COVID-19, with histopathologic and molecular evaluation of the specimens.

In this series, we were able to detect, by RT-PCR, the expression of SARS-CoV-2 genes in 14/16 (88%) of the thyroid specimens. To our knowledge, 3 studies reported to date the presence of the virus genome in thyroid; Wang *et al.* in 1 thyroid sample (21), Poma *et al.* in 9 out of 25 thyroids analyzed (36%) (22), and Wong *et al.* 2021 in 6 out of 8 thyroids (75%) (23). In the three previous studies, a sample was considered positive for SARS-CoV-2 when at least one of the tested genes was amplified by RT-PCR. We were more restricted in the present study; we performed RT-PCR with two different kits and considered positive only those cases with amplification for at least two viral genes. To evaluate the specificity of the signal, we also included 12 cases of thyroid tissue from the pre-pandemic period, and all were negative for all the viral genes tested. Taken together, these results suggest that thyroid cells can be infected by SARS-Cov-2 virus.

By RNA-ISH techniques, we were able to corroborate this observation since we could detect the presence of viral RNA transcripts in the thyrocytes in four cases *in situ* that were also positive for RT-PCR. In four other cases positive for RT-PCR, we were able to detect the RNA transcripts by ISH, but only in the adjacent tissue of the thyroid (muscle cells and adipocytes), and therefore, we cannot dismiss the possibility that the positivity detected in these cases by RT-PCR could be due to the infection of the adjacent tissue and not from the thyrocytes itself. Five cases that were positive for RT-PCR did not show positivity in the RNA-ISH. This discrepancy can be explained by the fact that ISH techniques are not as sensitive as RT-PCR and, consequently, miss some of the cases with low viral load. Similar results were also observed in a previous study (21).

The overall signals for RNA-ISH were very focal and discrete in our study. A similar pattern was described previously in studies reporting RNA-ISH in different human tissues (21, 23, 24, 25). So far, RNA-ISH data regarding SARS-CoV-2 in thyroid specimens are very scarce, and only two studies performed the aforementioned technique (21, 23). One study performed RNA-ISH in one thyroid sample, and, similar to our results, only rare positive clusters were detected in the specimen (21); as referred by the authors, this focal and discrete pattern can result from the possible low viral load in the thyroid samples. This possibility is in accordance with the relatively higher Ct values obtained in the RT-PCR for SARS-CoV-2 genes that we also observed in our study (Table 2). The other study uses fluorescent *in situ* hybridization (FISH)-based detection of SARS-CoV-2 RNA in six thyroids using two different probes: SARS-CoV-2 sense S gene probe and SARS-CoV-2 antisense of S gene probe that, according to Wong *et al.*, indicates viral

**Figure 3**

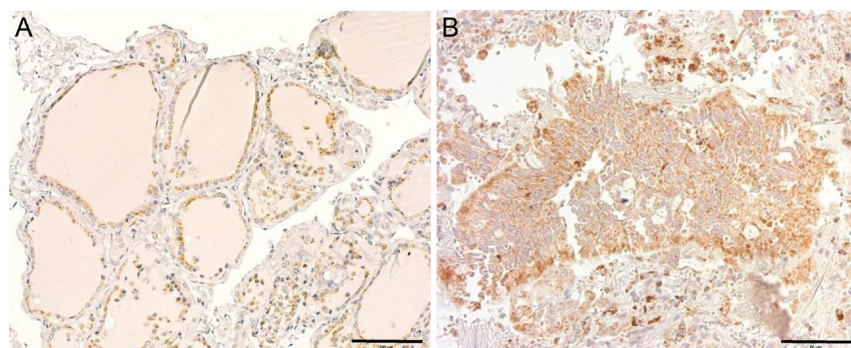
(A) SARS-CoV-2 nucleocapsid granular-like expression and (B) SARS-CoV-2 spike protein expressing cytoplasmic staining in the thyrocytes; (C) SARS-CoV-2 nucleocapsid immunohistochemical expression in the lung with focal cytoplasmic staining (arrows); (D) SARS-CoV-2 spike protein in the lung with focal cytoplasmic expression (arrows). Scale bars: 50  $\mu$ m.

replication activity (23). They report positive signals from both genomic and replicating RNA, in follicular epithelial cells, as well as in some stromal cells and endothelial cells (23), as we detected in our samples.

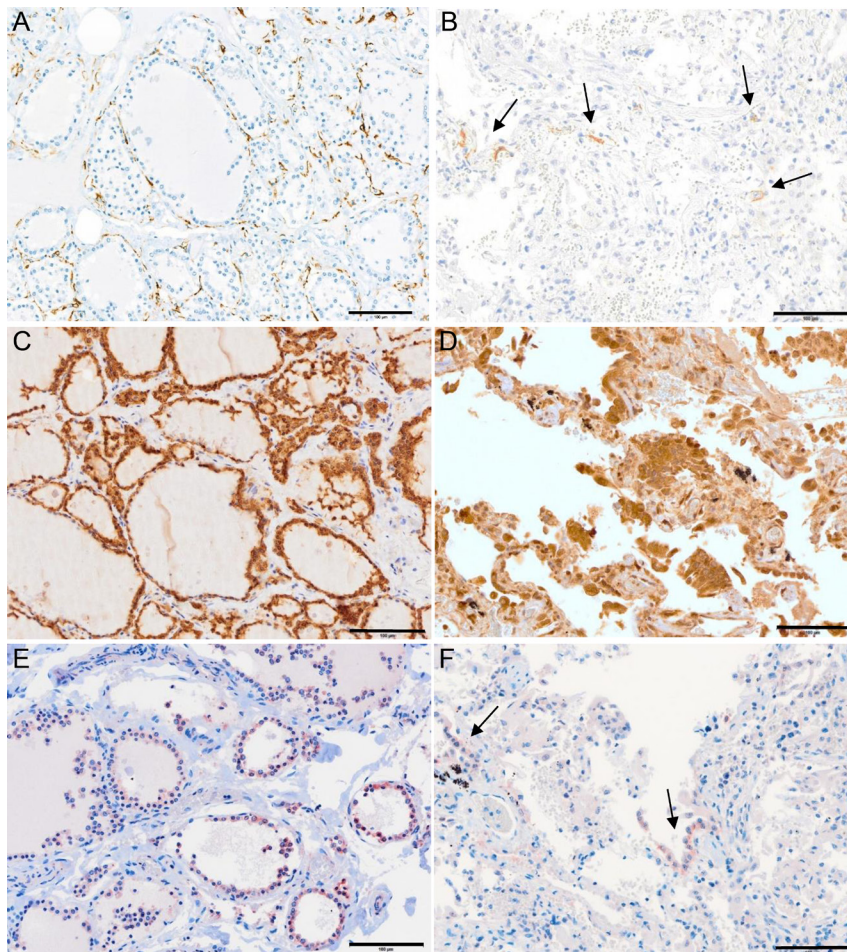
In the present study, SARS-CoV-2 variates were not sequenced, but SARS-CoV-2 B.1 clade, subclade B.1.1.33, was reported as the most prevalent genetic group circulating in the Latin America and Caribbean region until January 2021 (26). So, we can admit that, at least for the Brazil series, most of the patients were infected with the SARS-CoV-2 B.1 clade.

We have also performed IHQ for SARS-CoV-2 nucleocapsid and spike proteins, but the results obtained were not as demonstrative as the ones obtained with the previous techniques. We detected granular-like staining for the nucleocapsid protein in the thyrocytes of the majority of the samples; similar data have been reported for this protein in another study (22) in which the authors present a staining pattern very similar to the one shown in Fig. 4A.

In our series, we detected positive staining for the N protein in 11 cases, 10 of them were positive for the RT-PCR and 1 was inconclusive. Interestingly, the inconclusive case was the one that was positive for SARS-CoV-2 in the first extraction and negative in subsequent extraction. Once more, it is possible that viral load in the tissue of the second RNA extraction could not be sufficient for positive amplification of more than one gene. Immunohistochemistry for spike protein in thyroid has not been documented yet, and in our series, we found a very focal, weak cytoplasmic staining, and it was not possible to correlate N and S proteins expression through IHC (Supplementary Table 1). Hence, we observed that the nine cases positive for S protein were also positive in the RT-PCR amplification for the SARS-CoV-2, but in five cases that were positive in the RT-PCR, we could not detect S protein expression. The difficulty in the detection of the viral protein through IHC techniques has already been reported in other studies (23); besides the problem of viral load, it can also reside in antibody specificity, even

**Figure 4**

(A) Cleaved caspase-3 immunoeexpression in the thyroid (scale bar: 100  $\mu$ m) and (B) in the lung tissue (scale bar: 50  $\mu$ m).

**Figure 5**

(A) ACE-2 expression in a thyroid specimen with extensive staining, and (B) in the lung, with staining in <10% of the cells (arrows) (scale bar: 100  $\mu$ m); (C) TMPRSS2 expression in the thyrocytes with strong cytoplasmic expression (scale bar: 100  $\mu$ m) and (D) in the lung with strong cytoplasmic/nuclear staining (scale bar: 100  $\mu$ m); (E) Furin moderates cytoplasmic expression in the thyrocytes (scale bar: 100  $\mu$ m) and (F) in the lung with low cytoplasmic staining (arrows) (scale bar: 100  $\mu$ m).

more, when using FFPE material from autopsy. Finally, the negative case for the SARS-CoV-2 genome was also negative for both viral proteins N and S.

Another aim of our study was to evaluate the expression of cell entry factors such as ACE-2, TMPRSS2, and furin in the same specimens. ACE-2 expression was variable in the series, with RT-PCR-negative cases presenting high expression and RT-PCR-positive cases presenting low ACE-2 expression, and thus, it was not possible to establish any correlation. ACE-2 regulation upon SARS-CoV-2 entry is controversial and not yet well understood. A study using a cohort of COVID-19 patients detected high levels of Ang II in the blood of the patients, which was positively correlated with high viral load, thus, linking SARS-CoV-2 infection with ACE-2 downregulation (27). On the other hand, more recently, opposite results were observed in critically ill COVID-19 patients; van Lier *et al.* reported high levels of soluble ACE-2, low levels of Ang II and increased levels of Ang 1–7 when compared with healthy controls (28). Therefore, more studies are needed to clarify the putative relationship between ACE-2 expression and SARS-CoV-2

infection, especially since there are several other molecules that have been suggested as alternative cellular receptors for SARS-CoV-2 (e.g. lectins, phosphatidylserine receptors, CD147, neuropilin 1) (17). The aforementioned points may provide a justification for our variable data about ACE-2.

We observed in the present series a high expression of TMPRSS2, and this was also true for the negative cases in the RT-PCR for SARS-CoV-2 genes. On the contrary, furin protein expression was very low in our series, independent of the RT-PCR status. To our knowledge, no other study has reported IHQ protein expression of these two markers in thyroid of COVID-19 patients. Wang *et al.*, using RNA-ISH techniques, reported the presence of ACE-2 and TMPRSS2 mRNA in a series of pulmonary and non-pulmonary tissues of infected and deceased patients, and they reported that most tissues expressed both ACE-2 and TMPRSS2 transcripts, including the thyroid (21).

We observed high levels of apoptosis in the series, with high levels of expression of the activated caspase-3 in the thyroid and lung tissues. A co-localization of the viral RNA and activated caspase-3 was described in nasopharyngeal



swabs, indicating that viral infected cells had increased apoptosis (29). In our study, the activated caspase-3 expression was relatively high throughout the cases, independently of the results for SARS-CoV-2 RT-PCR, so we cannot exclude that this increase in the expression could be due to a *post-mortem* autolysis process.

Although infection by SARS-CoV-2 virus has been associated with cases of subacute thyroiditis (2, 15), we could not identify in our series any major histological signs of lymphocytic infiltration or inflammatory damage. Nevertheless, it would be interesting to evaluate the clinical data in terms of thyroid function of these patients.

Using a unique series of thyroid of COVID-19-deceased patients, the present study has some limitations. Since we studied autopsy specimens, the degree of autolysis in the tissues is variable, which can restrict the detection of the virus or even raise non-specific signals. To downgrade that possibility, we have used several positive and negative controls and pre-pandemic cases in our experiments. Furthermore, for most patients, we had only access to a single sample; this restricts the possibility of selecting a more representative thyroid sample of the SARS-CoV-2 infection.

Summing up, our results support that thyroid gland can be directly infected by the SARS-CoV-2 virus. We detected the amplification of SARS-CoV-2 genes in the thyroid specimens of 88% of the COVID-19-deceased patients, which was possible to confirm in the follicular cells through hybridization techniques *in situ*. On the other hand, protein detection of the SARS-CoV-2 nucleocapsid or spike protein does not seem to be a robust strategy to detect viral infection in FFPE tissues. Finally, we did not find any correlation between SARS-CoV-2 infection and the putative cellular 'doors' for virus entry.

#### Supplementary materials

This is linked to the online version of the paper at <https://doi.org/10.1530/ETJ-22-0074>.

#### Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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#### Author contribution statement

S M: performed ISH and IHQ staining, analysis of the results, reviewed the literature, and wrote the first version of the manuscript; A P: histological cuts, H&E staining, and IHQ staining; L S, C N, S G, F C: clinicopathological data and patients' material collection from Portugal; M D, P Sa: clinicopathological data and patients' material collection from Brazil; G A, R O, D C: SARS-CoV-2 RT-PCR amplification; MSS: histological evaluation of the H&E slides and wrote the manuscript; P So: study design and methodology, verification and discussion of data, and wrote the manuscript. All the authors contribute to the final version of the manuscript.

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